RESEARCH COMMUNICATION

Inducible expression of the γ-glutamylcysteine synthetase light subunit by t-butylhydroquinone in HepG2 cells is not dependent on an antioxidant-responsive element

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Mutation analysis of putative regulatory elements located in the 5′-flanking region of the gene encoding the regulatory subunit of γ-glutamylcysteine synthetase (*GLCLR*) revealed that neither an antioxidant-responsive element (ARE) nor an activator protein-1 (AP-1) site regulates inducible expression by t-butylhydroquinone (tBHQ). The AP-1 site was found to modulate basal expression of *GLCLR*. A 42 bp region between nucleotides −303 and −344, not containing an ARE, was found to regulate inducible expression of *GLCLR* by tBHQ.

Key words: activator protein-1, gene regulation, glutathione synthesis.

INTRODUCTION

Glutathione plays a major role in the detoxification of electrophilic xenobiotics, peroxides, reactive oxygen species and heavy metals, and is fundamental in maintenance of intracellular redox status [1]. The *de novo* biosynthesis of glutathione, catalysed by the sequential actions of γ-glutamylcysteine synthetase (GCS) and glutathione synthetase, is of principal importance in regulating intracellular glutathione homoeostasis [2]. GCS catalyses the rate-limiting step in glutathione synthesis, which is regulated physiologically by feed-back inhibition by glutathione and the availability of cysteine [3]. GCS is a heterodimer composed of a catalytic subunit (heavy subunit, GCS_h) and a regulatory subunit (light subunit, GCS_l), with the latter being responsible for modulating the K_m for glutamate and sensitivity to feed-back inhibition by glutathione [4–7].

Exposure of cells to environmental agents that result in oxidative stress causes an increase in glutathione levels, which has been attributed to an increase in GCS activity and expression of the GCS_h mRNA transcript [8,9]. Moreover, co-ordinate induction of the GCS_h and GCS_l subunits by oxidative stress, and by certain chemopreventive agents or their metabolites, has been reported [10–13]. In order to study the regulation of the GCS_l subunit, we recently isolated the GCS_l gene (*GLCLR*) and found that it is transcriptionally activated by t-butylhydroquinone (tBHQ) [13]. A number of putative enhancer sequences were identified within the 5′-flanking region of the *GLCLR* gene, including activator protein-1 (AP-1) and AP-1-like sites, an antioxidant-responsive element (ARE), a xenobiotic-responsive element and a metal-responsive element (MRE). AREs, AP-1 and AP-1-like sites have been reported to be important for both the constitutive and inducible expression of the GCS_h gene (*GLCLC*) [12,14–18].

The co-ordinate regulation of expression of the GCS_h and the GCS_l subunits by cellular stresses implies that similar regulatory mechanisms might control the transcriptional activity of the *GLCLC* and *GLCLR* genes. Recently, it was reported that mutation of the ARE, together with the AP-1 site, in the *GLCLR* gene abolishes inducible expression by β-naphthoflavone (β-NF) [19]. In the present study, we have examined induction of GCS_l by tBHQ, and in contrast with the findings of Moinova and Mulcahy [19], show that this is not dependent on an ARE. We have identified a 42 bp region of the *GLCLR* gene, which does not contain the ARE, which is capable of mediating inducible expression of the *GLCLR* gene by tBHQ.

EXPERIMENTAL

Recombinant plasmids

The reporter vectors, pCAT-Basic, pCAT-Promoter and pCAT-Control were purchased from Promega, Southampton, U.K. The construct pDGCAT1 contains an approx. 6.0 kb fragment of the *GLCLR* 5′-flanking region upstream of the promoterless pCAT-Basic reporter vector [13]. The plasmid pDGL5△205, which contains a 1.7 kb *GLCLR* fragment in pBluescript II SK(+) [13], was utilized for site-directed mutagenesis [20], and the mutations were confirmed by sequencing using a double-stranded DNA cycle sequencing kit (Gibco BRL, Paisley, Renfrewshire, U.K.). *Mlu*I/*Xba*I fragments (0.67 kb) containing the mutated sequences were substituted with the corresponding region of pDGCAT1, generating pDGCAT1mA1, pDGCAT1mA2, pDGCAT1mA3, pDGCAT1mA1:3 and pDGCAT1M1.

Nucleotides were progressively removed from the 3′-end of the *GLCLR* fragment in pDGCAT1 by exonuclease III [21], generating pDGCAT1.1 and pDGCAT1.2, which contain 5′-fragments upstream of nt −256 and −411 respectively. The *GLCLR* fragments of pDGCAT1.1 (approx. 6 kb) and pDGCAT1.2

Abbreviations used: AP-1, activator protein-1; ARE, antioxidant-responsive element; tBHQ, t-butylhydroquinone; CAT, chloramphenicol acetyltransferase; GCS, γ-glutamylcysteine synthetase; GCS_l, GCS light subunit; GCS_h, GCS heavy subunit; *GLCLC*, GCS_h gene; *GLCLR*, GCS_l gene; MRE, metal-responsive element; β-NF, β-naphthoflavone.

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(approx. 5.8 kb) were subcloned into the pCAT-Promoter vector, which contains the simian virus 40 promoter, generating pDGC-ATP1.1 and pDGCATP1.2. The nucleotides are numbered relative to A of the initiation codon of the GCS_1 cDNA [13,19].

The *GLCLR* region between nt −256 and −411 was amplified by PCR and ligated into the pCAT-Promoter vector, generating pDGCATP150. Synthetic oligonucleotides complementary to the *GLCLR* region between nt −346 and −303 were annealed and ligated into the pCAT-Promoter vector, generating pDGC-ATP44.

The 42 bp fragment between nt −303 and −344 or the 31 bp fragment between nt −303 and −333 was deleted in pDGL5△205 by a PCR-based deletion method [22], adding additional random nucleotides to retain the orientation of the remaining DNA. *Mlu*I/*Xba*I fragments containing the deletions were substituted with the corresponding region in pDGCAT1, generating pDGCAT1/42 and pDGCAT1/31 respectively. The 31 bp fragment was deleted in pDGCAT1mA3 by using the same method, to generate pDGCAT1/31m3.

Cell culture and transient transfections

The human hepatocyte carcinoma cell line, HepG2, was used for all experiments. Cells were maintained in Dulbecco's modified Eagle's medium with 862 mg/l Glutamax I™, 110 mg/l sodium pyruvate and 1 g/l glucose (Gibco BRL), supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Transient transfections were performed as described previously [13], and were carried out in triplicate on 2–3 separate occasions. Transfected cells were treated for 18–20 h with either 100 μM tBHQ or 75 μM β-NF or with DMSO at a concentration equivalent to that of drug-treated cells. Cells were harvested, and the β-galactosidase and chloramphenicol acetyltransferase (CAT) assays were performed and quantified as described previously [13]. Statistical analyses were performed using the Student's unpaired *t* test.

RESULTS AND DISCUSSION

Analysis of putative regulatory elements in the 5′-flanking region of the *GLCLR* gene

The transcriptional activity of the 5′-flanking region of the *GLCLR* gene has been shown previously to be increased in tBHQ-treated HepG2 cells [13]. Analysis of deletion mutants of a 6 kb fragment of the 5′ region of *GLCLR* showed that inducible expression by tBHQ resides within a 0.8 kb fragment of the *GLCLR* gene between nt −205 and −1007 [13]. Putative enhancer sequences identified within the 0.8 kb fragment include an ARE, a MRE, an AP-1 and an AP-1-like site [13,19], all of which have the potential to be involved in transcriptional regulation by chemical and oxidative stress [12,14,15,23]. Mutation and deletion analysis of putative enhancer sequences were undertaken to determine which regions are important for regulating constitutive and inducible expression of the *GLCLR* gene. Nucleotide substitutions were made in the proximal ARE and the AP-1 site of the 6 kb 5′-flanking region of *GLCLR* within the reporter construct pDGCAT1, as shown in Figure 1(B).

In transient transfection assays, the basal promoter activities of pDGCAT1mA1 and pDGCAT1mA2, which contain different base substitutions within the ARE, i.e. the terminal GC and initial TG residues respectively (Figure 1B), were comparable with the basal promoter activity of the control vector pDGCAT1 (Figure 1A). However, mutation of the AP-1 site revealed that this site contributes to the regulation of the constitutive expression of the *GLCLR* gene. The basal promoter activity of pDGCAT1mA3, which contains mutations of the initial TG residues of the AP-1 site, is approx. 50% lower ($P < 0.005$) than the basal promoter activity of pDGCAT1 (Figure 1A). Mutations of the AP-1 site, together with the ARE within the reporter construct pDGCAT1mA1:3, did not decrease the basal promoter activity to an extent significantly greater than that with pDGC-AT1mA3. These data suggest that, although the ARE is important for the constitutive expression of the *GLCLC* gene [12], it is not important for the basal expression of the *GLCLR* gene. The finding that the AP-1 site regulates the basal expression of the *GLCLR* gene is consistent with the findings of Moinova and Mulcahy [19]. Furthermore, an AP-1 site has been reported to be important for the constitutive expression of the *GLCLC* gene

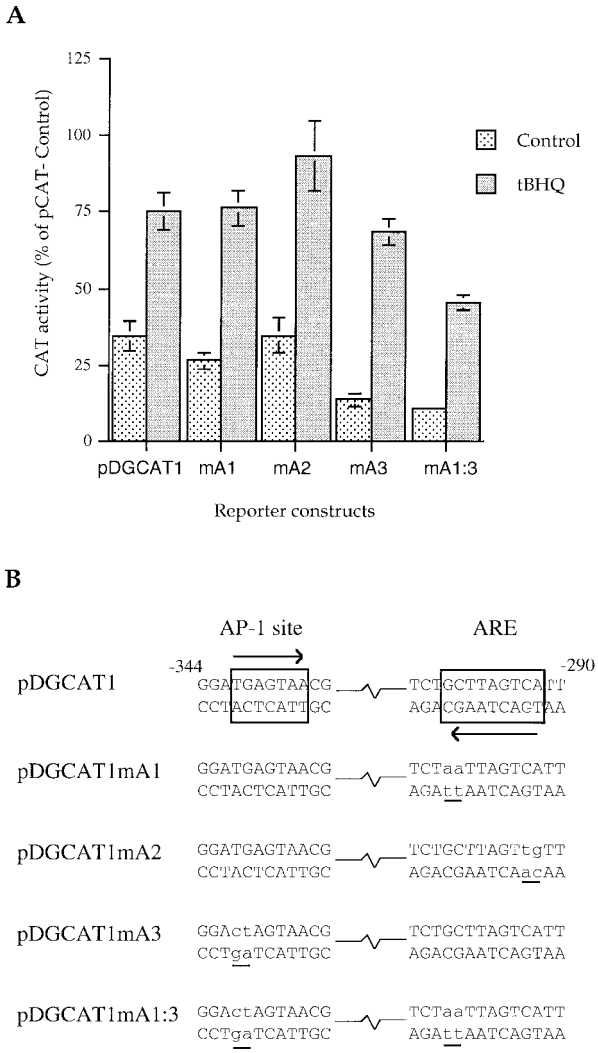

Figure 1 Mutation analysis of putative enhancers in the *GLCLR* gene promoter region

(**A**) The CAT activity of HepG2 cells transiently transfected with the reporter constructs pDGCAT1, pDGCAT1mA1, pDGCAT1mA2, pDGCAT1mA3 and pDGCAT1mA1:3, and the effect of tBHQ on activity. Transfections were performed in triplicate and values are the mean CAT activity ± S.E.M., presented as a % of the CAT activity of the control vector, pCAT-Control. When error bars appear to be absent, the S.E.M. was too small to be shown on the histogram. (**B**) A representation of the mutated bases within the ARE and AP-1 site in the *GLCLR* reporter constructs.

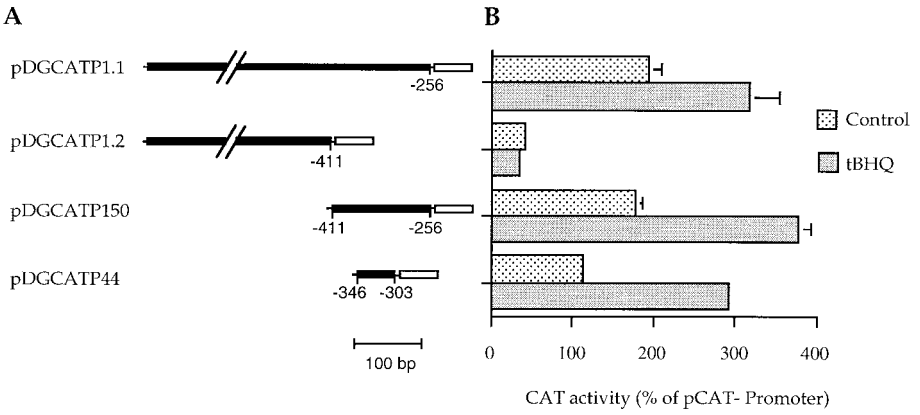

Figure 2 Regulation of enhancer activity of the 5′-flanking region of *GLCLR* by tBHQ

Portions of the 5′-flanking region of *GLCLR* were cloned into the pCAT-Promoter vector. A schematic representation of the clones is shown in (**A**), where filled boxes denote *GLCLR* DNA and open boxes denote the CAT reporter DNA. The nucleotide positions are numbered relative to the A residue of the initiation codon of the GCS_l cDNA. (**B**) The CAT activity of HepG2 cells transiently transfected with pDGCATP1.1, pDGCATP1.2, pDGCATP150 or pDGCATP44, as indicated in (**A**), and the effect of tBHQ treatment. Transfections were performed in triplicate and values are the mean CAT activity ± S.E.M., expressed as a % of the CAT activity of the pCAT-Promoter vector. When error bars appear to be absent, the S.E.M. was too small to be shown on the histogram.

[16,18], suggesting that similar mechanisms might influence basal expression of the genes encoding the GCS_h and GCS_l subunits.

The potential role of the ARE and AP-1 site in the tBHQ-inducible expression of the *GLCLR* gene was investigated. An increase (between 2- and 3-fold; $P < 0.001$) in transcriptional activity was found for pDGCAT1, pDGCAT1mA1 and pDGCAT1mA2 in tBHQ-treated cells (Figure 1). Thus alteration of the ARE sequence, which eliminates ARE function [24], does not abolish the inducible response to tBHQ. In tBHQ-treated cells, the transcriptional activities of pDGCAT1mA3 and pDGCAT1mA1:3 are also increased ($P < 0.0001$; Figure 1), showing that mutation of the AP-1 site alone, or together with the ARE, does not abolish the inducibility of the *GLCLR* reporter. These experiments imply that neither the ARE nor the AP-1 site regulate the inducible expression of the *GLCLR* gene by tBHQ, either on their own or in co-operation with each other.

The data suggest that *GLCLR* and *GLCLC* might be regulated by distinct mechanisms, as induction of *GLCLC* has been reported to be regulated by an ARE [12]. Our results, however, appear to be in contrast with the findings of Moinova and Mulcahy [19], who reported that mutation of the ARE in the *GLCLR* gene decreases the extent of inducibility by β-NF, and that mutations in the AP-1 site and the ARE together abolish induction [19]. Because of this apparent difference, we analysed the activity of the reporter constructs containing mutant ARE and AP-1 sites in the presence of β-NF. We did not find that mutations in the ARE (pDGCAT1mA1), or the ARE and AP-1 site together (pDGCAT1mA1:3), reduced the extent of induction of the *GLCLR* gene by β-NF (results not shown). It is unclear why different results have been obtained, but it is possible that the two HepG2 cell lines used in the different laboratories might differ in the levels of expression of transcription factors that regulate the expression of the *GLCLR* gene.

The MRE, reported to increase the expression of the metallothionein-1 gene by tBHQ-induced oxidative stress [23], was also analysed in the *GLCLR* gene. The promoter activity of pDGCAT1M1 containing a mutated MRE sequence (nt −482 to −488; altered from TGCGCTC to AATTCTC [23]) was comparable with the promoter activity of pDGCAT1 in both control and tBHQ-treated cells (results not shown). This suggests that the putative MRE does not contribute to either the basal or inducible expression of the *GLCLR* gene by tBHQ.

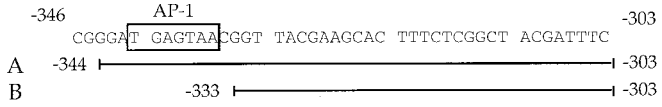

Figure 3 DNA sequence of the tBHQ-responsive region of *GLCLR*

The DNA sequence of the tBHQ-responsive region contained in pDGCATP44 is shown. The location of the AP-1 site is outlined, and the DNA regions eliminated in the constructs pDGCAT1/42 (**A**) and pDGCAT1/31 (**B**) are indicated underneath the sequence.

Enhancer regions of the *GLCLR* 5′-flanking sequence

The enhancer activity of the *GLCLR* 5′-flanking region was analysed by subcloning fragments of the 5′ region into the pCAT-Promoter vector. pDGCATP1.1, which contains an approx. 6 kb DNA fragment upstream of nt −256, and pDGCATP1.2, which contains the same DNA fragment, but upstream of nt −411 (Figure 2), were examined. Increased promoter activity of pDGCATP1.1, but not of pDGCATP1.2, was found in tBHQ-treated cells (Figure 2). Since removal of the fragment between nt −256 and −411 abolishes the induction of promoter activity by tBHQ, this 155 bp region is likely to mediate transcriptional activation in response to tBHQ. This proposal is supported by the finding that the promoter activity of pDGCATP150, which contains the *GLCLR* region between nt −256 and −411, is increased by approx. 2-fold ($P < 0.001$) in tBHQ-treated cells (Figure 2). The region responsible for induction by tBHQ was identified by analysis of portions of DNA within the 155 bp region, and a similar increase in transcriptional activity in tBHQ-treated cells was observed for pDGCATP44 ($P < 0.001$), which contains the region between nt −303 and −346 (Figure 2). The DNA sequence of this region is shown in Figure 3. The *GLCLR* gene fragment between nt −303 and −346 is therefore capable of enhancing transcriptional activity in response to

Table 1 A 42 bp fragment in the 5′ region of *GLCLR* regulates induction by tBHQ

The CAT activity in HepG2 cells transiently transfected with the reporter constructs pDGCAT1 and pDGCAT1/42 was measured in control and tBHQ-treated cells. The values are the means for triplicate transfections ± S.E.M. presented as a % of the CAT activity of the control vector, pCAT-Control.

	CAT activity (% of pCAT-Control)	
Reporter construct	Control	tBHQ-treated
pDGCAT1	40.0 ± 4.1	89.5 ± 3.0
pDGCAT1/42	22.7 ± 3.6	29.5 ± 0.4

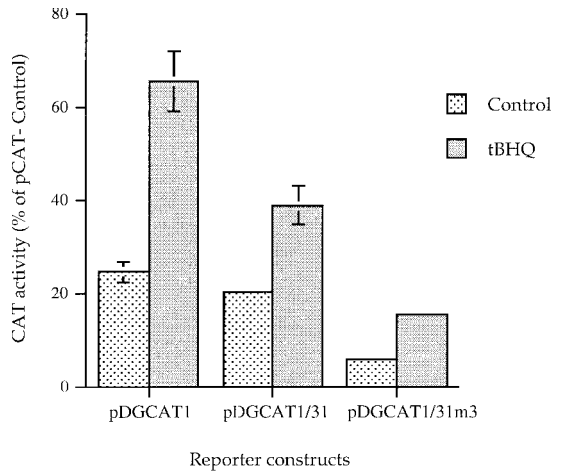

Figure 4 Analysis of the 42 bp region of *GLCLR* responsible for induction by tBHQ

The CAT activity from HepG2 cells transiently transfected with the reporter constructs pDGCAT1, pDGCAT1/31 and pDGCAT1/31m3 was measured in control and tBHQ-treated cells. The values are the means for triplicate transfections ± S.E.M., presented as a % of the CAT activity of the control vector, pCAT-Control. When error bars appear to be absent, the S.E.M. was too small to be shown on the histogram.

tBHQ, and might therefore regulate the tBHQ-inducible expression of the *GLCLR* gene. It is notable that the DNA fragment between nt −303 and −346 does not contain the ARE.

The importance of the region between nt −303 and −346 for the inducible expression of the *GLCLR* gene by tBHQ was demonstrated by deleting this DNA fragment from pDGCAT1 to create pDGCAT1/42, which lacks the 42 bp between nt −303 and −344 (Figure 3). Transcriptional activity of pDGCAT1/42 is not increased significantly in tBHQ-treated cells (Table 1). Since the ARE is still present within pDGCAT1/42, this supports the hypothesis that the ARE is not important for the induction by tBHQ. The AP-1 site, however, is deleted in pDGCAT1/42. To determine whether the elimination of this site in pDGCAT1/42 contributes to the loss of induction by tBHQ, pDGCAT1/31 (in which nt −303 to −333 are deleted but the AP-1 site is retained; Figure 3) was analysed. Figure 4 shows that the promoter activity of pDGCAT1/31 is increased in tBHQ-treated cells (1.7-fold; $P < 0.005$), but to a lesser extent than that of pDGCAT1 (2.3-fold; $P < 0.001$). The region between nt −334 and −344 therefore appears to modulate the inducible expression of the *GLCLR* gene, although the region between nt −303 and −333 is also likely to be important for obtaining maximal inducible expression.

Contributions by regions other than the AP-1 site within the *GLCLR* 5′-flanking sequence in regulating induction by tBHQ seem likely, because the promoter activity of pDGCAT1mA3, which contains a mutated AP-1 site, is inducible by tBHQ (Figure 1). In order to investigate further the involvement of the AP-1 site in induction of GCS_l by tBHQ, the promoter activity of pDGCAT1/31m3, in which nt −303 to −333 are deleted and the AP-1 site is mutated (the same mutation contained in pDGCAT1mA3), was analysed. Whereas the basal promoter activity of pDGCAT1/31m3 was found to be substantially lower than that of either pDGCAT1 or pDGCAT1/31 ($P < 0.001$), the activity was found to be inducible by tBHQ (approx. 2-fold; $P < 0.005$) (Figure 4). This supports further the hypothesis that the AP-1 site does not solely mediate the inducible expression of the *GLCLR* gene. Alternative transcription factors, which are transactivated by oxidative and chemical stress, might therefore bind to the region between nt −334 and −344 and contribute to the tBHQ-inducible expression. It is also feasible that the region between nt −303 and −333 has a modulatory effect on this inducible expression.

The AP-1 site has been shown to be important in regulation of the basal promoter activity of the *GLCLR* gene, and it is interesting to note that the basal promoter activity of pDGCAT1/31m3, which contains the mutant AP-1 site, is lower than that of pDGCAT1/42, in which the AP-1 site is removed. A possible explanation is that a repressor, which normally competes for a site that is overlapping with the AP-1 site, is capable of binding to that region in pDGCAT1/31m3, because the AP-1 site is mutated and AP-1 is unable to bind. In pDGCAT1/42, the binding site for the potential repressor might be disrupted, since an additional 11 bp are removed compared with pDGCAT1/31.

In conclusion, we have demonstrated that the constitutive expression of the *GLCLR* gene in HepG2 cells is regulated by an AP-1 site, but tBHQ-inducible expression is not regulated by either the ARE or the AP-1 site. A 42 bp region of the *GLCLR* gene, which does not contain the ARE, was found to mediate the tBHQ-inducible expression of the *GLCLR* gene.

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