Cystine/Glutamate Exchange Transporter Gene Expression* 

Electrophile Response Element-mediated Induction of the Cystine/Glutamate Exchange Transporter Gene Expression* 

Hiromi Sasaki, Hideyo Sato‡, Kazumi Kuriyama-Matsamura‡, Kanako Sato‡, Kanako Maebara§, Hongyu Wang‡, Michiko Tamba‡, Ken Itoh‡, Masayuki Yamamoto‡, and Shiro Bannai‡

From the Department of Biochemistry, Institute of Basic Medical Sciences, and Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan. Tel.: 81-298-53-3282; Fax: 81-298-53-3039; E-mail: hideyo-s@md.tsukuba.ac.jp

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In mammalian cultured cells, the cystine/glutamate exchange transport mediated by system x\textsuperscript{-}c\textsuperscript{\textsuperscript{\textsuperscript{-}}} is important to maintain intracellular GSH levels. System x\textsuperscript{-}c\textsuperscript{\textsuperscript{\textsuperscript{-}}} consists of two protein components, xCT and the heavy chain of 4F2 antigen. The activity of system x\textsuperscript{-}c\textsuperscript{\textsuperscript{\textsuperscript{-}}} is induced by various stimuli, including electrophilic agents like diethyl maleate. In the present study, we have investigated the mechanism of the transcriptional regulation of xCT mRNA by diethyl maleate. The xCT gene consisted of twelve exons and sequence analysis identified four electrophile response element (EpRE)-like sequences between −230 and −1 in the 5′-flanking region, designated EpRE-1 to EpRE-4. To identify sequences mediating the constitutive and induced expression of xCT, a series of 5′-deletion mutants created from the 5′-flanking region were cloned into a luciferase reporter vector and transfected into BHK21 cells. The 5′-deletion analysis revealed that the sequence between −116 and −82 is essential for the basal expression and the sequence between −226 and −116 containing EpRE-1 is essential in response to diethyl maleate. Other stress agents like arsenite, cadmium, and hydroquinone seemed to induce system x\textsuperscript{-}c\textsuperscript{\textsuperscript{\textsuperscript{-}}} activity via the same sequence. Furthermore, the experiments using the mouse embryonic fibroblasts derived from the Nrf2-deficient mice revealed that the induction of xCT gene by electrophilic agents is mediated by Nrf2. EpRE occurs in a broad spectrum of genes for the proteins that are involved in the defense against xenobiotics and regulates their expression. The present results have demonstrated that xCT is a novel member of this protein family.

GSH plays a prominent role in a cellular defense against reactive oxygen species and electrophiles. In mammalian cultured cells, we have previously characterized an anionic amino acid transport system, designated system x\textsuperscript{-}c\textsuperscript{\textsuperscript{\textsuperscript{-}}}, which mediates cystine influx coupled with the efflux of intracellular glutamate (1, 2). The intracellular GSH levels rapidly decrease by inhibiting the transport of cystine or depleting cystine from the culture medium (3). Therefore, this cystine/glutamate exchange transporter contributes to the maintenance of intracellular GSH levels (4). The activity of system x\textsuperscript{-}c\textsuperscript{\textsuperscript{\textsuperscript{-}}} is significantly induced by various stimuli, including diethyl maleate (DEM),\textsuperscript{1} arsenite, cadmium chloride, hydrogen peroxide, bacterial lipopolysaccharide, and TNF-α in mouse peritoneal macrophages (5, 6). Combinantly, intracellular GSH levels are increased, suggesting that system x\textsuperscript{-}c\textsuperscript{\textsuperscript{\textsuperscript{-}}} is fallen into the category of the stress proteins that are required for the metabolic response to oxidative stress. System x\textsuperscript{-}c\textsuperscript{\textsuperscript{\textsuperscript{-}}} is composed of two proteins, xCT and the heavy chain of 4F2 cell surface antigen (4F2hc) (7). 4F2hc is predicted to have a single transmembrane domain and has been recently demonstrated to be the complex component of some amino acid transporters such as systems L and y\textsuperscript{\textsuperscript{-}L} for the functional membrane expression of the counterpart protein components (8). xCT has 12 putative transmembrane domains, suggesting that xCT itself mediates the transport activity.

There is a family of enzymes called phase II detoxifying enzymes, which function as intracellular detoxication systems for mutagens, carcinogens, and other toxic compounds. The genes of these enzymes contain the cis-acting element, designated as electrophile response element (EpRE), also referred as antioxidant responsive element, responsible for the induction by electrophilic agents (9–11). Previous studies showed that the presence of a core sequence of 5′-RTGACnnnGC-3′ is necessary for an EpRE to be functional (12). Recently, Wasserman and Fahl (13) have identified the additional sequence necessary to define a sufficient and functional EpRE. The Nrf2 has been demonstrated to be an essential transcription factor for the expression of genes encoding the phase II enzymes, such as NAD(P)H:quinone oxidoreductase and glutathione S-transferase, by the electrophilic agents (14). When cells are treated with electrophilic agents, Nrf2 translocates to the nucleus, binds to EpRE in the presence of small Maf, another transcription factor, and up-regulates EpRE-mediated transcription (15). Previously, we demonstrated that in the macrophages derived from Nrf2-deficient mice the activity of system x\textsuperscript{-}c\textsuperscript{\textsuperscript{\textsuperscript{-}}} was not induced by the electrophilic and oxidative stress agents, although promoter sequences for xCT were not known at that time (16). It is highly likely that the induction of xCT mRNA by electrophilic agents is mediated via EpRE. In the present study, the gene for xCT has been isolated, and the function of 5′-flanking region of the gene has been analyzed. The data

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† To whom correspondence should be addressed: Dept. of Biochemistry, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan. Tel.: 81-298-53-3282; Fax: 81-298-53-3039; E-mail: hideyo-s@md.tsukuba.ac.jp

‡ The abbreviations used are: DEM, diethyl maleate; 4F2hc, 4F2 heavy chain; EpRE, electrophile response element; Nrf2, NF-E2-related factor 2; BSO, buthionine sulfoximine; γ-GCS, γ-glutamyl cysteine synthetase; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; RACE, rapid amplification of cDNA ends.
indicate that several EpRE-like sequences exist in the 5' -flanking region of xCT gene and that various electrophilic agents induce the expression of xCT mRNA via one of these sequences. 

**EXPERIMENTAL PROCEDURES**

**Materials—L-[^14]C]Cystine was obtained from PerkinElmer Life Sciences. Monobromobimane was purchased from Molecular Probes, Inc. (Eugene, OR). The luciferase assay kit (luciferase assay system with reporter lysis buffer) was from Promega (Madison, WI).**

**Cell Culture—** BHK21 cells originated from Syrian hamster kidney were cultured routinely in Dulbecco's modified Eagle's medium supplemented with 2.5% fetal bovine serum at 37 °C in 5% CO2 and 95% air. The Chinese hamster embryo fibroblasts from wild type and Nrf2-deficient mice (14) were prepared from 13.5 day embryos as described previously (17). The cells were cultured in Icosc's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 10 ng/ml human recombinant epidermal growth factor, and 1 × insulin-transferrin-selenium-G (Invitrogen) at 37 °C in 5% CO2 and 95% air.

**Cystine Uptake—** Cystine uptake was measured using techniques described previously (1). Cells were rinsed three times in warmed PBSG (10 mM phosphate-buffered saline (137 mM NaCl, 3 mM KCl), pH 7.4, containing 0.1% CaCl2, 0.01% MgCl2·6H2O and 0.1% glucose) and then incubated in 0.5 ml of the warmed uptake medium at 37 °C for specified time periods. The uptake medium was PBSG containing labeled cystine (0.1 μCi/ml). Uptake was terminated by rapidly rinsing the culture dishes three times with ice-cold PBS, and radioactivity associated with cell extracts was determined as described previously (18). Cystine uptake was determined under conditions approaching initial rates of uptake, i.e., measuring uptake for cystine at 120 s. The uptake of cystine increased linearly during this incubation interval. 

**Measurement of Intracellular Cystine**—The cystine content in the cells was determined by the method of Cotgreave and Moldeus (18) with a slight modification (19). The cells were rinsed three times with PBSG and incubated in the dark at room temperature for 10 min with 100 μCi of 8m M monobromobimane in 50 mM N-ethylmorpholine, pH 8, and 100 μl of 50 mM phosphate-buffered saline containing 0.01% CaCl2, 0.01% MgCl2·6H2O, and 0.1% glucose. Then 10 μl of 100% trichloroacetic acid was added. The protein precipitate was removed by centrifugation at 3000 × g for 5 min, and aliquots were analyzed for cystine-bimane adduct by HPLC. The HPLC separation was achieved on a steel column (4.6 × 100 mm packed with 3-μm octadecylsilica reversed-phase material. The fluorescence at 480 nm was monitored with the excitation at 394 nm. The elution was performed with 9% (v/v) acetonitrile in 0.25% (v/v) acetic acid, pH 3.7 for 8 min, and then with 75% (v/v) acetonitrile in water for 5 min. The flow rate was 1 ml/min throughout the process.

**Determination of Intracellular GSH Levels—** Intracellular GSH was extracted with 5% trichloroacetic acid and then treated with ether to remove the acid. The GSH content in the aqueous layer was measured using an enzymatic method described previously (20), which is based on the catalytic action of GSH in the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) by the GSH reductase system. The GSH extracted from the cells was mostly reduced GSH, and the content of the oxidized form, GSSG, was negligible throughout the experiments in this study.

**Isolation of Genomic Mouse xCT Clones and Sequence Analysis—** Mouse genomic library (Strategene) was screened using mouse xCT cDNA as a probe. Twelve positive clones were isolated, and one of the clones, which contained exon 1 and 5'-flanking region of the gene, was subcloned into pBluescript. The length of each intron was deduced from the restriction map of the gene or from the length of PCR products using the primers containing the sequences of its 5'- and 3'-flanking exons. The transcription initiation site was determined using 5'-RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen), and Primer Extension System (Promega) following the manufacturer's protocols, respectively.

**Construction of 5'-Deletion Mutants, Cell Culture, and Transfection into Cells—** A 4.7-kbp fragment was produced from the clone containing the first exon of xCT gene and its 5'-flanking region by cutting out with SacI and NotI. A 0.7-kbp fragment was similarly produced with PstI. These fragments were subcloned into pBluescript and cut out with SacI for 4.7-kbp fragment and with Xhol and SacI for 0.7-kbp fragment, and subcloned into pGL3-Basic (Promega). For smaller fragments, the insert was produced by PCR reaction using the primers containing mutations to generate appropriate restriction enzyme sites. The PCR products were cut out with the restriction enzymes and subcloned into pGL3-Basic. The constructs containing mutations in the EpRE-like sequence were produced using a QuikChange™ site-directed mutagenesis kit by following the manufacturer's protocol. The sequences of the final constructs were verified by dideoxynucleotide sequencing. BHK21 cells were transfected with the constructs using DEAE-dextran methods. Briefly, cells were plated at 2 × 10^5 cells/60-mm diameter dish. One day later, the culture medium was replaced with fresh one. After 4 h, the cells were washed twice with PBS and incubated at 37 °C for 4 h with 0.4 mg/ml DEAE-dextran solution containing 3.4 μg of pGL3-Basic or the constructs. To correct for transfection efficiency, 0.6 μg of the reporter plasmid pCMVβ (Clontech) containing the lacZ gene encoding β-galactosidase under the control of the human cytomegalovirus immediate-early promoter/enhancer was co-transfected with each constructs. 

**Measurement of Luciferase and β-Galactosidase Activities—** Luciferase activity was measured by the coloration assay using the LipofectAMINE PLUS reagent by following the manufacturer's instructions. The cells were plated at 2 × 10^5 cells/35-mm diameter dish, incubated for 24 h, transfected with the constructs, and incubated for further 24 h. Then, the cells were incubated with DEM for 2 h, and the cystine transport activity or luciferase activity was measured, or the nuclear extract was prepared. The nuclear extract was prepared using Nu-Clear™ Extraction kit (Sigma) by following the manufacturer's instruction. For cell harvest, transfectants were washed twice with PBS and incubated at room temperature for 15 min in 160 μl of reporter lysis buffer (Promega). Cells were then scraped from the plates and the resulting lysates were vortexed, frozen in liquid nitrogen, thawed at room temperature, vortexed again, and centrifuged at 12,000 × g for 15 s at 4 °C. The supernatants were stored at −80 °C until measuring the activities of luciferase and β-galactosidase.

**Northern Blot Analysis**—The cDNA probes for mouse xCT, human 4F2hc, and mouse β-actin were labeled using [α-32P]dCTP and Rediprime II random prime labeling system (Amersham Biosciences). RNA was electrophoresed on a 1% agarose gel in the presence of 2.2 mM formaldehyde, transferred to Hybond-N° membrane (Amersham Bio- sciences), and hybridized in a solution containing 50% formamide for 16 h at 42 °C. The membranes were washed twice for 15 min at room temperature with 1 × SSC, 0.1% SDS and then washed twice for 15 min at 68 °C with 0.1 × SSC, 0.1% SDS.

**Western Blot Analysis**—The nuclear extracts were lysed with SDS sample buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl, pH 6.8, and 0.1 mM phenylmethylsulfonyl fluoride. Sample total protein content was determined using the BCA protein assay reagents and 3% 2-mercapto-
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**Fig. 2.** Effects of various amino acids on the uptake of cystine in BHK21 cells. Cells were incubated for 24 h after plating. Then, the cells were further incubated with or without 100 μM DEM for 12 h. The rate of uptake of 0.65 mM L-[14C]cystine was measured in the absence (Control) or presence of 2.5 mM amino acids indicated. Each point represents the means ± S.D. (n = 4) and is expressed as a percentage of the control uptake.

**Fig. 3.** Expression of xCT and 4F2hc mRNAs in BHK21 cells cultured with diethyl maleate. Cells were incubated for 24 h after plating. Then, the cells were further incubated with or without 100 μM DEM for the time intervals indicated. Total RNA was isolated, and 10 μg each of the total RNA was loaded per lane. The hybridization was performed with 32P-labeled cDNAs of mouse xCT, 4F2hc, and β-actin.

**Fig. 4.** Intracellular cysteine and GSH levels in BHK21 cells incubated with various stress agents. Cells were incubated for 24 h after plating. Then, the cells were further incubated with 2.5 μM sodium arsenite (As), 10 μM CdCl2 (Cd), 100 μM DEM, and 25 μM hydroquinone (HQ) for 12 h, and intracellular cysteine and GSH levels were measured as described under “Experimental Procedures.” Each point represents the means ± S.D. (n = 4).

RESULTS

We investigated whether the activity of system x\textsubscript{c} occurs in BHK21 cells and is induced by electrophilic agents. Untreated cells significantly took up cystine, and the activity of cystine transport was further increased by treating the cells with sodium arsenite, CdCl\textsubscript{2}, DEM, and hydroquinone (Fig. 1). These reagents had no effect on the activity of system x\textsubscript{c}. Although the activity of system x\textsubscript{c} would be by phorbol myristate acetate, lipopolysaccharide, and TNF-α in the mouse peritoneal macrophages (6), these reagents had no effect on the activity of cystine transport in BHK21 cells. The cystine transport was strongly inhibited by glutamate and homocysteinate but to a lesser extent by aspartate (Fig. 2). Arginine and leucine were not inhibitory at all. In the cells treated with DEM, the pattern of the inhibition of the cystine transport was similar to that in untreated cells. From these results, we have concluded that the transport activity with the characteristics of system x\textsubscript{c} is expressed in BHK21 cells and that the activity of system x\textsubscript{c} is induced by DEM in these cells. Fig. 3 shows the expression of xCT and 4F2hc mRNAs in the cells cultured with DEM. The expression of xCT mRNA was strongly induced by DEM, whereas 4F2hc mRNA was relatively constant regardless of the presence of DEM. Intracellular cysteine levels were measured in these cells cultured with the stress agents. As shown in Fig. 4A, intracellular cysteine levels were increased concomitantly with the increase in the cystine transport activity in the cells treated with the stress agents. In these cells, intracellular GSH levels were also increased (Fig. 4B).

To characterize the genomic organization of the xCT gene, we screened the 129 SVJ mouse genome library by using digoxigenin-labeled mouse xCT cDNA as a probe, and 12 clones were isolated. Their inserts were further analyzed by restriction mapping, subcloning, and sequencing. The exon/intron organization of the mouse xCT gene is shown in Fig. 5A. The gene was encoded by 12 exons that were distributed over a region of more than 40 kbp of genomic DNA. The transcription initiation site was determined by 5′-RACE and the primer extension method (Fig. 5B). Four EpRE-like sequences were found and designated EpRE-1 to -4 (21). Thus, a series of 5′-deletion mutant/luciferase reporter fusion genes were generated by cloning various length of restriction fragments or PCR products into the luciferase reporter vector, pGL3-Basic, to localize the regions controlling basal and DEM-inducible expression of the xCT gene (Fig. 5B). These constructs were
transiently transfected into BHK21 cells, and luciferase activity was determined in the presence and absence of DEM. The results of the 5′-deletion studies indicated that the sequence between -226 and -117 including EpRE-1 contained the regulatory element responsible for maximum DEM-inducible expression and the sequence between -226 and -117 including EpRE-1 contained the regulatory element responsible for maximum DEM-inducible expression and the sequence between -116 and -81 regulated the basal expression (Fig. 6). To confirm that EpRE-1 influences response to DEM, the constructs containing mutations in this sequence were made and these constructs were transfected into BHK21 cells followed by treatment of DEM (Fig. 7). Although the basal luciferase activity was detected in the cells transfected with the constructs containing mutations, the effect of DEM was significantly decreased. The results indicate that EpRE-1 is mainly involved in the response to DEM. The effects of the EpREs on the induction of xCT by various stress agents. BHK21 cells were transfected with the construct containing a 0.7-kbp fragment of the 5′-flanking region of the xCT gene. The cells were then incubated with 2.5 μM sodium arsenite (As), 10 μM CdCl₂ (Cd), 100 μM DEM, and 25 μM hydroquinone (HQ) for 12 h, and the luciferase activities were measured. Values represent the means ± S.D. (n = 4–6).
agents were investigated. As shown in Fig. 8, the luciferase activity was significantly increased in the cells transfected with the pGL3−0.7 followed by the treatment of arsenite, cadmium chloride, or hydroquinone.

The electrophiles like DEM are known as the substrate of glutathione S-transferase and thus may cause GSH depletion. To investigate the effect of GSH depletion on the induction of system xct, treatment with BSO, an inhibitor of γ-glutamyl cysteine synthetase (γ-GCS), was added to BHK21 cells. As shown in Fig. 9A, intracellular GSH level was drastically decreased in the cells treated with BSO. However, the activity of cystine transport remained unchanged compared with that in the control cell (Fig. 9B). The luciferase activity also remained unchanged in the cells transfected with pGL3−0.7 followed by the treatment of BSO (Fig. 9C). These results suggested that the decrease in the intracellular GSH does not cause the induction of the activity of cystine transport.

We have investigated whether Nrf2 is involved in the upregulation of xCT gene expression by DEM. As shown in Fig. 10A, system xct activity was not induced at all by DEM in the embryonic fibroblasts derived from Nrf2-deficient mice. Then the expression of the reporter gene in these cells was examined. The cells were transfected with pGL3−0.23, treated with DEM, and the luciferase activity was measured (Fig. 10B). In the control cells (wild type), the luciferase activity was increased by DEM treatment in a dose-dependent manner, whereas the luciferase activity in the Nrf2-deficient cells remained unchanged by DEM treatment at the concentration of up to 50 μM. In the Nrf2-deficient cells, treatment of DEM at the concentration of higher than 100 μM caused profound cell damage. The effect of DEM on nuclear distribution of Nrf2 has been investigated in the wild type cells. As shown in Fig. 10C, Nrf2 moved into the nucleus significantly increased by treating the cells with DEM, suggesting that Nrf2 moved into the nucleus to increase the expression of xCT gene in these cells. Next, the wild type cells were co-transfected with the Nrf2 expression plasmid and the pGL3 reporter plasmids, and luciferase activity was measured. The luciferase activity in the cells transfected with the Nrf2 expression plasmid and pGL3−0.23 was increased in proportion to the dose of the Nrf2 expression plasmid despite absence of DEM (Fig. 10D). On the other hand, in the cells transfected with the Nrf2 expression plasmid and pGL3−0.12 or pGL3−0.08, the luciferase activity did not increase regardless of the dose of the Nrf2 expression plasmid. It is highly likely that overexpression of Nrf2 causes the movement of Nrf2 into the nucleus without the electrophilic agent. These results suggest that the movement of Nrf2 into the nucleus is crucial to the induction of xCT gene.

DISCUSSION

In the present study, we have found that the expression of xCT, but not 4F2hc, is strongly induced by DEM in BHK21 cells and that there are several EpRE-like sequences in the 5′-flanking region of xCT gene. We have demonstrated that the induction of xCT gene by DEM is mediated by these sequences. EpREs have been found in the promoter regions of various genes, e.g. those encoding glutathione S-transferase, NADPH: quinone oxidoreductase, the heavy and light subunits of γ-GCS, ferritin L subunit, metallothionein-1, and thioredoxin (9, 10, 22–25). The common function of these proteins is for cell defense against xenobiotics and oxidants. System xct mediates transport of cystine into the cells, and as shown here in BHK21 cells and in other cells previously, the intracellular GSH levels are regulated by the activity of this transport system (4). Since GSH is a key substance in antioxidant defense, the protein component of system xct, xCT, is deemed to be a member in the cell defense system. Therefore, it is reasonable that the expression of xCT is regulated through EpRE.

Recently, Erickson et al. (21) have investigated the promoter of the human γ-GCS modifier subunit gene in detail and proposed that the consensus sequence for the EpRE should be revised to 5′-RTKAAYnnnGCR-3′. EpRE-1 has a single nucleotide mismatch at −142, compared with the consensus EpRE proposed by them. The enhancement of the luciferase activity by DEM was significantly decreased in the cells transfected with pGL3−0.12, which does not contain EpRE-1 (Fig. 6). In addition, the luciferase activity in the cells transfected with the constructs containing mutations in EpRE-1 was significantly decreased (Fig. 7). EpRE-1 is completely conserved in the 5′-flanking region of the human xCT gene (26). Judging from these, the main region responsible for the DEM-inducible tran-
The transcriptional regulation of the xCT gene by EpRE-3 and EpRE-4 was assessed by luciferase assay. The constructs containing EpRE-3 and EpRE-4 were transfected into embryonic fibroblasts derived from wild type mice and treated with DEM. The luciferase activity in the cells transfected with constructs containing EpRE-3 and EpRE-4 was significantly increased as compared to the control cells. These results suggest that other EpREs, particularly EpRE-3 and EpRE-4, may contribute to the enhancement of the DEM-inducible transcription of the xCT gene.

Recently, Itoh et al. (15) have demonstrated that Keap1, the cytosolic protein, interacts with Nrf2 to form a complex in the cytosol and that DEM causes the dissociation of the complex and then Nrf2 moves into the nucleus to enhance the EpRE-mediated transcription of the xCT gene. The luciferase activity in the cells transfected with constructs containing EpRE-3 and EpRE-4 was significantly increased as compared to the control cells. These results suggest that other EpREs, particularly EpRE-3 and EpRE-4, may contribute to the enhancement of the DEM-inducible transcription of the xCT gene. The luciferase activity in the cells transfected with constructs containing EpRE-3 and EpRE-4 was significantly increased as compared to the control cells. These results suggest that other EpREs, particularly EpRE-3 and EpRE-4, may contribute to the enhancement of the DEM-inducible transcription of the xCT gene.

In the present study, we have demonstrated that the intracellular cystine levels are increased in BHK21 cells when they are treated with the stress agents. Most probably this increase is accounted for by the induction of system xc⁻ activity as shown previously in other cells (27). The concentration of the cysteine in the cells untreated and treated with DEM can be estimated to be ~0.1 and 0.3 mM, respectively, assuming that 1 mg of cell protein is equivalent to 5 μl of cell water (28). γ-GCS is known to catalyze the rate-limiting step in synthesis of GSH, and its apparent Km value for cysteine is reported to be 0.35 mM (29). Thus, the rate of GSH synthesis in BHK21 cells treated with DEM is much higher than that in the control cells even if γ-GCS per se remains unchanged. Mulcahy et al. (22, 23) have demonstrated that the EpREs in the 5′-flanking regions of the genes encoding γ-GCS heavy and light subunits regulate the expression of these genes in the cells treated with β-naphthoflavone, an electrophilic agent, which induces phase II enzymes. In the cells treated with DEM, both the increase in intracellular cystine levels caused by the induction of the activity of system xc⁻ and the induction of γ-GCS seem to contribute to the increase in GSH synthesis.
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