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Nrf2 and c-Jun Regulation of Antioxidant Response Element (ARE)-Mediated Expression and Induction of γ-Glutamylcysteine Synthetase Heavy Subunit Gene

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ABSTRACT. γ-Glutamylcysteine synthetase (γ-GCS) is a rate-limiting enzyme in the *de novo* synthesis of glutathione, a known scavenger of electrophiles and reactive oxygen species (ROS). The γ-GCS gene is expressed ubiquitously and induced coordinately with NAD(P)H:quinone oxidoreductase$_1$ (*NQO1*) and glutathione *S*-transferase Ya (*GST Ya*) in response to xenobiotics and antioxidants. The antioxidant response element (ARE) is required for expression and induction of these genes. In the current report, we demonstrated that ARE-mediated γ-*GCS* gene expression and induction is regulated by similar Nrf and Jun factors as reported earlier for the *NQO1* and *GST Ya* genes. The γ-*GCS* gene ARE competed with the binding of nuclear proteins (Nrf + Jun) to the *NQO1* gene ARE (hARE). In addition, the overexpression of Nrf2 and Nrf1 with c-Jun significantly up-regulated γ-*GCS* ARE-mediated basal expression and β-naphthoflavone induction of the chloramphenicol acetyltransferase gene in transfected HepG2 cells. Interestingly, Nrf2 + c-Jun was more effective than Nrf1 + c-Jun in the regulation of ARE-mediated γ-*GCS* gene expression. Further experiments demonstrated that the c-Jun level within the cells is an important determinant of the level of ARE-mediated γ-*GCS* gene expression. Therefore, at higher concentrations of c-Jun, γ-*GCS* gene expression is repressed, presumably due to generation of a sufficient amount of c-Jun + c-Fos complex that interferes with the binding of Nrf2 + c-Jun complex to the ARE. BIOCHEM PHARMACOL **59**;11:1433–1439, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. γ-glutamylcysteine synthetase; antioxidant response element; Nrf1; Nrf2; c-Jun; regulation of expression

γ-GCS† is a rate-limiting enzyme that is involved in the *de novo* synthesis of glutathione [1]. Glutathione is an effective scavenger of electrophiles and ROS that are generated during chemical metabolism within the cells [2]. This is an important step in the detoxification of various toxic chemicals and agents and is a part of the phase II reactions that serve as important protectors against the various electrophilic and ROS challenges [1–3]. Other enzymes in this phase II system include the NQOs, which catalyze two-electron reduction and detoxification of quinones, and the GSTs, involved in the conjugation of hydrophobic electrophiles and ROS with glutathione [4, 5].

The promoters of the *NQO1* and *GST Ya* genes contain a *cis*-acting DNA element designated as the antioxidant response element (ARE), also referred to as the electrophile response element (EpRE) [4, 5]. The ARE sequences are known to mediate expression and coordinated induction of the *NQO1* and *GST Ya* subunit genes in response to antioxidants, xenobiotics, oxidants, and hydrogen peroxide [4, 5]. The core sequence of the ARE as obtained by several mutational analyses is GTGAC***GC [6, 7]. This sequence bears close resemblance to the NF-E2 consensus sequence, which *trans*-activates β-globin gene promoters, and was used as a recognition site to clone Nrf1 and Nrf2 [8, 9]. Nrf1 and Nrf2 were demonstrated to positively regulate the ARE-mediated expression and induction of the *NQO1* gene [10]. More recent studies have shown that Nrf1 and Nrf2 heterodimerize with Jun (c-Jun, Jun-B, and Jun-D) proteins that bind with ARE and regulate expression and induction of *NQO1* and *GST Ya* genes [11]. Furthermore, it has been demonstrated that binding of *in vitro* translated Nrf2 and c-Jun proteins to the *NQO1* and *GST Ya* gene AREs requires an unknown cytosolic factor(s) [11]. Many other proteins, including YABP1, ARE-BP1, Ah receptor, estrogen receptor, mafK, and mafG, have also been shown to interact with the *GST Ya* gene ARE [12–17].

γ-GCS is a heterodimer made up of heavy and light chain subunits expressing catalytic and regulatory functions, respectively [18, 19]. It has been demonstrated that β-NF increases the steady-state mRNA levels of both these subunits in a time- and concentration-dependent manner

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† *Abbreviations*: γ-GCS, γ-glutamylcysteine synthetase; NQO, NAD(P)H:quinone oxidoreductase; GST, glutathione *S*-transferase; NF-E2, nuclear factor-erythroid 2; Nrf1 and Nrf2, NF-E2 related factors; c-Jun and c-Fos, AP-1 factors; β-NF, β-naphthoflavone; RSV, Rous sarcoma virus; ROS, reactive oxygen species; CAT, chloramphenicol acetyltransferase; and ARE, antioxidant response element.

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A.

Rat γ-GCS gene ARE

5′ AGC TTG CAC AAA GCG CTG AGT CAC GGG GAG GCG GTG CGC GCC CG3′
3' TCG AAC GTG TTT CGC GAC TCA GTG CCC CTC CGC CAC GCG CGG GC5'

Rat γ-GCS gene Mutant ARE

5′ AGC TTG CAC AAA GCA ACA CAG TCT GGG GAG GCG GTG CGC GCC CG3′
3' TCG AAC GTG TTT CGT TGT GTC AGA CCC CTC CGC CAC GCG CGG GC5'

Human NQO1 gene ARE (hARE)

5′ GAT CCA GTC ACA GTG ACT CAG CAG AAT CTG 3′
3' CTA GGT CAG TGT CAC TGA GTC GTC TTA GAC 5'

B.

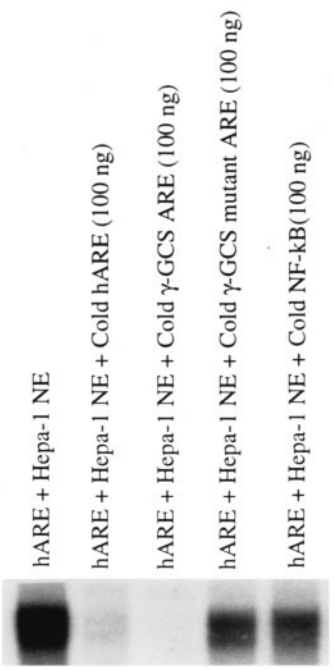


FIG. 1. (A) Nucleotide sequence of the rat γ-GCS gene ARE, the rat γ-GCS gene mutant ARE, and the human *NQO1* gene ARE (hARE). (B) Band shift assay. The human *NQO1* gene ARE (hARE) was end-labeled with [γ-^{32}P]ATP. One hundred thousand cpm of the hARE was incubated with 15 μg of Hepa-1 nuclear extract, and a band shift assay was performed as described in Materials and Methods. The binding of nuclear proteins to the hARE was competed with 100 ng of unlabeled (cold) hARE, γ-GCS ARE, γ-GCS mutant ARE, or NF-κB. Only DNA-shifted bands are shown.

[19]. In addition, mutagenesis experiments have demonstrated that the ARE identified in γ-GCS is required for induction in response to β-NF [19, 20]. However, the transcription factors that mediate this effect on γ-GCS gene expression remain unknown.

In the present study, we demonstrated that the heavy subunit γ-GCS gene ARE competes with the *NQO1* gene ARE for binding to similar (Nrf–Jun) nuclear transcription factors. We also showed that overexpression of Nrf2 and c-Jun up-regulate ARE-mediated expression and β-NF induction of γ-GCS gene expression. We further demonstrated that the c-Jun level within the cells is an important determinant of positive and negative regulation of ARE-mediated γ-GCS gene expression. These results led to the conclusion that coordinated induction of the heavy subunit γ-GCS gene with the *NQO1* and *GST Ya* genes in response to xenobiotics and antioxidants is mediated by Nrf and Jun proteins.

MATERIALS AND METHODS

Construction of ARE-tk-CAT Plasmids

The nucleotide sequences of the human *NQO1* gene ARE (hARE), the rat γ-GCS gene ARE, and the γ-GCS gene mutant ARE are shown in Fig. 1A. The construction of hARE-tk-CAT has been described [12]. Both strands of the γ-GCS ARE and γ-GCS mutant ARE were synthesized with *Hin*dIII and *Bam*HI ends and annealed to generate double-stranded oligonucleotides. The double-stranded γ-GCS ARE and mutant ARE were kinased and subcloned between *Hin*dIII and *Bam*HI sites of pBLCAT2 to generate

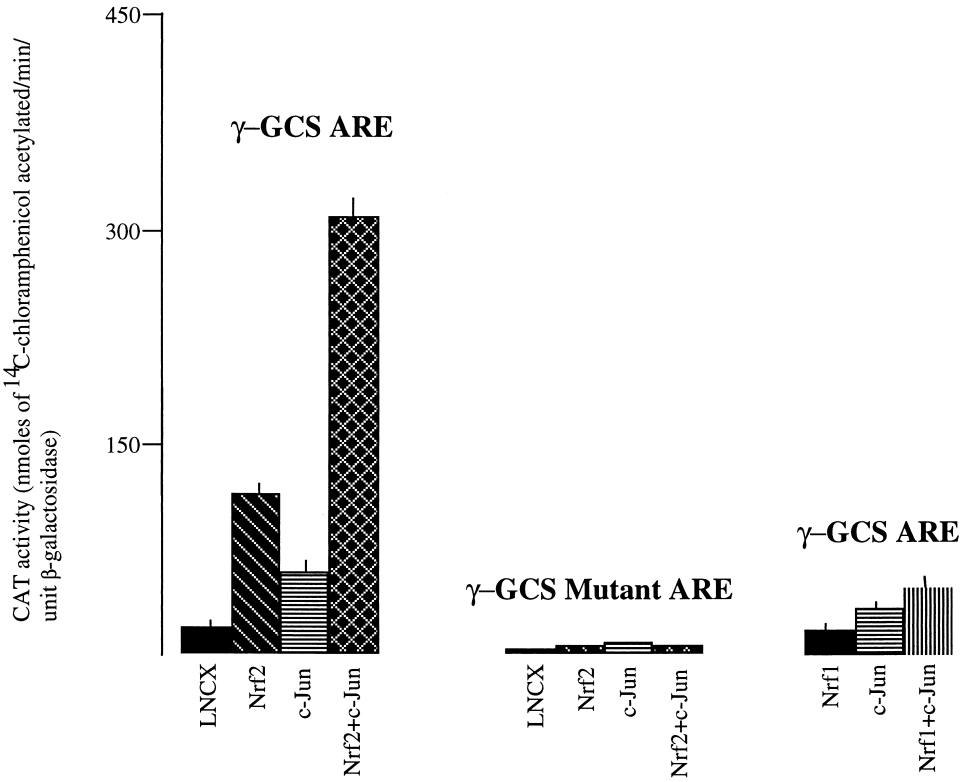


FIG. 2. Effect of overexpression of Nrf and Jun proteins on the expression of γ-GCS gene ARE-mediated CAT gene expression. Five micrograms of γ-GCS ARE-tk-CAT or γ-GCS mutant ARE was cotransfected with similar amounts of LNCX–Nrf2, LNCX–Nrf1, or LNCX–c-Jun individually and in combination. Five micrograms of the RSV-β-galactosidase plasmid was included in each case as a control of transfection efficiency. The cells were scraped 48 hr after transfection, homogenized, and assayed for β-galactosidase and CAT activities by procedures described previously [12]. The values are means ± SEM of three independent transfection experiments.

γ-GCS ARE-tk-CAT and γ-GCS mutant ARE-tk-CAT plasmids.

Cell Culture and Cotransfection of Reporter Plasmids

Human hepatoblastoma (HepG2) cells were grown in monolayer cultures as described [12]. HepG2 cells were transfected with 5 μg of the reporter plasmid γ-GCS ARE-tk-CAT alone or in combination with the expression plasmids LNCX–Nrf1, LNCX–Nrf2, and LNCX–c-Jun by the calcium phosphate procedure [12]. Five micrograms of RSV-β-galactosidase plasmid was used in each individual experiment to normalize for transfection efficiency. In related experiments, the transfected cells were treated with β-NF and *tert*-butylhydroquinone for 16 hr. The cells were harvested, homogenized, and analyzed for β-galactosidase and CAT activities by procedures described previously [12].

Band Shift Assay

Nuclear extracts from mouse hepatoma (Hepa-1) cells were prepared according to the procedure of Kadonaga and Tjian [21]. Band shift and competition assays were performed by procedures as described [12].

RESULTS

The nucleotide sequences of the rat γ-GCS gene ARE, the γ-GCS gene mutant ARE, and the human *NQO1* gene ARE are shown in Fig. 1A. The ^{32}P-labeled *NQO1* gene ARE bound with a complex of nuclear proteins (Fig. 1B). The binding of nuclear proteins to the *NQO1* gene ARE was competed by the γ-GCS ARE but not by the mutant γ-GCS ARE and NF-κB oligonucleotides (Fig. 1B). The transfection of HepG2 cells with LNCX–Nrf2, LNCX–Nrf1, and LNCX–c-Jun led to the overexpression of the respective proteins as determined by western analysis (data not shown). These results were similar to those reported by us earlier [10]. The results of cotransfection of HepG2 cells with the reporter plasmid γ-GCS ARE-tk-CAT and expression plasmids (LNCX–Nrf2, LNCX–Nrf1, and LNCX–c-Jun individually and in combination) are shown (Fig. 2). The overexpression of Nrf2 and c-Jun individually resulted in 5.8- and 2.9-fold increases in γ-GCS ARE-mediated *CAT* gene expression, respectively (Fig. 2). The overexpression of Nrf2 and c-Jun combined produced a 16.2-fold increase in γ-GCS ARE-mediated *CAT* gene expression as compared with the LNCX (vector) control in transfected HepG2 cells. The replacement of γ-GCS ARE-tk-CAT with γ-GCS mutant ARE-tk-CAT resulted in the loss of basal and increased effects of overexpression of Nrf2 and c-Jun (Fig. 2). In similar experiments, the overexpression of Nrf1 + c-Jun also led to increased expression of γ-GCS gene ARE-mediated *CAT* gene expression (Fig. 2). However, Nrf1 was significantly less effective than Nrf2 in inducing ARE-mediated expression of the γ-GCS gene. The treatment of transfected HepG2 cells with β-NF showed a further increase in γ-GCS ARE-mediated *CAT* gene expression (Fig. 3). The β-NF-induced expression of γ-GCS ARE-mediated *CAT* gene in HepG2 cells overexpressing Nrf2 and c-Jun was significantly greater than in the HepG2 cells expressing endogenous levels of Nrf2 and

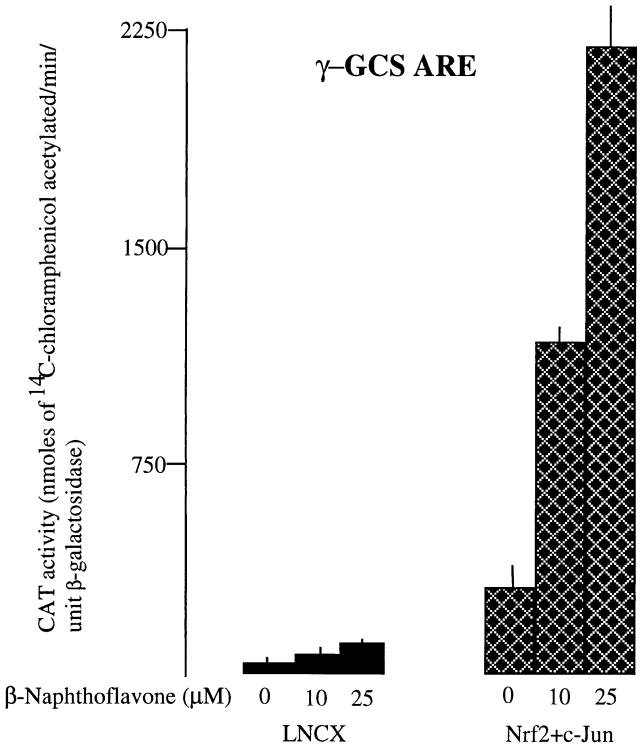


FIG. 3. Effect of overexpression of Nrf2 and c-Jun on the induction of γ-GCS gene ARE-mediated CAT gene expression in response to β-NF. Five micrograms of γ-GCS gene ARE-tk-CAT was mixed with similar amounts of LNCX–c-Jun and LNCX–Nrf2 and transfected into HepG2 cells. Five micrograms of the RSV-β-galactosidase plasmid was included in each transfection as a control of transfection efficiency. The Nrf and Jun transfected cells were treated with β-NF for 16 hr before harvesting and analysis of β-galactosidase and CAT activities. The data are means ± SEM of three independent transfection experiments.

c-Jun. Interestingly, γ-GCS gene ARE-mediated *CAT* gene expression was sensitive to the ratio of Nrf2 and c-Jun proteins in the transfected HepG2 cells (Fig. 4). A constant amount (2.5 μg) of the LNCX–Nrf2 plasmid was cotransfected with various amounts of LNCX–c-Jun plasmid in HepG2 cells. The transfection of HepG2 cells with 2.5 μg of the expression plasmid LNCX–Nrf2 alone resulted in a 5.2-fold increase in γ-GCS ARE-mediated *CAT* gene expression as compared with vector (LNCX)-transfected control (Fig. 4A). Cotransfection of 2.5 μg of Nrf2 with 1.25 and 2.5 μg of c-Jun plasmid led to 12.3- and 15.3-fold increases in γ-GCS ARE-mediated *CAT* gene expression, respectively, as compared with the vector control (Fig. 4A). Interestingly, the increase in c-Jun plasmid from 2.5 to 5 μg resulted in significant repression of the γ-GCS gene ARE-mediated *CAT* gene expression (Fig. 4A). In a similar experiment, the *NQO1* gene ARE-mediated *CAT* gene expression was >2-fold higher than that mediated by the γ-GCS gene ARE. In a related experiment, a constant (2.5 μg) amount of LNCX–c-Jun plasmid was cotransfected with the various concentrations of LNCX–Nrf2 plasmid into HepG2 cells (Fig. 4A). The maximum expression of the *CAT* gene mediated by the γ-GCS gene ARE was observed with a 1:1 ratio of LNCX–Nrf2 and LNCX–c-Jun transfected plasmids (Fig. 4A). An increase in the amount of the LNCX–Nrf2 plasmid from 2.5 to 5 μg showed a similar pattern of results as observed with 2.5 μg of Nrf2 plasmid (compare panels A and B of Fig. 4).

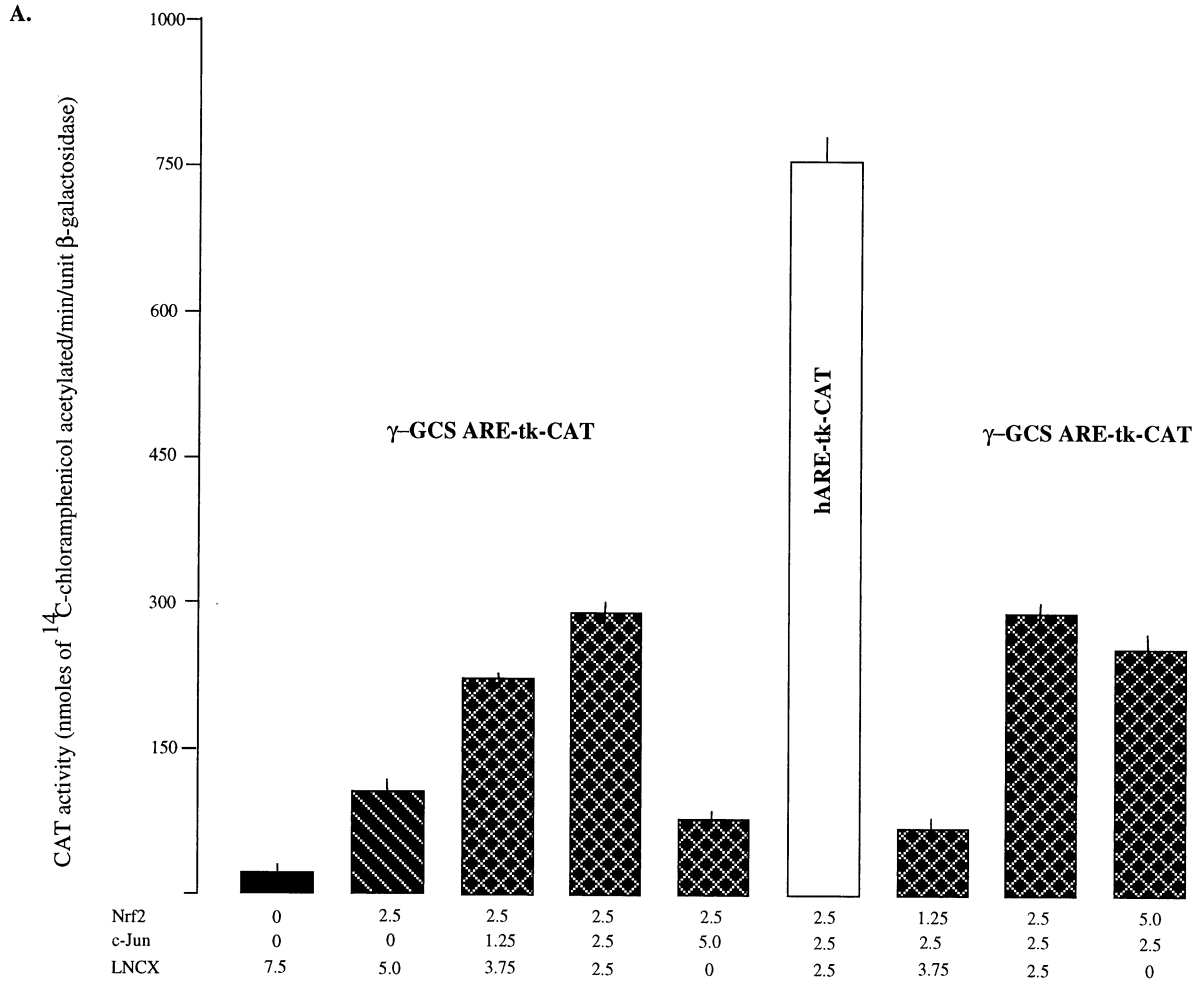
DISCUSSION

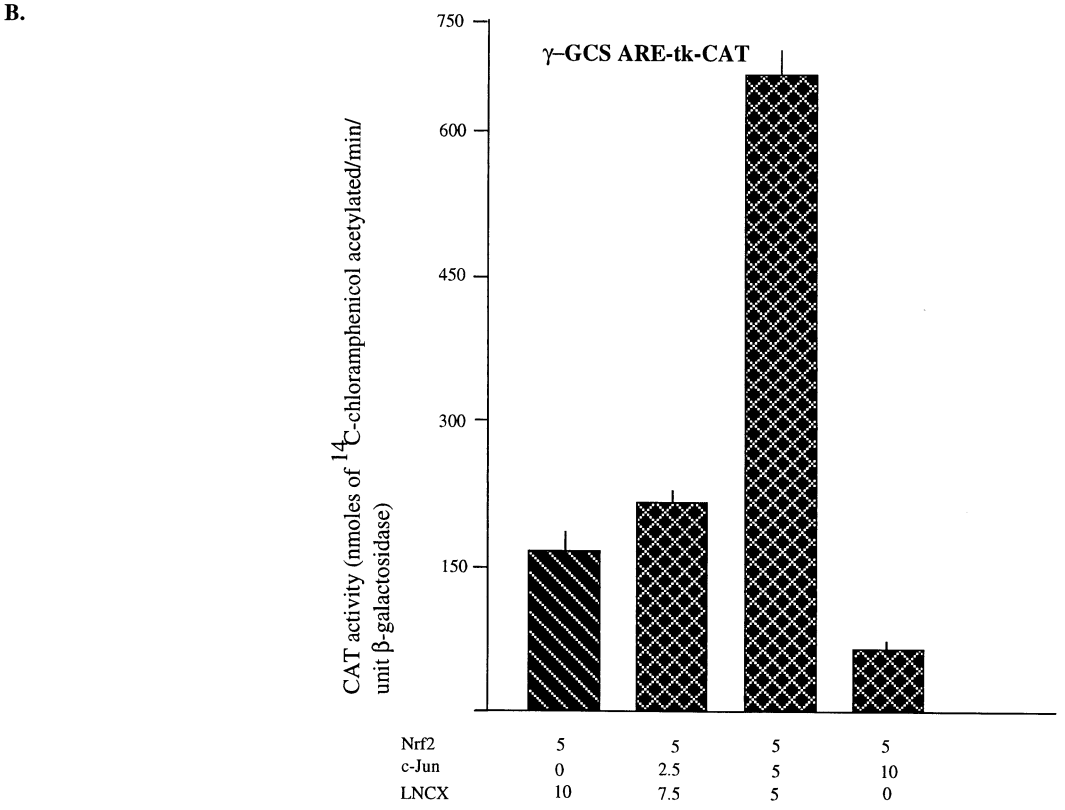
Cellular exposure to xenobiotics and antioxidants leads to coordinated induction of a battery of genes encoding detoxifying enzymes, including *NQO1*, *GST Ya*, and γ-*GCS* [4, 5]. The induction of detoxifying enzymes is a mechanism of critical importance to protect cells against adverse effects of oxidative and electrophilic stress [4, 5]. The expression and coordinated induction of detoxifying enzyme genes are mediated by the ARE [4, 5].

We recently demonstrated that Nrf proteins in association with Jun proteins bind with the *NQO1* gene ARE (hARE) and mediate ARE-regulated expression and induction of the *NQO1* gene in response to xenobiotics and antioxidants [11]. Similar transcription factors have also been demonstrated to regulate ARE-mediated expression and induction of the *GST Ya* gene [11].

In the present report, the results from several experiments demonstrated that ARE-mediated γ-*GCS* gene expression and induction is regulated by similar Nrf and Jun factors as reported earlier in the case of the *NQO1* and *GST Ya* genes. The γ-*GCS* gene ARE competed with the binding of nuclear proteins (Nrf + Jun) to the *NQO1* gene ARE. In addition, the cotransfection of Nrf2 with c-Jun significantly up-regulated γ-*GCS* ARE-mediated basal expression and β-NF induction of the *CAT* gene in transfected HepG2 cells. The combined effect of Nrf2 + c-Jun was significantly greater than the additive effect of individual proteins. These results clearly indicated that Nrf + Jun proteins regulate ARE-mediated expression and induction of the γ-*GCS* gene. A comparison of Nrf2 with Nrf1 indicated that Nrf2 + c-Jun is a more effective combination than Nrf1 + c-Jun in the regulation of ARE-mediated γ-*GCS* gene expression. This result is in agreement with previous observations for the *NQO1* gene ARE [11]. The

FIG. 4. γ-GCS gene ARE-mediated CAT gene expression: Effect of different levels of cDNA derived c-Jun and Nrf2. (A) A constant amount (2.5 μg) of Nrf2 plasmid was cotransfected with various amounts of c-Jun and γ-GCS ARE-tk-CAT in HepG2 cells. In a related experiment, the c-Jun concentration was kept constant at 2.5 μg, and cells were transfected with various amounts of Nrf2. Five micrograms of the RSV-β-galactosidase plasmid was included in each case as a control of transfection efficiency. hARE-tk-CAT was used as a positive control in one lane. The cells were harvested 48 hr after transfection, homogenized, and analyzed for β-galactosidase and CAT activities. (B) The amount of Nrf2 was increased from 2.5 to 5 μg in the experiment as described above. Data are the means ± SEM of three independent transfection experiments.


A.
CAT activity (nmoles of 14C-chloramphenicol acetylated/min/unit β-galactosidase)
1000
750
600
450
300
150
γ–GCS ARE-tk-CAT
hARE-tk-CAT
γ–GCS ARE-tk-CAT
Nrf2 0 2.5 2.5 2.5 2.5 2.5 1.25 2.5 5.0
c-Jun 0 0 1.25 2.5 5.0 2.5 2.5 2.5 2.5
LNCX 7.5 5.0 3.75 2.5 0 2.5 3.75 2.5 0


B.
CAT activity (nmoles of 14C-chloramphenicol acetylated/min/ unit β-galactosidase)
750
600
450
300
150
γ–GCS ARE-tk-CAT
Nrf2 5 5 5 5
c-Jun 0 2.5 5 10
LNCX 10 7.5 5 0

greater efficiency of Nrf2 as compared with Nrf1 might be related to the number of mutated leucines in their leucine zipper regions [11]. Nrf2 contains only one mutated leucine as compared with two mutated leucines in Nrf1. Therefore, it is possible that Nrf2–c-Jun heterodimers are more stable than Nrf1–c-Jun heterodimers, resulting in better performance of Nrf2 than Nrf1. The various results also indicated that the c-Jun level in the cells may be important for up- and down-regulation of ARE-mediated γ-*GCS* gene expression and induction. The overexpression of equimolar ratios of Nrf2 and c-Jun led to maximum induction of γ-*GCS* gene expression. However, further increase in overexpression of c-Jun led to repression of Nrf2–c-Jun activation. This repression may be due to formation of a sufficient amount of c-Jun + c-Fos complex, which is known to interfere with the binding of Nrf2 + c-Jun complex to the ARE [10]. The role of c-Fos in the repression of *NQO1* and *GST Ya* gene expression in c-Fos-/- mice has been reported [22]. The positive and negative role of c-Jun in ARE-mediated γ-*GCS* gene expression is also consistent with the similar regulation of urokinase gene expression [23]. Preliminary results also indicated that an unknown cytosolic factor(s) that is required for binding of *in vitro* translated Nrf + Jun proteins to *NQO1* and *GST Ya* gene AREs is also required for binding to the γ-*GCS* gene ARE (data not shown).

The complete mechanism of signal transduction from xenobiotics and antioxidants to the ARE-mediated induction of γ-*GCS* and other detoxifying enzyme genes remains unknown. However, there is enough evidence to suggest that Nrf and Jun proteins associate and bind to the ARE in different genes, leading to their increased expression. These transcription factors may serve as oxidative stress response factors within the cells. The current hypothesis is that ROS generated during the metabolism of xenobiotics and antioxidants produce a signal. The signal, directly or through intermediary proteins, is received by Nrf and Jun proteins, which bind to the ARE. This leads to the coordinated activation of detoxifying enzyme genes. The identity of the intermediary proteins remains unknown. In addition to Nrf and Jun proteins, several other factors including YABP1, ARE-BP1, Ah receptor, estrogen receptor, mafK, and mafG also have been shown to interact with the *GST Ya* gene ARE [12–17]. However, the role of these factors in the regulation of ARE-mediated expression and induction of *GST Ya* gene expression remains unknown. In addition, the role of these proteins in ARE-mediated expression and induction of the *NQO1* and γ-*GCS* genes also remains unknown. More recently, a third member of the Nrf family of transcription factors, designated as Nrf3, has been reported [24]. The role of Nrf3 in ARE-mediated expression and induction of γ-*GCS* and other detoxifying enzyme genes is expected but remains unknown.

In conclusion, we have demonstrated that ARE-mediated γ-*GCS* gene expression and induction in response to xenobiotics and antioxidants are regulated by Nrf and Jun proteins. The Nrf2 + c-Jun combination was found to be more effective than Nrf1 + c-Jun. The c-Jun level within the cells plays an important role in determining the level of induction of γ-*GCS* gene expression.

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