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Identification and Characterization of an Nrf2-Mediated ARE Upstream of the Rat Glutamate Cysteine Ligase Catalytic Subunit Gene (*GCLC*)

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ABSTRACT

The antioxidant response element (ARE) is an essential component of upstream regulatory sequences present on genes for most phase II detoxification enzymes, including the glutamate cysteine ligase catalytic subunit (GCLC). NF-E2-related factor 2 (Nrf2) is a principal transcription factor that binds to the ARE and plays a key role in cellular responses to stress via the Keap1-Nrf2-ARE pathway. However, the ARE that mediates human GCLC gene expression has not been found in the rat. Thus, how the ARE-mediated Keap1-Nrf2-ARE pathway regulates glutathione homeostasis in the rat remains a puzzle. We have identified a putative ARE sequence ~4 kb upstream in the rat *GCLC*. We further defined the rat GCLC-ARE in the category with the most ARE characters, that is, this rat GCLC-ARE is a sequence-specific site that significantly enhances promoter activity in reporter genes. The rat GCLC-ARE is an Nrf2-mediated element to which binding has been demonstrated in nuclear extracts and induced by *tert*-butylhydroquinone. Given the central role that rat models play in toxicology and pathology, this first discovery of the rat GCLC-ARE enhancer similar to that found in the human gene has broad implications for the study of antioxidant defenses and their regulation in a number of different fields. J. Cell. Biochem. 107: 944–954, 2009.

KEY WORDS: GCLC; RAT; ARE/EpRE; Nrf2; GSH; TRANSCRIPTION; AP-1

The antioxidant response element (ARE), which is now generally referred to as the electrophile response element (EpRE) [Dickinson et al., 2004], regulates the expression and coordinated induction of genes for many phase II detoxifying enzymes in response to antioxidants and xenobiotics [Dhakshinamoorthy et al., 2000; Jaiswal, 2004]. The ARE "core" sequence was initially proposed to be 5′-RTGACnnnGC-3′ [Rushmore et al., 1991] and then further extended to 5′-TMAnnRTGAYnnnGCRwwww-3′ [Wasserman and Fahl, 1997]. Following a series of comprehensive mutational analyses of the mouse NADPH dehydrogenase quinone1 (NQO1) gene, Nioi et al. [2003] suggested that the element was 5′-gagTcACaGTgAGtCggCAaaatt-3′. Although the ARE sequence usually contains an embedded Activator Protein-1 (AP-1) element, it has been shown that the ARE is unique and responds independently from AP-1 [Xie et al., 1995]. Many basic leucine zipper (bZip) proteins, such as AP-1, ATF/CREB, Mafs, as well as cap'n'collar (CNC) proteins, which are homologous to the Drosophila "cap'n'collar," including NF-E2-related factors, p45NFE2, and Bach, have been implicated in the regulation of the ARE. However, the underlying mechanism for ARE activation appears consistent with a predominant role for Nrf2, which is essential for the positive regulation of ARE-mediated gene expression [Venugopal and Jaiswal, 1996; Itoh et al., 1997]. Nrf2 is normally localized in the cytoplasm and bound to the cytoskeleton-associated protein Keap1 [Itoh et al., 1999]. Upon stimulation by electrophiles, such as a phenolic "antioxidant" *tert*-butylhydroquinone (TBH) [Li et al., 2002], Nrf2 is released from Keap1 and translocated into the nucleus.

Unlike the AP-1 family of transcription factors that can bind to either the AP-1 site, also known as the PMA-responsive element (TRE), or ARE site, the Nrf2 transcription factor is reported to only bind to the ARE and is unable to form a homodimer with itself.

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Therefore, Nrf2 forms heterodimers with other bZip proteins in order to achieve ARE-binding. As a consequence of different pairing of proteins, the heterodimer binding to the ARE can either enhance or inhibit gene expression. For example, the Nrf2-small Maf heterodimer activates the transcription of many cytoprotective genes through the ARE [Motohashi et al., 2004]. Heterodimers of Nrf2 with Jun proteins (c-Jun, Jun-B and Jun-D) act to up-regulate ARE-mediated expression [Jeyapaul and Jaiswal, 2000] whereas heterodimers of Nrf2 with Fos proteins, such as c-Fos and Fra-1, negatively regulates ARE-mediated gene expression [Venugopal and Jaiswal, 1996; Wilkinson et al., 1998]. Small Maf (Maf F, G, and K) proteins lack a transcriptional activation domain, and Maf–Maf homodimers repress ARE-mediated gene transcription [Dhakshinamoorthy and Jaiswal, 2000; Nguyen et al., 2000]. In addition, Bach1 competes with Nrf2 to dimerize with Maf leading to the down-regulation of the ARE [Dhakshinamoorthy et al., 2005]. It appears that the differences in the expression of signaling components and in the metabolism of different cell types results in significant heterogeneity in the cells' response to the same compounds [Dickinson et al., 2003].

GCLC is the catalytic subunit of the rate-limiting enzyme in GSH synthesis. The induction of human *GCLC* gene can be mediated by either ARE or AP-1 elements, which are both located in its 5′-flanking region [Jardine et al., 2002; Dickinson et al., 2003]. Deletion mutagenesis studies of the human *GCLC* gene promoter identified two distal AREs between positions −3802 and −2752 [Mulcahy et al., 1997] which are far from several proximal AP-1/TRE sites. However, the AREs which are present in the 5′-flanking region of the human *GCLC* genes were not found in the rat *GCLC* promoter. Yang et al. [2001] cloned and characterized a 1.76 kb 5′-flanking region of the rat *GCLC* (GenBank accession number AF218362), but no ARE was found. They only found AP-1 sites that are required for constitutive induction of *GCLC* gene expression by TBH [Yang et al., 2002]. Recently, these investigators reported that Nrf1 and Nrf2 indirectly regulated TBH-induced rat *GCLC* gene expression through modulation of the expression of key AP-1 and NF-kB family members [Yang et al., 2005b]. Acknowledging the genetic conservation between human, mouse and rat species, it is unlikely that the rat *GCLC* gene has a separate regulatory mechanism without an ARE. Since many *cis*-acting enhancers can act anywhere in the gene [Macfarlane, 2000], the AREs may not necessarily be located within the promoter region. For example, an *AKRIC2* ARE was recently found to be 5.5 kb upstream of the open reading frame (ORF), far away from its related gene [Lou et al., 2006].

Based on a search for an ARE candidate sequence in the 5′-flanking region of rat *GCLC* gene, we identified an ARE-like sequence about 4 kb upstream of the GCLC-ORF. To determine whether this "GCLC-ARE" sequence influences *GCLC* gene expression, we characterized its function through the gene reporter assay, Chromatin Immunoprecipitation Assay, and Electrophoretic Mobility Shift Assay. We demonstrate for the first time an indispensable role of this "GCLC-ARE" in the Nrf2-mediated regulation of rat *GCLC* gene expression. Given the central role that rat models play in toxicology and pathology, this is important information for researchers studying the role of antioxidant defenses and their regulation in a broad range of fields.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENTS

Explanted vascular smooth muscle cells (VSMC) were isolated from a group of F344 rats (24 months) and grown in Dulbecco's Modified Eagle Media (DMEM), supplemented with 10% (v/v) FBS and 100 unit/ml penicillin/streptomycin (PS) (Invitrogen, Carlsbad, CA), as previously described [Li et al., 2006a]. Cells were treated with TBH (Sigma–Aldrich, St. Louis, MO) at 40, 80, 120, and 160 μM for 4 or 16 h, respectively.

WESTERN BLOTTING

The antibody for GCLC was purchased from Neomarkers (Fremont,CA), for β-actin from Abcam (Cambridge, MA). Cytoplasmic proteins were extracted from VSMC after each of the treatments. Twenty micrograms of cytoplasmic protein were electrophoresed on 10% SDS–PAGE gels and then electroblotted onto nitrocellulose membranes, as described previously [Li et al., 2006b]. The images were captured with a GS-700 Imaging Densitometer (Bio-Rad, Richmond, CA) and analyzed with the Quantity One Software Version 4.2 (Bio-Rad).

PLASMID CONSTRUCTION

Four reporter plasmids were constructed by inserting selective fragments into the corresponding pGL3-luciferase reporter vectors (Promega, Madison, WI). All insertions were confirmed by nucleotide sequencing.

The double-stranded oligonucleotides (oligos) containing the rat GCLC-ARE enhancer (underlined), shown below, was inserted into a pGL3-promoter vector at *Sma*I site using the in-Fusion Cloning kit (Clontech, Mountain View, CA):

5′-GCCAGCCCGCGCAGAGTGTTGAGTCACGGTGAGGCGGCAC-3′
3′-CGGTCGGGCGCGTCTCACAACTCAGTGCCACTCCGCCGTG-5′

The oligos with three nucleotides deleted (in bold above) was also inserted into a pGL3-promoter at *Sma*I site:

5′-GCCAGCCCGCGCAGAGTGTTGAGCGGTGAGGCGGCAC-3′
3′-CGGTCGGGCGCGTCTCACAACTCGCCACTCCGCCGTG-5′

The additional 15-bases on the 5′-end of each strand were designed to be homologous to the vector sequences for infusion and are not shown above.

A 1.76-kb fragment of the full rat GCLC-promoter (−1756 to +2) was PCR-amplified using the following primer set:

Full-F1: 5′-GGAGAATCTCCAGCATCCAG-3′
Full-R2: 5′-CATGGCCGCGTCCTCCTCCT-3′

This fragment was inserted into a pGL3-basic vector at *Sma*I site.

A 170-bp ARE-containing fragment within the 5′-flanking region of *GCLC* from −4061 to −3892 was also PCR-amplified using the primer set:

GCLC-ARE-F: 5′-CACAGGATCTCAGCGAAG-3′
GCLC-ARE-R: 5′-TCACCGTGACTCAACACT-3′

and inserted into above constructed pGL3-basic plasmid carrying on a GCLC-promoter between *Sac*I and *Mlu*I sites.

TRANSIENT TRANSFECTION AND REPORTER ASSAYS

VSMC grown overnight in 12-well plates at a density of $2\text{-}3 \times 10^5$ per well were co-transfected in triplicate with 1.6 μg of each pGL3-luciferase reporter together with 0.1 μg of pRL-TK, an expression plasmid encoding *Renilla* luciferase (Promega). In some cases, 1.6 μg of Nrf2 expression vector, described previously [Chan and Kwong, 2000], was also included in the co-transfection. The transfection was delivered by Lipofectamine™ 2000 transfection reagent (Invitrogen). After 24 h, the cells were refreshed with normal medium for another 24 h; or alternatively, further treated with 160 μM of TBH for 16 h. The cells were then lysed with 150–200 μl/per well of 1 ×Passive Lysis Buffer (Promega). The luciferase gene activity (luc-activity) was measured using the Dual-Luciferase reporter assay system kit (Promega) using a Lumat LB 9507 Luminometer (Berthold, Bad Wildbad, Germany), to assess firefly and *Renilla* luciferase activity sequentially.

CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

VSMC histones were natively cross-linked to DNA by adding 1% formaldehyde directly to the culture medium for 15 min at 37°C. The ChIP Assay Kit was from Upstate (Chicago, IL). Cells were lysed with 200 μl per 1×10^6 of SDS lysis buffer. Cell lysates were then sonicated with a 600-watt ultrasonic processor setting at 30% of maximum power to shear the DNA in a range of 200 to 1,000 bp. Twenty microliters of chromatin in the supernatant of sonicated cell lysates was saved as input/starting material, while the rest of the supernatant chromatin was diluted in a 2 ml volume with ChIP Dilution Buffer and pre-cleaned by the Protein Agarose Slurry. The chromatin DNA–protein complexes were immunoprecipitated individually by 4 μg each of Nrf2 and c-Fos antibodies (Santa Cruz, CA), and then the chromatin proteins were dissociated from DNA through the procedures recommended by the manufacturer. In addition, anti-mouse IgG and anti-RNA polymerase II antibodies were included in the IP to process DNA for a negative control or a ChIP quality control, respectively. The purified ChIP DNA from Nrf2- or c-Fos- bound chromatin, together with controls, was finally used as templates to amplify the ARE-containing region in the 5′-flanking region of the rat *GCLC* or AP-1-containing region in *GCLC* promoter. The primer set used for the rat GCLC-ARE region was:

GCLCARE-F3: 5′-AGAGCTTCTTCTCGTCGGAA-3′ (NW_047799: 52340044)
GCLCARE-R2: 5′-CACGAATCAACGCTCAGCAA-3′ (NW_047799: 52340295)

The primer set for the ARE region in the rat *NQO1* promoter [Favreau and Pickett, 1995] was chosen as an efficiency-indicator for Nrf2 immunoprecipitation as follows:

NQOARE-P1: 5′-TGAAGAGACCCAAGCGTGTA -3′ (M58495: 541)
NQOARE-P2: 5′-CTGGCCTAGGACTTCTGTAT-3′ (M58495: 948)

The primer set used for AP-1 in the rat GCLC-promoter [Yang et al., 2001] was designed as:

GCLCAP1F: 5′-ACCACTGCTGGACGAATCTT-3′ (AF_218362:731)
GCLCAP1R: 5′-TGAATGTTGTGTGGCTCCAC-3′ (AF_218362:979)

The primer set spanning 100 bp of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in length, which was cross-linked to Acetyl Histone H3, was also designed as a quality control:

GAPDH-F: 5′-CATTGACCTCAACTACATGG-3′ (NM_017008)
GAPDH-R: 5′-TGACCAGCTTCCCATTCTCA-3′

Up to 1 μg template was added to a 50 μl PCR mixture containing 1.25 units Ampli Taq Gold (Applied Biosystem, Foster City, CA), 0.2 mM dNTPs, 1× PCR buffer with $MgCl_2$, and 200 nM of each primer. The PCR was run under the following conditions: 94°C 14 min for 1 cycle; 94°C 1 min, 68°C 1 min, 72°C 2 min for 14 cycles; 94°C 1 min, 55°C 1 min, and 72°C 2 min for 20 cycles; and a final extension at 72°C for 8 min.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Double-stranded oligos from the 5′-flanking region of the rat *GCLC* gene, containing the rat GCLC-ARE enhancer (underlined), were synthesized by Invitrogen as probe (NW_047799: 52339471):

5′-GCGCAGAGTGTTGAGTCACGGTGAGGCGGCACGG-3′
3′-CGCGTCTCACAACTCAGTGCCACTCCGCCGTGCC-5′

The same double-stranded oligos, but with a three-nucleotide deletion shown in bold above, was synthesized as GCLC-del-ARE (dARE); and double-stranded oligos containing the following human GCLC-ARE4 [Mulcahy et al., 1997] was also synthesized as an ARE consensus (underlined).

5′-GCACAAAGCGCTGAGTCACGGG-3′
3′-CGTGTTTCGCGACTCAGTGCCC-5′

The AP-1 and NF-kB consensus oligos were purchased from Promega. The oligonucleotide probes were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. EMSA and supershift EMSA were performed as described previously [Li et al., 2006a]. Antibodies for the Nrf2, c-Fos (specifically recommended for supershifts), c-Jun, and Mafs proteins were purchased from Santa Cruz. For competition experiments, the competitive double-stranded oligos were added at 100:1 molar ratio 15 min prior to the addition of labeled probes.

RESULTS

IDENTIFICATION OF A PUTATIVE ARE ON THE 5′-REGION OF THE RAT GCLC

The rat *GCLC* gene is located on chromosome 8 genomic contig (NW_047799). The locus spans 37286-bp in length, from nt. 52343716 to nt. 52381001. The nucleotide analysis of 6 kb upstream *GCLC* for ARE candidates revealed a single putative site around nt. 52339801, which is up to 20 bp in length and 3,915-bp upstream of the gene coding region (Fig. 1A). The sequence of one strand contains a potential ARE site in which 16 of 20 nucleotides match the human GCLC ARE4, while the sense strand clearly contains an embedded TRE/AP-1 site (Fig. 1B). This GCLC-ARE contained an A instead of the G in the common ARE core sequence 5′(TGA****GC) 3′; however, this single nucleotide variance was not as critical as previously expected, since it has been modified to a lower case "g" in the latest version of the core sequence [Nioi et al., 2003].

RESPONSIVENESS OF VSMC TO TBH AND Nrf2-OVEREXPRESSION

We have previously shown higher constitutive GCLC expression in VSMC explanted from old rats [Li et al., 2006a]. Changes in GCLC in these VSMC in response to 40, 80, and 160 μM of TBH-treatments for 16 h, or to transfection with the Nrf2 expression vector for 24 h were examined by Western blots using cytoplasmic proteins extracted from the cells (Fig. 2). Figure 2 shows that cellular GCLC protein was significantly increased with 40–80 μM TBH treatments ($P \leq 0.01$), reaching a 73% increase when exposed to 160 μM TBH ($P < 0.04$). The 50% induction of GCLC expression was also seen in VSMC transfected with the Nrf2 expression vector ($P \leq 0.01$). These data suggest that VSMC actively respond to electrophilic stress.

THE ENHANCEMENT OF REPORTER GENE ACTIVITY

Four reporter plasmids constructed by inserting selective fragments into the corresponding pGL3-luciferase reporter vectors are illustrated in a schematic representation in Figure 3. To verify whether this rat GCLC-ARE acts as an enhancer in the up-regulation of gene expression, a 40-bp double stranded oligo composed of the rat GCLC-ARE sequence was inserted into a pGL3-promoter luciferase reporter vector containing the SV40 promoter and named pGL3-promoter-ARE (Fig. 3A-1). The oligos with three nucleotides deleted was also inserted into a pGL3-promoter at *Sma*I site and named pGL3-promoter-del-ARE (Fig. 3A-2). To further determine if the GCLC-ARE had the same effect on the 1.76 kb of the GCLC promoter, two additional plasmids were constructed for that purpose. The pGL3-basic-full reporter was composed of the pGL3-basic with an insertion of the 1.76 kb GCLC-promoter (Fig. 3B-3). The other plasmid should be composed of the GCLC-ARE containing region together with the GCLC-promoter. From a review of the previous literature, we found that only those reporter plasmids with ARE-containing fragments alone could contribute to the clarification of ARE function. For example, the fact that the human GCLC gene is modulated by a distal ARE4 was determined by the plasmids with an isolated ARE4 insertion [Mulcahy et al., 1997]. In addition, an enhancer, as described previously, can operate from any position in the gene (upstream, downstream, or even in the coding region) because the transcription factors can bend and warp the DNA to "loop" until everything comes together in the correct position to activate transcription. This implies that enhancers operate independent of its position. The key feature of an enhancer is the enhancement of promoter capacity and efficiency [Blackwood and Kadonaga, 1998; Macfarlane, 2000]. Therefore, instead of the full length of the 2.3 kb region, a 170-bp PCR fragment containing only the rat GCLC-ARE enhancer was inserted into the pGL3-basic-full at the 5′-side of the GCLC-promoter, which we have named the pGL3-basic-full-ARE (Fig. 3B-4).

Transient transfection of VSMC with the constructed pGL3-promoter-ARE greatly increased luc-activity in comparison with the pGL3-promoter alone (5.6-fold, $P < 0.01$), as shown in Figure 4A. In addition, the luc-activity of the ARE-containing vector was higher than the luciferase reporter positive-control vector from Promega, which carries both the promoter and enhancer from SV40. The pGL3-promoter-del-ARE failed to increase luc-activity, suggesting that the integrity of GCLC-ARE is sequence-specific. The insertion of GCLC-ARE into the pGL3-basic-full also significantly increased luc-activity compared to the vector without the ARE ($P < 0.01$), as shown in Figure 4B. This provides support for the contention that the GCLC-ARE could enhance GCLC promoter function as it did for the SV40 promoter. Interaction with Nrf2 and the response to electrophiles are two important features of the ARE. To test these two features on the GCLC-ARE, transiently transfected cells with pGL3-promoter-ARE were either co-transfected with an Nrf2 expression vector or treated with TBH, 160 μM, for 16 h after transfection. The results showed that over-expression of Nrf2 significantly increased luc-activity of the pGL3-promoter-ARE than vehicle alone ($P < 0.05$) while 160 μM of TBH induced luc-activity to an even greater degree (5.9-fold, $P < 0.01$) than in untreated cells (Fig. 4C).

BINDING OF Nrf2 TO THE GCLC-ARE IN ChIP ASSAY

Binding of Nrf2 to the human GCLC ARE has been reported to mediate human *GCLC* gene induction [Wild et al., 1999; Dickinson et al., 2004]. If the rat GCLC-ARE does interact with Nrf2 and is involved in Nrf2-mediated *GCLC* expression, it should be possible to immunoprecipitate the ARE-containing DNA from cross-linking chromatin with the anti-Nrf2 antibody. In addition to Nrf2, c-Fos is another transcription factor known to bind to both ARE and AP-1 sites. Therefore, ARE-containing ChIP DNA was isolated using both antibodies. PCR was performed to amplify the GCLC-ARE containing region and compared with the AP-1 containing region. The rat NQO1 ARE was chosen as a positive control for Nrf2-ARE binding. The amplification results from the ChIP DNA are shown in Figure 5.

As shown in lanes 1 and 4 in Figure 5, the quality of the ChIP DNA immunoprecipitated by both Nrf2 and c-Fos antibodies was confirmed by the amplification of the rat NQO1 ARE-containing region. Rat GCLC-ARE (lanes 2 and 5) was also amplified from ChIP DNA generated with either Nrf2 or c-Fos antibodies, suggesting that both transcription factors are involved in the binding complex. However, the AP-1 containing band was only seen in the c-Fos generated ChIP sample (lane 6), but not in the Nrf2-generated one (lane 3), thus eliminating the possibility of Nrf2 binding to the AP-1 site.

In lanes 8 and 9 of Figure 5, ChIP DNA immunoprecipitated by RNA polymerase II resulted in the desired bands for the ARE or the

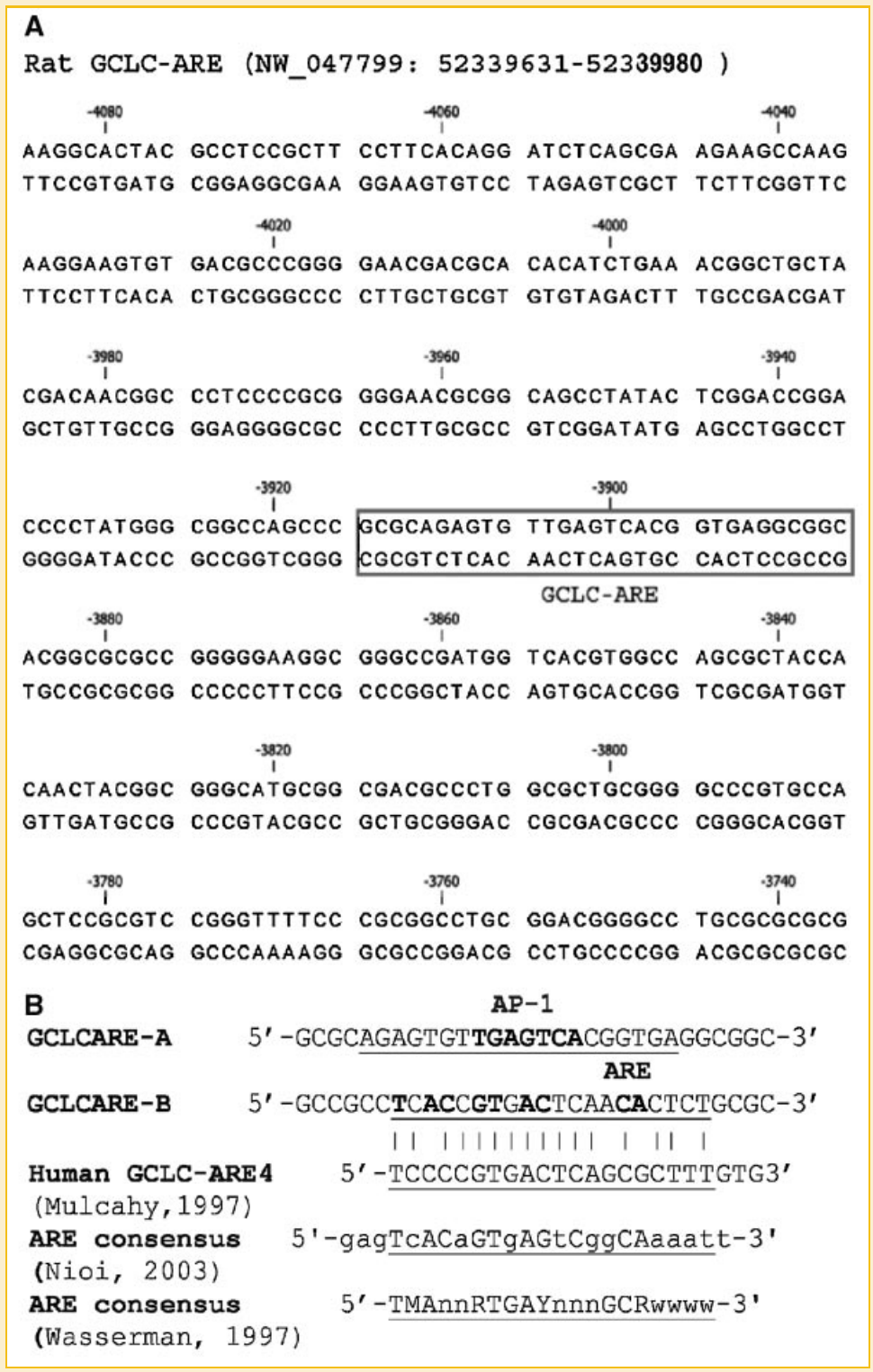

Fig. 1. Identification of the rat GCLC-ARE sequence. A: The top area presents a partial 5′-flanking region of rat *GCLC* gene located on chromosome 8 genomic contig (NW_047799: nt. 52339631–52340080). The boxed nucleotides indicate the position of the putative regulatory element. B: The two strands of the rat GCLC-ARE are shown in A (sense) and B (anti-sense). The ARE core sequences with internal AP-1/TRE motifs, specifically underlined, and the nucleotides matched to the human GCLC-ARE4 [Mulcahy et al., 1997] are denoted. The ARE consensus sequence from Nioi et al. [2003] and Wasserman and Fahl [1997] are listed at the bottom for comparison. The capital and lowercase letters denote the key and non-key nucleotides respectively, as described in the original publications.

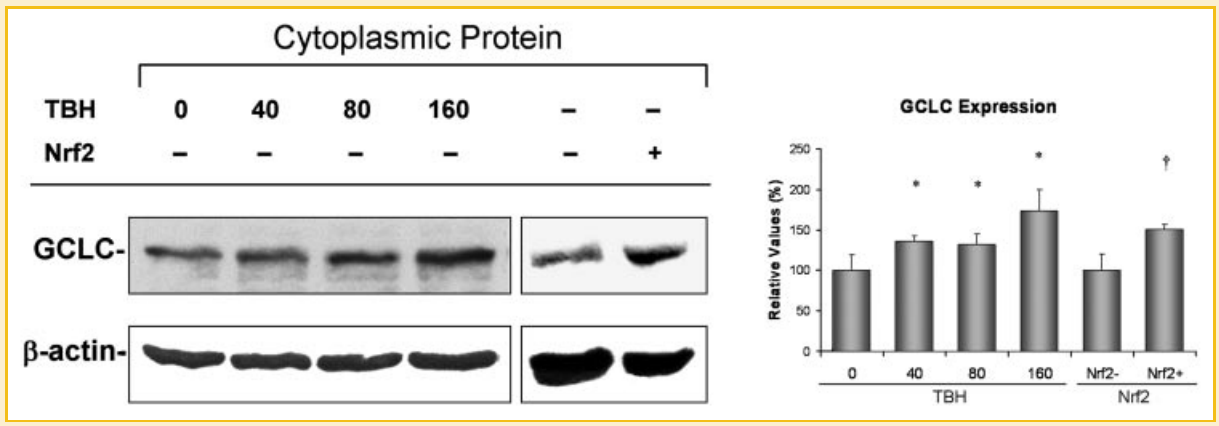

Fig. 2. Response of VSMC to TBH and Nrf2-overexpression. VSMC were treated with 40, 80, and 160 μM of TBH for 16 h, or transfected with 24 μg of pEF1/v5-Nrf2 per100 mm dish for 48 h and compared to the corresponding vehicle controls. Cytoplasmic proteins were extracted from cells. The representative Western blots for GCLC protein levels are shown in the left panel with β-actin as a loading control and the corresponding bar graph which summarizes data from 4 blots is shown in the right panel. "*" indicates $P \leq 0.05$ when comparing TBH treatments with vehicle control; "†" indicates $P \leq 0.05$ when comparing Nrf2-overexpression with vehicle control.

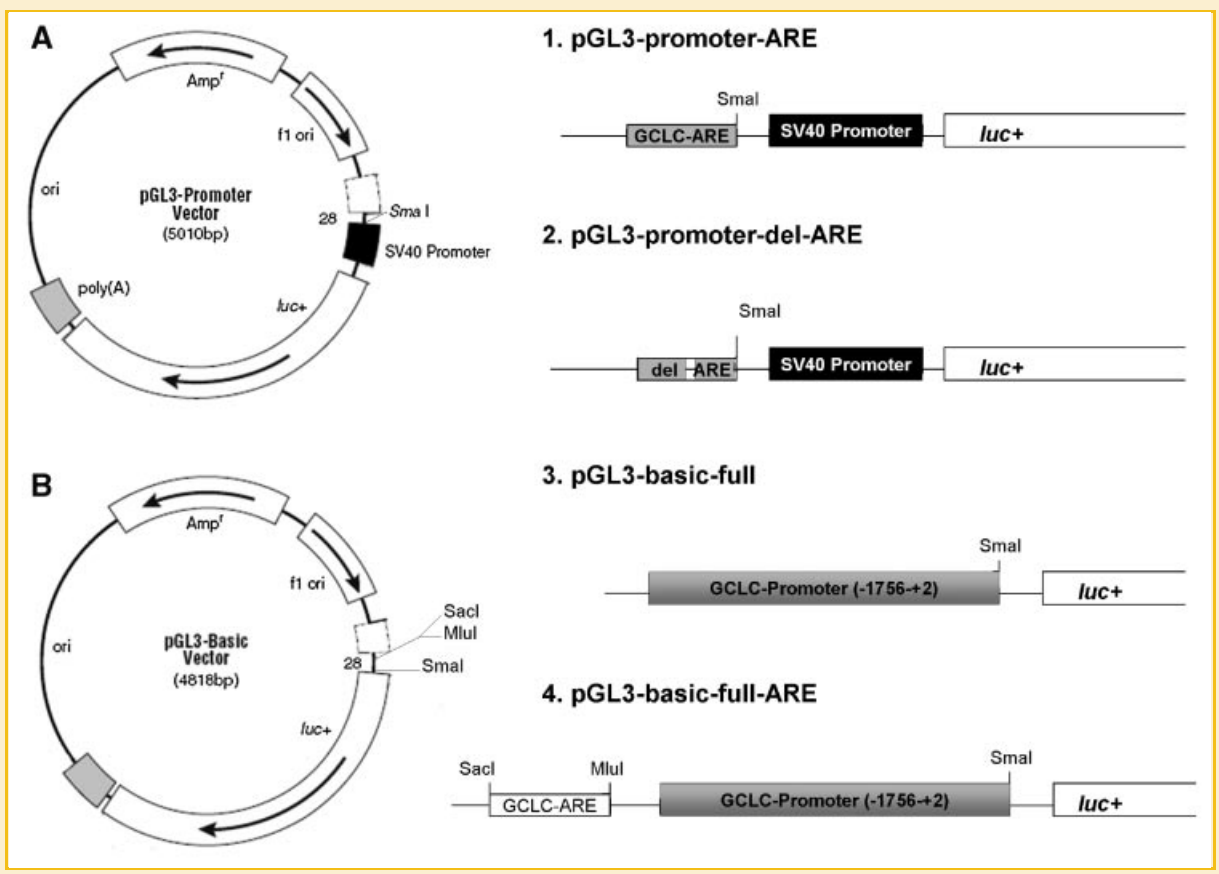

Fig. 3. A schematic representation of reporter constructs. A: The pGL3-Promoter Vector circle map (Promega) is shown on the left; the *Sma*I indicates the site where GCLC-ARE or del-ARE was inserted. The linearized enhancer/promoter/gene relationship between GCLC-ARE, SV40 promoter and luciferase gene is shown on the right. B: The pGL3-Basic Vector circle map (Promega) is shown on the left. The full size of GCLC-promoter was inserted into the indicated *Sma*I site of that reporter vector, while the 170-bp fragment containing GCLC-ARE was inserted between the *Sac*I and *Mlu*I sites. On the right, a linearized enhancer/promoter/gene relationship between GCLC-ARE, GCLC-promoter and luciferase gene is displayed.

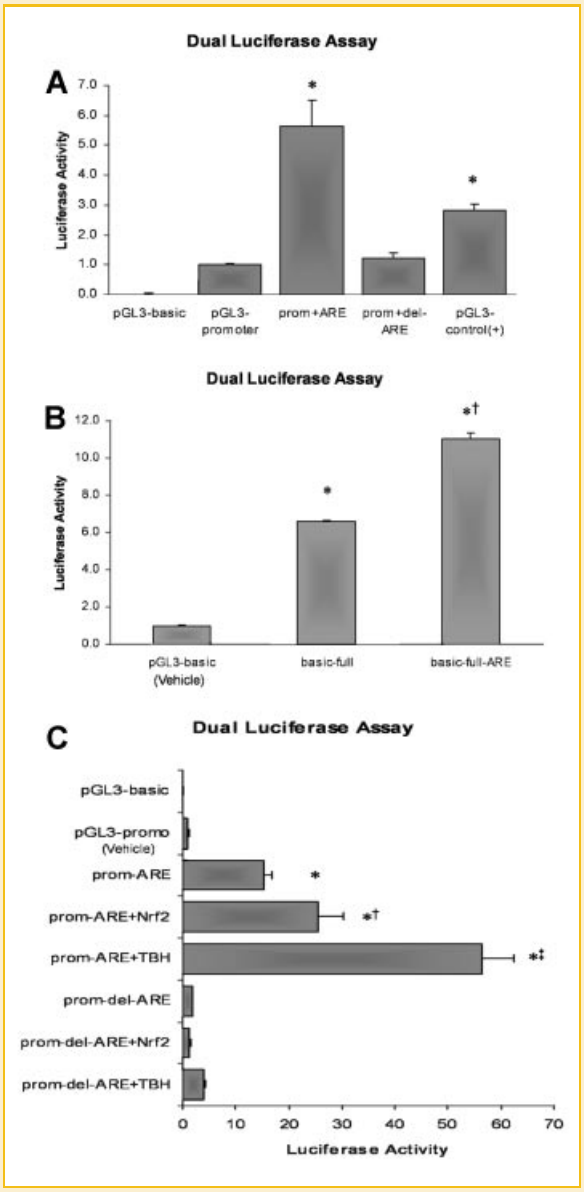

Fig. 4. Luciferase activities of reporter plasmids constructed with rat GCLC-ARE. Rat VSMC were transiently transfected with reporter genes for 24 h in triplicate and lysates measured for luciferase activities. The results are summarized as mean ± SE in each of the bar-graphs as follows: A: luc-activity for the pGL3-promoter–ARE, pGL3-promoter-del-ARE, and positive (pGL3-control +) control was each compared with a pGL3-promoter vehicle. Both the pGL3-promoter–ARE and positive control are significantly higher than the vehicle ($^{*}P < 0.01$). B: luc-activity for pGL3-basic-full was significantly higher than a pGL3-basic vehicle ($^{*}P < 0.01$); luc-activity for pGL3-basic-full-ARE was not only significantly higher than the pGL3-basic vehicle ($^{*}P < 0.01$), but also greater than the pGL3-basic-full reporter without ARE ($^{\dagger}P < 0.01$). C: luc-activities for treated or untreated pGL3-promoter–ARE (prom-ARE) were all greater than vehicle of the pGL3-promoter ($^{*}P < 0.01$). The luc-activities for Nrf2 co-transfected pGL3-promoter–ARE ($^{\dagger}P < 0.05$) or TBH treated pGL3-promoter–ARE ($^{\ddagger}P < 0.01$) were higher than untreated pGL3-promoter–ARE. Luc-activities for treated or untreated pGL3-promoter-del-ARE (del-ARE) show no differences.

GAPDH control (lane 10), which is consistent with that these sequence-specific DNA binding transcription factors constitute the core RNA polymerase II machinery [Kadonaga, 2004].

BINDING OF NUCLEAR PROTEINS TO RAT GCLC-ARE

Nuclear proteins were extracted from normal rat VSMC (control), VSMC treated with TBH for 16 h, or transfected with Nrf2 expression vector for 48 h, respectively. Binding of these nuclear proteins to the GCLC-ARE was detected by EMSA. The results are summarized in Figure 6. In Figure 6A, EMSA was performed using two [γ-^{32}P] ATP labeled probes to compare the binding complexes of the rat GCLC-ARE with the ARE consensus. Binding of nuclear proteins to the ARE consensus shows only a single strong band, but binding of the nuclear extracts to the GCLC-ARE consisted primarily of two bands: a major upper band, similar to the ARE consensus, and a faint lower band. To determine if both bands were related to the rat GCLC-ARE, we examined the response of nuclear proteins to the 16 h TBH treatments, as shown in Figure 6B. Both bands showed a dose dependent increase in binding activity, suggesting that both are GCLC-ARE binding complexes. The binding activities achieved a 1.36 ± 0.27-fold increase at 40 μM TBH; $1.49.47 \pm 0.15$-fold increase at 80 μM ($P < 0.05$) and 1.65 ± 0.17-fold increase at 160 μM ($P < 0.02$) relative to the vehicle control. Since small Maf proteins, only 18 kDa in size, are involved in ARE binding complex, it would not be unexpected if the relative smaller complexes with Mafs are observed.

In Figure 6C, different cold competitors were tested for their effects on GCLC-ARE binding. The NF-kB consensus in lane 2 did not show any effect on the GCLC-ARE hot probe, whereas, in lane 3, the AP-1 consensus does show competition with the upper band of the GCLC-ARE, which may result from the embedded AP-1 site. The human ARE4 (lanes 6 and 7) successfully attenuated rat GCLC-ARE binding in a dose-dependent manner. In contrast, the GCLC-del-ARE in lane 4, which lacks the three key nucleotides, failed to show any effect on GCLC-ARE binding.

We further examined binding of nuclear Nrf2 protein to the GCLC-ARE through supershift assays using the Nrf2 antibodies recommended for supershift assays from Santa Cruz. In lanes 1–3 of Figure 6D, the Nrf2 (C-20) antibody did not shift any Nrf2 bands in response to TBH treatments. In contrast, the Nrf2 (H-300) antibody displayed Nrf2-shifted bands induced by TBH treatments (lanes 4–6) as well as over-expression of Nrf2 (lanes 7–8). The Nrf2 C-20 antibody recognizes the C-terminal of Nrf2, which contains the DNA-binding domain and CNC/bZip domain. Hence, in the supershift assay, binding of Nrf2 protein to DNA may interfere with the affinity of the antibody to its recognition epitope. To the contrary, the anti-Nrf2 (H-300) antibody is raised against amino acids 37–336, which keeps the recognition region away from the DNA binding domain. We are uncertain whether this antibody has full affinity to be able to shift of all of the Nrf2 heterodimer because the band shift was not very strong. However, these data suggest that the source of antibody selected for a specific component may influence the detection of band shifts in the EMSA. For this reason, one cannot compare the quantitative assessment of the binding activities of different components in a complex.

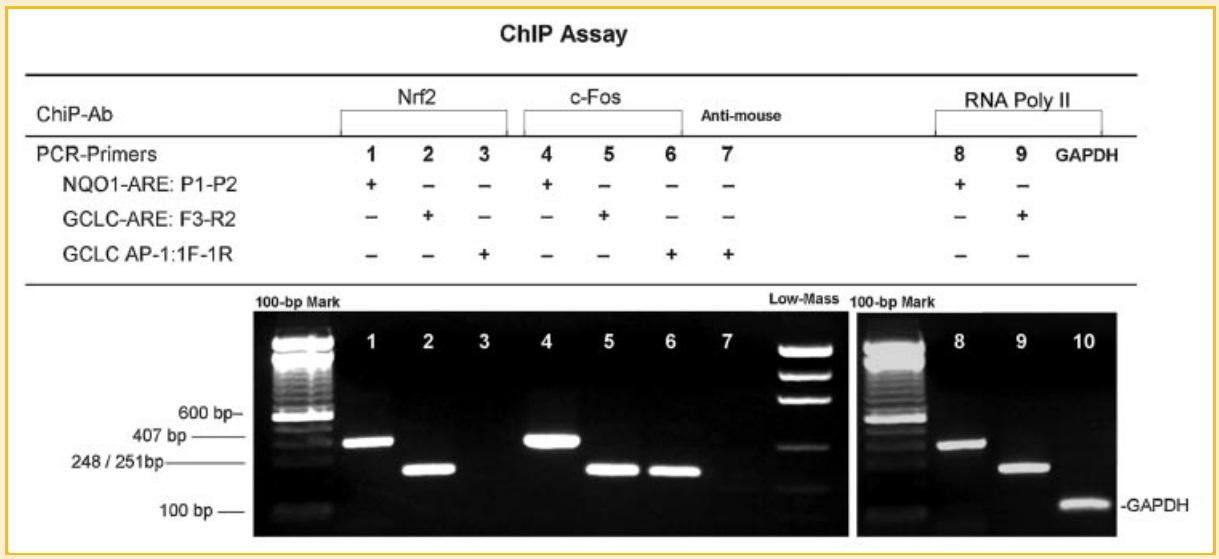

Fig. 5. ChIP assay for PCR amplification of rat GCLC-ARE. ChIP DNA was immunoprecipitated and purified from cross-linked chromatin. PCR-amplified bands are shown in groups with immunoprecipitated antibodies (ChIP Ab): Nrf2 (lanes 1–3), c-Fos (lanes 4–6), anti-mouse IgG (lane 7) or RNA polymerase II (lanes 8 and 9). The primer sets for NQO1-ARE (407 bp), GCLC-ARE (251 bp), GCLC-AP-1 (248 bp) and GAPDH (100 bp) containing regions are indicated on the right, respectively. Both the 100 bp marker and low-mass marker were included to determine the corresponding size of PCR products.

Finally, a set of EMSAs that compared the DNA binding activity between control and Nrf2 over-expression nuclear proteins is shown in Figure 6E. Over-expression of Nrf2 (lane 2 vs. 1) increased rat GCLC-ARE binding activity and therefore it was less efficient in the attenuation of the ARE4-competition when the same amount of competitor was applied (lane 4 vs. 3). The Nrf2-supershift shows that over-expressed Nrf2 strengthened the Nrf2-shifted band by 0.49 ± 0.08-fold ($P < 0.01$) relative to control and confirmed the effect of Nrf2 on the up-regulation of GCLC-ARE binding activity (Fig. 6E, lane 6 vs. 5 and Fig. 6D, lane 8 vs. 7). In addition, the supershift using antibodies to probe other ARE components are included in Figure 6E, lanes 7–12. The c-Fos antibody shifted almost the entire upper band of the ARE (lanes 7–8, Fig. 6E). The faint c-Jun band shift, which was strengthened by overexpressing Nrf2, was also observed using a c-Jun antibody that was nonspecific for the EMSA (lane 10 vs. 9). Furthermore, the supershift with a MafF/G/K antibody did not show any band shift, but rather appeared to attenuate the lower ARE-band (lanes 11–12).

Interestingly, the supershifted c-Fos band in nuclear proteins from Nrf2 over-expressed cells is weaker than control, directly opposite from the Nrf2 results. Similar results were observed after exposure of cells to the 16 h TBH treatments (Fig. 6F, lanes 1–4) that the c-Fos-shifted band was down regulated. These results support our previous findings that Nrf2 and c-Fos are competing with each other [Li et al., 2006a]. This is also consistent with a reported negative role of the c-Fos on ARE-mediated gene transcription, especially with respect to the over expression of c-Fos down-regulating ARE reporter gene activity [Venugopal and Jaiswal, 1996; Wilkinson et al., 1998]. In addition, the fact that the c-Fos antibody showed such a significant shift of the c-Fos heterodimer (i.e., almost all of the ARE upper band) raises the following questions: (1) If c-Fos binds to DNA through its DNA binding domain, is it complete, partial or not at all? (2) What is the c-Fos partner in the heterodimer? and (3) Is the c-Fos heterodimer a constitutional component in the ARE binding complex and down-regulated when the Keap1-Nrf2 pathway is activated? c-Fos is a multifunctional transcription factor that forms heterodimers with other members of the leucine zipper family to function independent of AP-1 [Sassone-Corsi et al., 1988; McBride et al., 2003; Seldeen et al., 2008]. The mechanisms underlying the pleiotropic effects of c-Fos remain largely undefined.

DISCUSSION

Cellular mechanisms for detoxification are receiving more attention because of the growing threats from environmental hazards that contribute to long-term negative health effects. Cellular responses to environmental stresses via the Keap1-Nrf2-ARE pathway play a key role in protecting cells from endogenous and exogenous stresses [Surh, 2003; Kensler et al., 2007]. It is also known that the Keap1-Nrf2-ARE pathway is a signaling pathway for maintaining human GSH homeostasis through the transcriptional modulation of GCLC expression. The AREs which are present in the 5′-flanking region of the human *GCLC* genes were not found in the 1.76 kb of rat *GCLC* promoter, raising the question of how the Keap1-Nrf2-ARE pathway could up-regulate rat GCLC transcription without an actual ARE present. Several efforts have been made to find the mechanisms responsible for the control of rat *GCLC* transcription [Yang et al., 2005a,b], but all of these investigations were related to other pathways rather than directly probing the Nrf2-ARE relationship. To answer this question, we searched the rat genome for the candidate

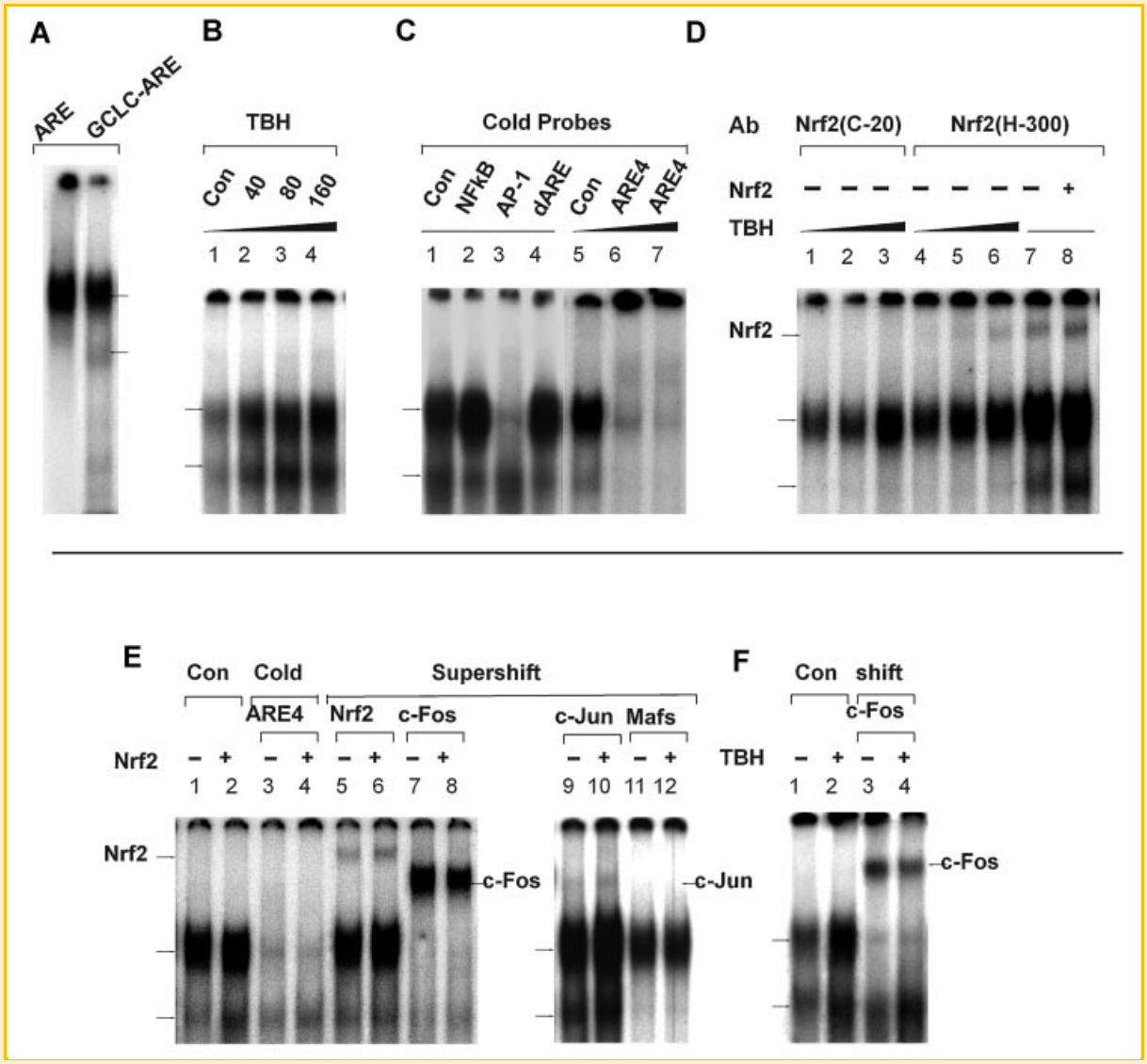

Fig. 6. Binding of nuclear proteins to GCLC-ARE. Nuclear proteins were extracted from cultured rat VSMC grown in control medium, transfected with Nrf2 expression vector, or treated with TBH. EMSA was performed with a [γ-^{32}P] ATP-labeled GCLC-ARE probe. The representative EMSA was selected from three or more gels run on different days. A: Rat GCLC-ARE probed bands compared to those probed with the ARE consensus. B: Example of an EMSA showing the response of nuclear proteins extracted from cells treated with doses of 40, 80,and 160 μM TBH for 16 h. C:A series of cold competitors: NFκB (lane 2), AP-1 (lane 3), GCLC-del-ARE (lane 4) and ARE 4 (lanes 5–7) were used in the representative EMSA to compete with rat GCLC-ARE probe, respectively. The dose of ARE4 used in the lane 7 is 1.3-old of that used in lane 6. D: Example of a supershift EMSA showing different shift-efficiencies with selected Nrf2 antibodies: the C-20 is against the C-terminus of Nrf2 and the H-300 is against the N-terminus (37–336). The cells were treated with TBH (lanes1–6: 80 μM in lanes 2, 5 and 160 μM in lanes 3, 6) or over-expressed Nrf2 (lane 8 vs. 7). E: Representative EMSA comparing GCLC-ARE binding activity between control (lanes 1, 3, 5, 7, 9, 11) and over-expression of Nrf2 (lanes 2, 4, 6, 8, 10, 12), including ARE4 cold competition (lanes 3 and 4) and supershift (lanes 5–12). The antibodies used in the supershift are Nrf2 (lanes 5 and 6), c-Fos (lanes 7 and 8), c-Jun (lanes 9 and 10) and MafF/G/K respectively. Arrows indicate the corresponding shifted bands. F: An additional EMSA for supershift with c-Fos antibody in response to TBH treatments (lanes1–4). The TBH used in lanes 2 and 4 was 40 μM for 16 h.

ARE sequence around the *GCLC* gene and identified a putative fragment about 4 kb upstream of *GCLC* that could function as an enhancer for processing Nrf2-ARE interaction. We characterized this rat GCLC-ARE as consistent with the following reported roles for the ARE:

1. *The rat GCLC-ARE should function as an enhancer.* When inserted into a luciferase-reporter gene, it significantly enhanced either SV40 or *GCLC* promoter activity.
2. *The rat GCLC-ARE should interact with nuclear extracts to form binding complexes.* The binding complexes were formed and composed of several bZip proteins.
3. *The rat GCLC-ARE should be a sequence-specific site.* When missing three key nucleotides, the GCLC-ARE totally lost its enhancer function in the reporter gene and prevented nuclear extracts binding to it in a competitive EMSA.
4. *The rat GCLC-ARE should be an Nrf2-mediated element.* The binding of Nrf2 to the ARE was detected by the Nrf2 antibody

through both immunoprecipitation in a ChIP assay and supershifting in DNA binding assays. Furthermore, over-expression of Nrf2 increased the GCLC-ARE luc-activity in a reporter gene and strengthened DNA-binding band in the supershift EMSA.

5. *Finally, the rat GCLC-ARE should be a real electrophile response element.* Treatment of cells with TBH greatly induced its reporter gene activity as well as DNA binding activity.

In conclusion, the present data demonstrate for the first time that there is indeed an ARE which is capable of modulating the transcription of the rat *GCLC* gene. Furthermore, a direct Nrf2-ARE interaction regulates the rat *GCLC* and suggests that the ARE-mediated Keap1-Nrf2-ARE pathway plays the same important role in maintaining GSH homeostasis for the rat as it does for the human. Given the central role that rat models play in a variety of studies, this is important finding will enhance the ability of investigators to further our understanding of the role of antioxidant defenses and their regulation. However, other questions arise not just for the GCLC-ARE alone but for all AREs: How do the heterodimerized proteins bind to the ARE? How do the heterodimerized protein components change with upstream signaling to accommodate responses to different stressors? Is the composition of the binding complex tissue- or cell-type specific? There is still much to be done to fully understand the nature of ARE binding complexes but now have an approach in a common model.

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