Identification of a Variant Antioxidant Response Element in the Promoter of the Human Glutamate-Cysteine Ligase Modifier Subunit Gene

REVISION OF THE ARE CONSENSUS SEQUENCE*

Constitutive and inducible expression of the gene encoding the modulator subunit of human glutamate-cysteine ligase (GCLM) is regulated by either of two regions of the promoter: an antioxidant response element (ARE) at −302–291 and a 44-bp fragment (−346–303) upstream of the ARE. This second region includes a consensus AP-1 site previously considered responsible for the enhancer activity of the upstream fragment. Deletion of a 165-bp fragment (−348–183) including the ARE and upstream 44-bp fragment totally ablated t-butyldihydroquinone (tBHQ) inducibility of a GCLM promoter/luciferase transgene. Mutation analyses confirmed that both the ARE and the −346–303 fragment could support induction following tBHQ exposure but demonstrated that induction in the latter case did not involve the AP-1 site at −341–335. A region sharing significant homology with the consensus ARE sequence except for a single nucleotide mismatch at −330 (5′-TTACnnnGCA-3′ versus 5′-TGACnnnGCA-3′) was identified at the 5′-end of the 44-bp fragment immediately adjacent to the AP-1 site. A G in this position has been considered an invariant requirement of functional ARE sequences. Mutation of T−330 to A (a substitution known to ablate ARE function) or C eliminated basal and inducible expression. Substitution of a G at −330 enhanced basal expression relative to the wild-type sequence, but induction following tBHQ exposure was comparable, indicating that either sequence (5′-TTACnnnGCA-3′ versus 5′-TGACnnnGCA-3′) may function as an ARE, although the former sequence is less effective at directing basal expression. This possibility was confirmed by similar mutational analyses of the core sequence of hNQO1, a prototypic ARE. Electrophoretic mobility shift competition assays revealed that the 5′-TTACnnnGCA-3′ sequence could compete with the hNQO1 ARE for protein binding but was less effective than a similar probe containing the 5′-TGACnnnGCA-3′ motif. Probes including the T(−330)A or T(−330)C mutations were ineffective. These results reveal that the GCLM promoter includes two functional AREs, one having a variant sequence. The results indicate that the consensus ARE sequence should be revised to 5′-RTKAYnnnGCR-3′.

Glutathione is an abundant cellular sulphydryl compound that functions as an important antioxidant contributing to the protection of cells from free radical, oxidative, and electrophilic damage. Glutathione is synthesized in two ATP-dependent steps, the first catalyzed by the rate-limiting enzyme glutamate-cysteine ligase (GCL), also referred to as γ-glutamylcysteine synthetase or γ-GCS), and the second by glutathione synthetase. GCL is a heterodimer composed of a catalytic subunit (GCLC, 73 kDa) and a modifier subunit (GCLM, 28 kDa), each encoded by separate genes. The catalytic subunit is responsible for synthetic activity (1) and is the site of feedback inhibition by GSH (2). The modifying subunit lacks catalytic activity but regulates the kinetic properties of the catalytic subunit, rendering it more efficient under physiological concentrations of glutamate and glutathione (3).

In many cases adaptive changes in glutathione homeostasis are associated with an elevation of GCLC enzyme activity. Recent studies have revealed that the genes encoding the catalytic and modifier subunits are commonly induced along with a battery of other drug metabolizing and antioxidant defensive genes, all contributing to an adaptive response to cellular stress. Many of the protective enzymes encoded by these adaptive response genes, including NQO1 (4), heme oxygenase 1 (5), glutathione-S-transferase Ya (6, 7), and both subunits of GCL (8, 9), have been shown to be coordinately up-regulated through antioxidant-responsive elements (AREs) (10), also known as electrophile-responsive elements or EpREs (6), located within the promoters of their respective genes.

The presence of consensus AREs in the promoter of each GCL subunit gene provides an appealing mechanism to explain the apparent coordinate regulation of these two genes detected in early studies of cellular responses to oxidative and electrophilic challenges. However, recent evidence demonstrating differential expression of the two GCL subunit genes following exposure to certain chemical and physical agents suggests that regulation of the two genes is complex (11). Although induction of GCLC by β-NF (9) and lactacystin (12) has been shown to be regulated through a distal ARE, other studies have reported that GCLC induction following exposure to the pro-oxidants menadione or hydrogen peroxide is mediated through an AP-1 site (13, 14), and induction following exposure to ionizing radiation can be mediated via an NF-κB site (15). In the case of transcriptional activation of the human GCLM subunit gene we reported that two distinct regions of the promoter, an ARE located −300 base pairs upstream of the ATG start site and a

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† To whom correspondence should be addressed: KA/554 CSC, 600 Highland Ave., Madison, WI 53792. Tel.: 608-263-3695; Fax: 608-262-8430; E-mail: mulcahy@bascom.wisc.edu.
putative AP-1 site located 33 base pairs upstream of the ARE (8), can direct induction following exposure to β-NF. In contrast, Galloway and McLellan (16) reported that neither the ARE nor the AP-1 site was capable of supporting GCLM induction following exposure to the phenolic antioxidant tBHQ or β-NF. They concluded that one or more unidentified cis-elements located within a 42-base pair region of the GCLM promoter immediately upstream of the ARE site, but overlapping the nucleotides that we identified as an AP-1 site, mediates induction (16).

To better define the transcriptional regulation of GSH homeostasis in general and GCLM subunit gene control in particular, we re-evaluated the functional significance of the region between −345 and −209 of the GCLM promoter. Our results confirm that the ARE is capable of supporting induction of GCLM expression following exposure to tBHQ, as was previously reported for β-NF. However, as suggested by Galloway and McLellan (16), the upstream cis-element capable of supporting GCLM induction is not an AP-1 site per se. Our results indicate that the upstream regulatory element responsible for up-regulation of the GCLM gene is in fact a second ARE, unidentified in previous analyses because of a single nucleotide mismatch within the ARE consensus sequence in a position previously considered to be invariant. In addition to reconciling fundamental differences in previous experimental observations and identifying specific regulatory sequences capable of directly inducing expression of the GCLM gene, the findings reported strongly suggest that the ARE consensus sequence needs to be revised.

**EXPERIMENTAL PROCEDURES**

**Transient Transfection**—Transient transfection assays were performed as described previously (8). Briefly, cultures of HepG2 liver hepatoblastoma cells were grown overnight in 6-well plates in complete (10% fetal bovine serum and 50 μg/ml gentamicin) Dulbecco’s modified Eagles’ medium (DMEM) and then aspirated and refed with fresh complete DMEM. Two hours later, each well was transfected with 1–2 μg of luciferase reporter DNA plus 0.5 μg of a plasmid encoding β-galactosidase, using a standard calcium phosphate transfection procedure. 4–6 h after transfection, cells were shocked with 10% glycerol in the medium for 3 min. Cells were washed twice with Ca2+/Mg2+-free phosphate-buffered saline and then refed with complete DMEM. Approximately 24 h later, cells were treated with media containing MeSO or 100 μM tBHQ dissolved in MeSO. 18 h after addition of tBHQ, cells were washed twice in phosphate-buffered saline and harvested using Promega reporter lysis buffer. Cellular extracts were analyzed for luciferase activity. Luciferase activity was normalized to β-galactosidase activity and protein concentration, compared with activity in cells transfected with control vectors, and expressed as relative luciferase activity.

**Reporter/Enhancer Vector Construction**—Creation of GCLM reporter constructs containing specific deletions or point mutations was described previously (8). The −1927/GCLMΔ−348:−183 construct was made by digesting the full-length −1927/GCLM−luc transgene with SacII and AgeI, blunt-ends the cut sites, and re-ligating. Single mutants mA1 and mA3, as used by Galloway and McLellan (16), were created using Promega Gene Editor in vitro site-directed mutagenesis system, using the wild-type −1927/GCLM−luc transgene as a template (16). The double ARE/AP-1 mutant, mA1:3, was made with the Stratagene QuikChange XL site-directed mutagenesis kit using the mA3 construct as a template. All recombinant vectors were sequenced to confirm the sequence of regions of interest.

To make the enhancer/reporter transgenes (−346:−303, −345:−315, −346:−334, −344:−329, −346:−324, −346:−303m, and −346:−303mA3), complementary pairs of oligonucleotides (see Fig. 5A) were synthesized at the University of Wisconsin-Madison Biotechnology Center. The oligonucleotides were annealed as described (17), phosphorylated, and then ligated into the Smal site of the pT81-luciferase vector (ATCC 37584), creating the various enhancer vectors shown. The T(−330)A mutant was created with the Stratagene QuikChange XL site-directed mutagenesis kit using the −346:−303 enhancer/reporter transgene as a template.

The −312 and −302 inserts (see Fig. 4) were created using a PCR strategy and were cloned into the SmalNeol site of a pGL3-Basic variant antioxidant response element...
vector, which had been mutated by introduction of a point mutation into an AP-1 site contained in the parental pGL3-Basic as described previously (18). The 5' primer for the 313 construct was 5'-TGCTAGCAATTGTCATGGTCCACCG-3' and for the 303 construct it was 5'-GTTAGCAATTGTCATGGTCCACCG-3', and both utilized the same 3' primer, 5'-GGCGTCTTCCATGGCAGCGG-3'. The PCR reaction was performed using Pfu polymerase utilizing the full-length GCLM luc reporter construct as a template. The ARE consensus sequence of the 312 construct was mutated from ATGACTCAGCA to AGGACTCAGCA using the 5'-3'MORPH DNA site-directed mutagenesis kit to create the 312m construct.

Electromobility Shift Assays—Complimentary oligonucleotides containing the human hNQO1 ARE sequence (hNQO1) were prepared for use as double-stranded probes as described previously (19). The sequence of the sense strand was 5'-CAGTCACAGTACTCGAATTGTCATGGTCCACCG-3'. Nuclear extracts were prepared as described by Digman et al. (20) from HepG2 cells following treatment with Me2SO or 100 μM tBHQ for 18 h. Incubation conditions and non-denaturing gel analysis were performed as reported previously (19).

**RESULTS**

**Induction Following tBHQ Exposure Is Mediated by Sequences between −345 and −209 of the GCLM Promoter**—To localize cis-elements capable of mediating tBHQ induction, a series of promoter/luciferase transgenes containing progressive deletions of the full-length (1927 bp) GCLM promoter were transfected into HepG2 cells. As observed previously for β-NF, maximal induction by tBHQ (−2.5–4-fold) was achieved with a transgene containing the −345–209 promoter fragment (Fig. 1). No further induction was observed in cells transfected with transgenes including longer GCLM promoter fragments. Regulation of basal expression was also similar to previous findings (8, 16). Elements within the −312 construct were mutated from ATGACTCAGCA to AGGACTCAGCA using the 5'-3'MORPH DNA site-directed mutagenesis kit to create the −312m construct.

**Fig. 2. Effect of ARE and/or AP-1 mutations on the induction of −1927/GCLM5'-luc transgenes by tBHQ.** A. the sequence of the fragment between −349 and −287 of the wild-type GCLM promoter and the mutations of the ARE (−302−292) and the AP-1 site (−341−335) are shown (8). An open box enclosing a response element signifies the wild-type sequence, whereas absence of the box indicates a mutation of the element. Arrowheads and lowercase letters indicate mutations. B. the effect of transfected GCLM mutations on basal and tBHQ-induced expression. Each transgene is represented by an illustration to the left of the plot; open boxes symbolize wild-type response elements and filled boxes represent mutated elements. Wild-type and mutant transgenes were transiently transfected, treated, and harvested as done for Fig. 1. Results are reported as means ± S.E. of at least three independent determinations.
ibility of the GCLM gene was solely mediated by the −345:−209 fragment of the promoter, a mutant transgene in which the region between −348:−183 was deleted (−1927/GCLM5−luc) was engineered. Transfection of the −1927/GCLM5−luc transgene into HepG2 cells completely eliminated the induction of reporter gene expression following tBHQ exposure.

The −345:−209 promoter fragment contains both an ARE (−302:−292) and an AP-1 site (−341:−335), suggesting that one or both elements may mediate tBHQ induction as reported previously for β-NF (8). To examine the functional capabilities of these elements further, mutations in the ARE (m1, m2), upstream AP-1 site (m4), or both sites (m1m4, m2m4) were introduced into the wild-type −1927/GCLM5−luc transgene (Fig. 2A). When either the ARE (m3, m4, m3m4) or the upstream AP-1 site (m1, m2) were left intact, full induction (2−2.5-fold) of the transgene by tBHQ was retained. Induction was only lost when both sites were introduced simultaneously (m1m4, m2m4). Mutation of the consensus AP-1 site contained within the GCLM ARE (m1, m3, m1m4, and m3m4) did not significantly influence induction of reporter gene expression. These outcomes were entirely consistent with our previous reports with β-NF (8).

Because the mutations of the ARE and AP-1 sites used by Galloway and McLellan (16) differed slightly from the mutations used for our studies, transgenes replicating the ARE mutant (mA1), AP-1 mutant (mA3), and ARE/AP-1 double mutant (mA1:3) (Fig. 3A) used by these authors were created and transfected into HepG2 cells. These new mutants are similar to the m2, m4, and m2m4 transgenes, respectively, used in our previous study except for the specific base substitutions used to disrupt the AP-1 and ARE sequences (compare Figs. 2A and 3A). Like their counterparts (the m2 and m4 mutants) mA1 and mA3 were fully inducible by tBHQ (Fig. 3B) as reported previously (16). So, too, was the double mutant mA1:3. This observation replicated Galloway and McLellan’s (16) experience with the double mutant but differed dramatically with the m2m4 double mutant, which failed to respond to tBHQ exposure (Fig. 2B). Thus, these experiments confirmed the previously reported results from each laboratory, suggesting that the very
slight differences between the m2m4 and mA1:3 mutants are sufficient to either confer or ablate inducibility.

**The GCLM ARE Supports Induction by tBHQ**—Based on previous analyses of GCLM promoter functions, elements capable of supporting increased expression in response to β-NF or tBHQ were proposed to be the ARE (−302 to −292), the upstream AP-1 site (−341 to −335), and a 42-bp region immediately upstream of the ARE (−344 to −303) (16). To further delineate the role of these three elements in induction of the GCLM gene, a series of additional promoter/reporter transgenes were developed and transfected into HepG2 cells. Two new transgenes, each containing the GCLM ARE sequence, the first terminating at the 3′-limit of the core ARE sequence (−302; Fig. 4A) and the second including an additional 10 bp of 3′-flanking sequence from the GCLM promoter (−312) were generated by deleting the GCLM promoter sequence further upstream. Although the longer of the two transgenes (−312) supported higher levels of basal expression (Fig. 4B), both were induced by tBHQ treatment. Introduction of a single base mutation within the consensus ARE core (−312m) completely eliminated tBHQ responsiveness. Collectively these data demonstrate that the GCLM ARE is capable of directing increased expression following exposure to this agent.

**Induction Following tBHQ Does Not Involve the Upstream AP-1 Sequence**—The 42-base pair fragment (−344 to −303) immediately upstream of the ARE sequence, but not the AP-1 site contained therein, has been hypothesized to mediate induction of the GCLM gene (18). Expression of two enhancer/reporter transgenes (−346 to −303 and −345 to −315, Fig. 5A) containing this region of the promoter was induced (3-fold) by exposure to 100 μM tBHQ (Fig. 5B). However, three different AP-1 enhancers, each including an intact AP-1 site and variable lengths of flanking sequence (−346 to −334, −344 to −329, and −346 to −324), failed to respond to tBHQ by directly increased reporter expression, demonstrating that the AP-1 sequence present in the 44 bases upstream of the GCLM ARE (sequence of synthetic oligonucleotides corresponding to enhancers of various lengths between −346 and −303 of the GCLM promoter region. Oligonucleotides were annealed, phosphorylated, and ligated into the Smal site of the pT81-luciferase vector. Two different mutations of the AP-1 site in the 44-bp enhancer/reporter transgene (arrowheads and lowercase letters) were also ligated into the pT81 vector. The various enhancer/reporter constructs were transiently transfected with pCMVβ into HepG2 as done for Fig. 2. Cells were incubated in media containing Me2SO or 100 μM tBHQ dissolved in Me2SO for 18 h prior to harvest. Results are reported as means ± S.E. of at least three independent transfection experiments.

**Identification of a Variant ARE**—These enhancer studies suggest that the −345 to −315 region of the GCLM promoter includes one or more response elements other than the AP-1 site capable of supporting full induction of the gene, independently of the downstream GCLM ARE. However, sequence analysis failed to identify any candidate cis-elements within this region. When the sequence of the 44-bp fragment from −346 to −303 was aligned with the expanded consensus ARE motif defined by Wasserman and Fahl (21), a remarkably strong match was identified (Fig. 6A). The region of the GCLM promoter between positions −337 and −318 satisfies the ARE core motif requirements with a single exception, a T instead of a G at position −330. Although the alignment depicted in Fig. 6A
suggests an additional departure in the 3'-end of the expanded consensus ARE sequence, Wasserman and Fahl (21) determined that a minimum of three of the last four positions (wwww) needed to be occupied by either an A or a T, with no particular requirement for specific order. The 3'-end of the 337:318 fragment therefore satisfies this requirement.

To evaluate the possibility that the 337:318 fragment might function as an ARE despite this single base difference with the defined ARE consensus, the T at 330 within the 345:315 enhancer was mutated to an A, a nucleotide previously shown to ablate ARE function (22, 23). The T(330)A mutation completely eliminated induction by tBHQ (Fig. 6B). To complete the analysis, T3G and T3C mutations were introduced at the 330 residue in the 345:315 enhancer. The T(330)G enhancer was capable of directing a 4-fold greater constitutive expression relative to the wild-type sequence but was similarly responsive (~2.5-fold) to induction by tBHQ. The T(330)C enhancer lacked both constitutive and inducible activity. These studies suggested that the wild-type 337:318 sequence constitutes a functional ARE and that the TTAC motif was less potent at directing constitutive expression than the TGAC motif but that either motif was capable of directing full induction following exposure to tBHQ. Because the GCA-box at the 3'-end of the core ARE sequence has also been shown to be essential for ARE function, we mutated the GCA in the wild-type 345:315 enhancer to TTT and eliminated basal and inducible properties.

To further test the hypothesis that the TTAC may function as an ARE, we introduced the same series of mutations described above into an enhancer/reporter vector containing the ARE from the human NQO1 gene, transfected the vectors into HepG2 cells, and examined luciferase expression following exposure to tBHQ. As expected, the wild-type hNQO1 enhancer directed constitutive expression of the luciferase gene (Fig. 6C). Expression was induced ~2.5-fold by tBHQ. Substitution of an A or C for G in the TGAC core motif eliminated both the basal and inducible responses. Interestingly, however, the G3T mutation, rather than ablating ARE function, resulted in an enhancer with reduced basal expression but full inducibility. As was true for the wild-type 345:315 enhancer, mutation of the hNQO1 GCA-box eliminated basal and inducible enhancer activity.
FIG. 7. Wild-type GCLM −345:−315 probe can compete with the hNQO1 ARE for protein binding. Nuclear extracts were prepared from HepG2 cells exposed to 0.1% Me2SO or 100 μM tBHQ for 18 h. Synthetic oligonucleotides containing the hNQO1 ARE sequence were annealed, endlabeled, and incubated with 10 μg of nuclear extract. Extracts were then separated by gel electrophoresis, and gels were visualized using a Molecular Dynamics PhosphorImager. Prior to electrophoresis some extracts were incubated with the labeled hNQO1 probe as well as unlabeled probes as indicated at 100-fold excess (lanes 3–8).

Because the TTAC motif retained ARE function, albeit reduced relative to the properties of the TGAC sequence, the ability of the wild-type −345:−315 sequence and each of the three T−330 mutants to compete for binding of proteins to an hNQO1 ARE probe in electrophoretic mobility shift assays were compared. Unlabeled probes corresponding to the hNQO1 and GCLM AREs both effectively competed with the labeled hNQO1 probe for protein binding following tBHQ exposure (Fig. 7; compare lanes 3 and 4 with lane 2). The GCLM −345:−315 TGAC and TCAC mutants were ineffective as competitors (lanes 6 and 8). Not surprisingly the effectiveness of the TGAC mutation was comparable with the two consensus ARE probes (lanes 3, 4, and 7). The wild-type −345:−315 GCLM sequence (TTAC) partially competed with the hNQO1 probe (lane 5 versus lanes 6, 7, and 8).

DISCUSSION

Two independent investigations have demonstrated that constitutive expression of the human GCLM subunit gene is mediated by an AP-1 site located at −341:−335 in the promoter of the gene (8, 16). Additional sequences upstream of the AP-1 site also contribute to maximal basal expression (8). Both studies also mapped elements responsible for induction of the GCLM gene to a common region of the GCLM promoter, between nucleotides −345 and −209 in one case and between −411 and −256 in the other. The region of overlap, −345 to −256, contains a consensus ARE as well as the AP-1 site involved in directing basal expression. The functional relevance of this region of the promoter was confirmed in the present study by loss of reporter gene inducibility in cells transfected with the −1927/GCLM5Δ−348:−183 deletion mutant (Fig. 1). Although both studies localized inducible trans-activating activity to this common region, the conclusions about the role of the AP-1 and ARE sequences in inducible expression of the gene differed significantly. Our laboratory reported that either the ARE or upstream AP-1 sites was sufficient to support induction, whereas Galloway and McLellan (16) concluded that induction was mediated by a 42-bp region immediately upstream of the ARE, including nucleotides comprising the AP-1 site but not the AP-1 site per se.

Paradoxically, the results summarized in Figs. 2 and 3 confirm the previous observations from both laboratories. From Fig. 2 it is obvious that elimination of induction requires simultaneous mutation of both the ARE and AP-1 sites (m1m4 or m2m4), suggesting that either is capable of supporting induction independently of the other, as we reported previously. In contrast, the double mutant (mA1:3) employed by Galloway and McLellan (16) continued to support inducible expression (Fig. 3) as had been reported, suggesting that neither element was required for induction. These results, therefore, failed to unequivocally resolve the question regarding whether the ARE and AP-1 sites are involved in GCLM induction in response to these two agents.

To better define possible roles for these cis-elements in GCLM gene regulation the properties of promoter/reporter transgenes or enhancers containing only one or the other of the two sequences were evaluated. The −302/GCLM5−luc and −312/GCLM5−luc promoter/reporter transgenes (each containing the GCLM ARE at −302:−292) supported basal expression and full induction of the luciferase gene following tBHQ exposure (Fig. 4). A single base mutation disrupting the consensus ARE sequence abolished this response, firmly establishing that the GCLM ARE is capable of mediating induction. Favreau and Pickett (23) demonstrated previously that nucleotides contiguous to the 3′-end of the minimal ARE sequence, particularly multiple adenines, exert a profound enhancing effect on basal expression, as was observed for the −312/GCLM5−luc transgene (Fig. 4B). Furthermore, the elimination of 3′-flanking sequences decreased tBHQ induction by −30% as described by Wasserman and Fahl (21). Hence, the GCLM ARE at −302:−292 exhibits all of the functional characteristics typical of an ARE.

Assessment of the potential role of the AP-1 site using a series of enhancer/reporter constructs spanning the 44-bp region between −346 and −303 (upstream of the ARE; Fig. 5) clearly indicates that induction by sequences upstream of the ARE does not involve the AP-1 site per se, contrary to our earlier conclusion. This is clearly evident by the failure of the −346:−334, −344:−329, and −346:−324 enhancers, each containing the intact AP-1 motif, to respond to tBHQ exposure. Although the introduction of the specific AP-1-disrupting mutation (−346:−303m; TGAATAA to gGAGTAA) into the inducible −346:−303 enhancer completely abolished the responsiveness of the enhancer, in aggregate the data support Galloway and McLellan’s (16) conclusion that the 42-bp fragment immediately upstream of the ARE can support induction of the GCLM gene but does not involve the AP-1 site as such.

We also examined the functional consequences of the specific AP-1-disrupting mutation (−346:−303mA3; TGAATAA to cGAGTAA) examined by Galloway and McLellan (16). Even though this double base mutant and the single base −346:−303m mutant described above each contained a mutation at nucleotide position −341 (see Fig. 5A), the mA3 mutant enhancer/reporter was still inducible by tBHQ (Fig. 5B), in contrast to the single base mutant. Why two strikingly similar mutants would manifest such profoundly different consequences is currently unknown. However, the failure of the mA3
mutation to ablate induction following tBHQ exposure provides an explanation for the paradoxical results reported in the two original publications describing GCLM promoter characterizations. Galloway and McLellan (16) based their conclusion that induction of the GCLM gene was not regulated by the ARE at −302 to −292 largely on the observation that the GCLM promoter mutant (mA1:3) containing a mutation of the ARE (mA1) as well as the AP-1-disrupting mA3 mutation continued to support induction following tBHQ and β-NF exposures. However, as shown in the current study, the specific base substitutions employed to create the mA3 mutant are of little functional consequence in and of themselves. Hence, the mA1:3 mutant was functionally not a double mutant; it retained an inducible upstream element. In contrast, the current enhancer studies confirm that both inducible activities were functionally disrupted in the mA1:4 mutant used by Moinova and Mulcahy (8). The functional difference between these two mutants therefore accounts for the most significant difference in the findings reported in the two previous reports.

The enhancer studies summarized in Fig. 5 were also particularly informative for identifying the cis-element(s) within the 44-bp −346 to −303 region of the promoter that is operative in induction of the gene, enabling functional mapping of potential regulatory sites to the region between nucleotides −345 and −315. Subsequent analyses revealed that this region of the promoter comprises a previously unrecognized functional ARE spanning nucleotides −337 to −318 (Fig. 6). Prior recognition of this site as an ARE was masked by the presence of a T instead of a G at position −330 within the sequence, which otherwise satisfies the expanded ARE consensus (Fig. 6) as defined by Wasserman and Fahl (21). Designation of this sequence as an ARE, despite the single base difference with the core motif, is supported by the otherwise high degree of homology with the expanded ARE consensus sequence, by the disruption of function following introduction of a specific single base mutation (5′-TGAC-3′ to 5′-TAAC-3′) known to abolish ARE function (22), by the observation that mutation of the TGAC motif in the prototypic hNQO1 ARE to TTAC preserves ARE function (albeit with lower basal potency), and by the ability of the TTAC-containing sequence to partially compete with the hNQO1 ARE sequence for protein binding (Fig. 7).

Prior to the current study, the G within the 5′-TGACnnnGCA-3′ core consensus ARE motif was considered to be an invariant requirement for basal and inducible activity of AREs. In fact, following a comprehensive analysis of ARE sequence requirements, Favreau and Pickett (23) concluded that the TGA sequence within the 5′-TGACnnnGCA-3′ was absolutely required for high basal and inducible expression. However, in defining the sequence requirements for functional AREs, all previous studies only considered the influence of a substitution of an A for the G at this position within the TGA sequence. Although this particular substitution was shown to ablate induction following exposure to various ARE-inducers, the functional consequences of a T or C substitution were not previously considered, leaving open the possibility that an operative ARE could accommodate either of these nucleotides at this position within the ARE core motif. Our findings with both the GCLM ARE at −337 to −318 and the hNQO1 ARE demonstrate that substitution of an A or C for the G residue in the core ARE sequence (5′-TGACnnnGCA-3′) eliminates ARE function. More importantly, the sequence 5′-TTACnnnGCA-3′ supports ARE function. The two functional ARE sequences are not, however, functionally equivalent with respect to constitutive expression; the 5′-TTACnnnGCA-3′ motif is only −25% as effective as an enhancer of basal expression. Nevertheless, in terms of gene induction following exposure to tBHQ the two sequences are equipotent.

Based on the studies described in this report we conclude that the GCLM promoter contains two adjacent AREs (Fig. 7A), each capable of supporting induction of the gene in response to tBHQ, β-NF, and most likely other Phase II enzyme inducers. It is also important to note that residues flanking the variant ARE must also contribute in some way to GCLM gene induction because site-directed mutagenesis of position −341 effectively eliminated induction by the −346 to −303 promoter fragment (Fig. 5).

The identification of the variant ARE rationalizes differences reported in the two previous analyses of GCLM promoter function because this sequence is contained within the functional 42-base pair fragment identified by Galloway and McLellan (16). In addition to resolving issues specifically related to the control of expression of the human GCLM gene, these studies further demonstrate that the consensus ARE core sequence should be revised to 5′-RTKAYnnnGCR-3′. Such revisions of the consensus sequence will undoubtedly culminate in identification of additional ARE-regulated genes.

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Aileen M. Erickson, Zulimar Nevarea, Jerry J. Gipp and R. Timothy Mulcahy

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