Regulation of human γ-glutamylcysteine synthetase: co-ordinate induction of the catalytic and regulatory subunits in HepG2 cells

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We have shown that in HepG2 cells treatment with 75 μM t-butylhydroquinone (tBHQ) results in a 2.5-fold increase in glutathione concentration, as part of an adaptive response to chemical stress. In these cells the elevation in intracellular glutathione level was found to be accompanied by an increase of between 2-fold and 3-fold in the level of the 73 kDa catalytic subunit of γ-glutamylcysteine synthetase (heavy subunit, GCS_h) and the 31 kDa regulatory subunit (light subunit, GCS_l). Levels of GCS_h and GCS_l mRNA were increased by up to 5-fold in HepG2 cells in response to tBHQ. To study the transcriptional regulation of GCS_l, we subcloned 6.7 kb of the upstream region of the human GCS_l gene (*GLCLR*) from a genomic clone isolated from a P1 lymphoblastoid cell line genomic library. HepG2 cells were transfected with *GLCLR* promoter reporter constructs and treated with tBHQ. This resulted in an induction of between 1.5-fold and 3.5-fold in reporter activity, indicating that transcriptional regulation of *GLCLR* is likely to contribute to the induction of GCS_l by tBHQ in HepG2 cells. Sequence analysis of the promoter region demonstrated the presence of putative enhancer elements including AP-1 sites and an anti-oxidant-responsive element, which might be involved in the observed induction of the *GLCLR* promoter.

INTRODUCTION

Glutathione is of fundamental importance in the cellular detoxification of many drugs, environmental chemicals and oxidants [1]. Elevated levels of glutathione and glutathione-metabolizing enzymes have frequently been observed as part of an adaptive response to environmental stress as well as being associated with resistance to a spectrum of chemotherapeutic drugs, including redox-cycling agents, alkylating agents and platinum-based drugs in tumours and cancer cell lines [2–6].

Many different biological parameters have the potential to influence glutathione homeostasis. A large body of evidence suggests that γ-glutamylcysteine synthetase (GCS) (glutamate:cysteine ligase, EC 6.3.2.2), which catalyses the first step in the pathway for glutathione synthesis from its constituent amino acids, is of central importance in regulating glutathione levels [7–10]. GCS is a heterodimer comprising a heavy subunit (GCS_h, 73 kDa) and a light subunit (GCS_l, 31 kDa) [11]. GCS_h is responsible for the ligase activity, whereas GCS_l is responsible for regulating the catalytic activity of the heavy subunit [12–14]. Data from studies *in vitro* suggest that GCS_l might interact with GCS_h *in vivo* to enhance the catalytic efficiency of GCS when glutathione levels decrease; interaction between GCS_h and GCS_l causes a decrease in the K_m for glutamate and lowers the sensitivity to feedback inhibition by glutathione.

So far, regulation of GCS activity at a transcriptional level has been studied with respect to the catalytic subunit alone. Levels of mRNA encoding GCS_h have previously been shown to be increased substantially (up to 50-fold) in tumour cell lines made resistant to different chemotherapeutic drugs [4,15–18]. This was found to be associated with increased glutathione levels and is a consequence of transcriptional activation of the GCS_h gene (*GLCLC*). Furthermore transcriptional activation of the gene encoding GCS_h has been shown to occur in rat lung epithelial cells and bovine pulmonary artery endothelial cells in response to quinone-induced oxidative stress [19,20].

The promoter region of human *GLCLC* has recently been reported to contain putative regulatory elements that have been shown previously to be present in other genes that are inducible by different cellular stresses, as well as in genes that are aberrantly expressed in drug-resistant cancer cells [17,21]. These putative enhancer sequences include an AP-1 site and a sequence similar to the AP-1 site, termed an AP-1-like site [17], a xenobiotic-responsive element (XRE), an antioxidant-responsive element (ARE) and an NF-κB-like site. Of these, the AP-1-like site has been implicated in the overexpression of GCS_h in cisplatin-resistant ovarian cells, as well as in the induction of GCS_h in response to oxidants [17,22,23]. The functional importance of the other putative enhancer elements is not yet clear.

The human GCS_l subunit is encoded by the gene *GLCLR*, which is located on a separate chromosome from *GLCLC* [24–26]; little is known about its transcriptional regulation. It is unknown whether the induction of GCS_h protein levels is accompanied by the induction of GCS_l protein, or indeed whether the genes are subject to co-ordinate transcriptional regulation in response to stress or in drug-resistant cell lines.

During the present study we have shown that both GCS_h and GCS_l mRNA are induced by t-butylhydroquinone (tBHQ) in the human hepatocellular carcinoma cell line HepG2 and that this is accompanied by a corresponding increase in levels of both

Abbreviations used: ARE, antioxidant-responsive element; BHA, butylated hydroxyanisole; CAT, chloramphenicol acetyltransferase; GCS_h, GCS catalytic heavy subunit; GCS_l, GCS regulatory light subunit; *GLCLC*, GCS_h gene; *GLCLR*, GCS_l gene; GCS, γ-glutamylcysteine synthetase; tBHQ, t-butylhydroquinone; XRE, xenobiotic-responsive element. *GLCLR* nucleotide positions are numbered relative to the open reading frame such that +1 denotes the A residue of the translation initiation codon.

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The nucleotide sequence depicted in Figure 4 has been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases and appears under the accession number BankIt145510 AF028815.

polypeptides. We have isolated and characterized the *GLCLR* promoter and found that transcriptional regulation is likely to have a role in the induction of GCS_l by tBHQ.

EXPERIMENTAL

Chemicals

All chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.) unless otherwise stated.

DNA and plasmids

Oligodeoxyribonucleotides HL1 (5′-GGCACGAGGCTGCGG-CCGCAGTAGCCGGAG CCGGAGCCGCAGCCACCGGT-3′) and HL2 (5′-CGGAAGAAGTGCCCGTCCACGCACAG-CGAGGAGCTTCATGATTGTATCCA-3′) correspond to nt −253 to −202 and 93 to 143 of the human GCS_l cDNA [27] respectively, where nucleotide +1 denotes the A residue of the translation initiation codon.

Human GCS_l cDNA (EST clone no. 133938) was obtained from the I.M.A.G.E. consortium (St. Louis, MO) [28] and its identity was verified by dideoxynucleotide sequencing and comparison with the cDNA clone described by Mulcahy and co-workers [27].

A full-length human GCS_h cDNA clone was isolated by screening a λZAP human liver cDNA library (Stratagene, Cambridge, U.K.) with a human GCS_h cDNA fragment, which was a gift from Professor R. T. Mulcahy (University of Wisconsin, Madison, WI, U.S.A.).

Nucleotide sequences were obtained by dideoxynucleotide sequencing [29] modified for use with Sequenase 2.0 (US Biochemicals, Cleveland, OH, U.S.A.) or *Taq* DNA polymerase (Gibco BRL, Paisley, Renfrewshire, U.K.).

Library screening and isolation of *GLCLR* genomic clone

A gridded P1 human lymphoblastoid cell line genomic library [Resourcenzentrum im Deutschen Humangenomprojekt am Max-Planck-Institut für Molekulare Genetik (former Reference Library Database), Berlin-Charlottenburg, Germany] [30] was screened by using an adaptation of the method of Church and Gilbert [31] with a probe derived from the human GCS_l cDNA. The screened filters were washed twice for 10 min with 160 mM sodium phosphate, pH 7.2, containing 0.1 % (w/v) SDS at 65 °C before being exposed to X-ray film (Fuji, Tokyo, Japan). A single positive clone was identified (ICRFP700O1492QDB) and obtained for further characterization.

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 15 % (v/v) heat-inactivated fetal calf serum, 500 i.u./ml penicillin and 500 mg/ml streptomycin at 37 °C in a humidified air/CO_2 (19:1) atmosphere.

HepG2 cells were seeded at a density of 10^6 cells per 10 cm plate 24 h before transfection. HepG2 transfection was performed by the method of calcium phosphate co-precipitation [32]. For each transfection 10 μg of pDGCAT reporter or pCAT-control (Promega, Southampton, UK) and 5 μg of pSV-β-gal (Promega) were used.

For measurement of β-galactosidase and chloramphenicol acetyltransferase (CAT) activity, cells were lysed in 0.25 mM Tris/HCl, pH 7.8, by repeated freeze–thaw cycles. Insoluble material was removed by centrifugation. The β-galactosidase activity of extracts was determined with the method described by Sambrook et al. [33] adapted for use on a Cobas Fara centrifugal analyser. β-Galactosidase activities were used to normalize samples to take into account variations in transfection efficiency. CAT assays were performed by the method of Gorman et al. [34]. CAT activity was quantified by phosphorimaging with a Bio-Rad GS-525 Molecular Imager System (Bio-Rad, Hemel Hempstead, Herts., U.K.).

Chemical treatment of cells

HepG2 cells were plated out at 2.5×10^6 per 10 cm plate and treated with 75 or 100 μM tBHQ (Aldrich, Poole, Dorset, U.K.) (from 200 mM stock in DMSO). Control cells were treated with an equivalent concentration of vehicle. For glutathione determination and Western blotting studies, cells were lysed in 10 mM sodium phosphate, pH 7.2, containing 2 mM $MgCl_2$ and 1 mM EDTA by repeated freeze–thaw cycles. Insoluble material was removed by centrifugation.

Analytical

Protein concentrations and glutathione levels were determined by the methods of Bradford [35] and Tietze [36] respectively. These methods were modified for use on a Cobas Fara centrifugal analyser.

Western blot analysis

Polyclonal antibodies were raised in female New Zealand White rabbits against synthetic peptides corresponding to regions of human GCS_h and GCS_l. The peptides were coupled to keyhole limpet haemocyanin and resuspended in 0.9 % NaCl before immunization using standard protocols [37]. The peptides that produced a specific immune response were those corresponding to residues 1–20 (MGLLSQGSPLSWEETKRHAD) and 82–101 (GERTNPNHPTLWRPEYGSYM) for GCS_h and 165–184 (SD-LDKTQLEQLYQWAQVKPN) for GCS_l. These antibodies were used at a dilution of 1:500 for Western blot analysis. Proteins were resolved by SDS/PAGE [10 % (w/v) gel] and electrotransferred to nitrocellulose membrane [38]. Rat kidney cytosol, which contains relatively high levels of each of the GCS subunits, was used as a standard to confirm the identity of the GCS subunits in HepG2 cells. The relative intensities of bands on autoradiographs were estimated by scanning densitometry.

RNA isolation and Northern blot analysis

Total RNA from HepG2 cells was prepared by the method of Chomczynski and Saachi [39]. RNA (25 μg per lane) was electrophoretically fractionated through a 2.2 M formaldehyde/1 % (w/v) agarose gel and transferred to Qiabrane nylon membrane (Qiagen, Dorking, Surrey, U.K.), and cross-linked by UV irradiation. Membranes were prehybridized for at least 1 h at 42 °C in 50 % (v/v) formamide/10 % (w/v) dextran sulphate/1 % (w/v) SDS/0.6 M NaCl containing denatured salmon sperm DNA (0.1 mg/ml). The membranes were hybridized with ^{32}P-DNA probes derived from human GCS_l cDNA, human GCS_h cDNA or a 1.2 kb *Eco*R1/*Hin*dIII fragment of rat glyceraldehyde-3-phosphate dehydrogenase cDNA for 24 h at 42 °C. Membranes were washed twice for 15 min each at room temperature in 0.3 M NaCl/30 mM sodium citrate (2 × SSC) containing 0.1 % (w/v) SDS, then at 65 °C in 0.2 × SSC containing 0.1 % (w/v) SDS followed by 0.1 × SSC containing 0.1 %

(w/v) SDS for 15 min each, with a final wash for 1 h in 0.05 × SSC containing 0.1 % (w/v) SDS. The membranes were exposed to X-ray film (Fuji). The relative intensities of bands on autoradiographs were estimated by scanning densitometry.

RESULTS

Effect of tBHQ on glutathione levels and expression of GCS subunits in HepG2 cells

Cancer chemopreventive antioxidants such as butylated hydroxyanisole (BHA) have previously been shown to induce hepatic detoxification systems in rodents (reviewed in [6]). This has also been shown to be associated with an increase in glutathione levels, and Borroz et al. [40] have demonstrated that GCS_h is induced by BHA in mouse liver. tBHQ is a principal metabolite of BHA that causes induction of detoxification enzymes [41], so we tested the hypothesis that tBHQ would cause the induction of GCS and increase glutathione levels in the human hepatocyte carcinoma cell line HepG2. Treatment of HepG2 cells with tBHQ (75 and 100 μM for 18 h) increased total glutathione levels to approx. 2.5-fold those in untreated cells (Table 1). No difference in effect was seen between the drug concentrations used. To determine whether the increase in glutathione concentrations was associated with an increase in GCS protein, Western blotting with antibodies raised against peptides from GCS_h and GCS_l was performed. Figure 1 shows that both the catalytic and regulatory subunits of GCS were induced by tBHQ (approx. 2-fold and 3-fold respectively) after 18 h of treatment. Northern blotting was performed to establish whether the co-ordinate increase in the levels of the two protein subunits was associated with a corresponding increase in each mRNA (Figure 2). Increases of approx. 4-fold in GCS_l mRNA and 5-fold in GCS_h mRNA levels were observed. In accordance with previous observations [4,16,27], we also detected two distinct transcripts (3.5 and 4.1 kb) that hybridized with the GCS_h cDNA probe. Both of these transcripts were found to be induced to a similar extent.

Table 1 Effect of tBHQ on glutathione levels in HepG2 cells

Results are the means ± S.D. for three separate experiments.

Treatment	[Glutathione] (nmol/mg of protein)
Control	71.33 ± 10.11
75 μM tBHQ	193.58 ± 37.34
100 μM tBHQ	184.99 ± 33.61

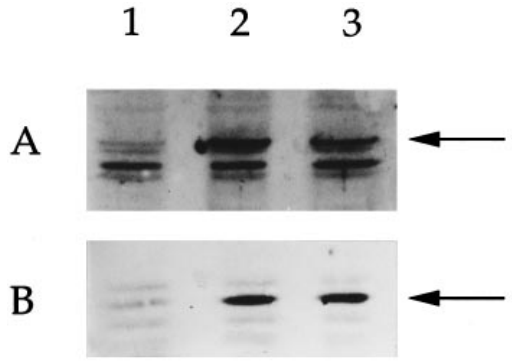

Figure 1 Induction of GCS subunits by tBHQ in HepG2 cells

HepG2 cells were cultured in the presence of tBHQ for 18 h; cell lysate supernatants were prepared as described in the text. Proteins (20 μg per lane) were resolved by SDS/PAGE [10% (w/v) gel] and electrotransferred to nitrocellulose membrane. Membranes were probed with antisera raised against peptides from the GCS_h subunit (**A**) or the GCS_l subunit (**B**) as described in the text. Arrows indicate the positions of the GCS subunits. Lanes were loaded with extracts from cells that had been treated as follows: lane 1, no treatment; lane 2, 75 μM tBHQ; lane 3, 100 μM tBHQ.

Isolation of genomic clones containing upstream regions of human *GLCLR*

It has been shown previously that GCS_h is subject to transcriptional activation in a human alveolar epithelial cell line as an adaptive response to the oxidants menadione and H_2O_2 [23]. Nothing is known about the transcriptional regulation of human GCS_l. To study the transcriptional regulation of GCS_l, we isolated a human *GLCLR* genomic clone as described in the Experimental section. Southern blot analyses of the isolated genomic clone with oligonucleotides HL1 (containing a sequence from the 5′ untranslated region of the GCS_l cDNA; nt −253 to −202) and HL2 (containing a sequence from the open reading frame of the GCS_l cDNA; nt 93–141) was used to identify restriction fragments containing upstream regions of *GLCLR* but excluding most of the open reading frame. Appropriate *GLCLR* fragments were subcloned into pBluescript II SK(+) (Stratagene) generating pDGL5 containing a 1.9 kb *Nco*I fragment and pDGL6 containing a 5.7 kb *Eco*RI fragment (Figure 3). A 1.0 kb *Eco*RI/*Nco*I fragment of pDGL5 was subcloned into pDGL6 to produce pDGL9. pDGL9 contains 6.7 kb of the upstream region of *GLCLR*, a partial restriction map of which is shown in Figure 3.

Exonuclease III digestion [42] was used to make deletions in pDGL5. Deletion product pDGL5Δ205 contains nt −1927 to −205 of *GLCLR* and was chosen as the DNA fragment around which to assemble the *GLCLR* promoter reporter constructs because it lacked the ATG initiation codon but contained a portion of the 5′ untranslated region of the GCS_l cDNA [27].

Restriction fragments from pDGL9 and pDGL5Δ205 were assembled in pCAT-basic (Promega) to give the series of reporter constructs shown in Figure 3. These constructs comprised the following *GLCLR* DNA fragments ligated into pCAT-basic: pDGCAT1 contained a 4.3 kb *Pst*I/*Nco*I fragment of pDGL6 ligated to the 1.7 kb *Nco*I/*Xba*I fragment of pDGL5Δ205, and thus contained 6.0 kb of consecutive *GLCLR* upstream sequence; pDGCAT2 contained a 4.3 kb *Eco*RV/*Xba*I fragment of pDGCAT1; pDGCAT3 contained the 1.7 kb *Nco*I/*Xba*I fragment from pDGL5Δ205; pDGCAT4 contained a 0.8 kb *Hin*dIII/*Xba*I fragment of pDGCAT1; and pDGCAT6 contained a 2.7 kb *Hin*dIII fragment of pDGCAT1. Thus pDGCAT1, pDGCAT2, pDGCAT3 and pDGCAT4 contained *GLCLR* gene fragments of decreasing length (6, 4.3, 1.7 and 0.8 kb respectively) from the distal end. Unlike pDGCAT1, pDGCAT2, pDGCAT3 and pDGCAT4, pDGCAT6 lacked nt −205 to −1007.

Functional analysis of the promoter region of *GLCLR*

Basal promoter activity of *GLCLR* in HepG2 cells

The restriction fragments of *GLCLR* subcloned into the promoterless pCAT-basic plasmid were used to examine basal and inducible expression in HepG2 cells. In transient transfection assays, the pCAT-basic derivatives pDGCAT1, pDGCAT2, pDGCAT3 and pDGCAT4 all demonstrated basal promoter

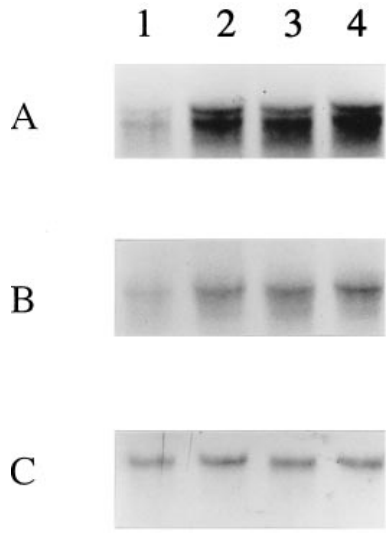

Figure 2 Induction of mRNA encoding GCS subunits by tBHQ in HepG2 cells

HepG2 cells were cultured in the presence of tBHQ for 6 h; total RNA was prepared as described in the text. RNA (25 μg per lane) was fractionated electrophoretically through a 2.2 M formaldehyde/1% (w/v) agarose gel, transferred to nylon membrane and cross-linked with UV. Hybridization with ^{32}P-DNA probes derived from human GCS_h cDNA (**A**), human GCS_l cDNA (**B**) or rat glyceraldehyde-3-phosphate dehydrogenase cDNA (**C**) was performed as described in the text. Lanes were loaded with RNA from cells that had been treated as follows: lane 1, no treatment; lane 2, 50 μM tBHQ; lane 3, 75 μM tBHQ; lane 4, 100 μM tBHQ.

Table 2 Induction of *GLCLR* promoter activity by tBHQ

Four separate sets of transfection experiments were performed. The results shown are from a single experiment and are representative of the relative values found in each experiment. The values for relative CAT activity are expressed as a percentage of the CAT activity from the pCAT-control vector in control and tBHQ-treated cells. Abbreviation: n.d., not detectable.

	Relative CAT activity (%)	
Reporter construct	Control	tBHQ-treated
pCAT-basic	n.d.	n.d.
pDGCAT1	26.9	92.2
pDGCAT2	34.0	97.8
pDGCAT3	115.1	174.1
pDGCAT4	86.0	180.9
pDGCAT6	n.d.	n.d.

activity in HepG2 cells (Table 2). pDGCAT1 and pDGCAT2 were found to have lower transcriptional activity than pDGCAT3 and pDGCAT4, suggesting that there are elements upstream of nt −1927 that regulate basal expression. No promoter activity was detected with pDGCAT6, indicating that regions within nt −205 to −1007 are essential for basal promoter activity.

Inducible promoter activity of *GLCLR* in HepG2 cells

Promoter activity of each of the pDGCAT1, pDGCAT2, pDGCAT3 and pDGCAT4 constructs was found to be induced by tBHQ (Table 2). pDGCAT1 and pDGCAT2 were inducible by approx. 3-fold, whereas pDGCAT3 and pDGCAT4 were inducible by 1.5-fold and 2-fold respectively. This apparent

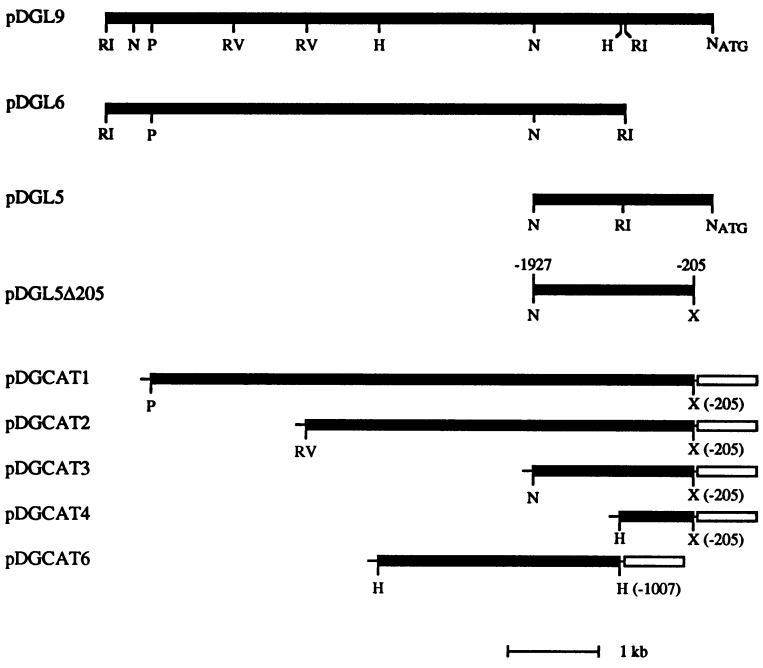

Figure 3 Subcloning of the human *GLCLR* promoter region

Filled boxes denote *GLCLR* DNA; open boxes denote the CAT open reading frame. A partial restriction map is shown for the pDGL9 insert, with restriction endonuclease sites denoted as follows; RI, *Eco*RI; RV, *Eco*RV; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; X, *Xba*I. The *Xba*I site in pDGL5Δ205 lies in the plasmid multiple cloning site, adjacent to the exonuclease III deletion point. N_{ATG} indicates the *Nco*I site overlapping the start of the *GLCLR* open reading frame. The downstream end of the *GLCLR* DNA in CAT reporter constructs is indicated by the nucleotide position, numbered relative to the A residue of the *GLCLR* initiation codon.

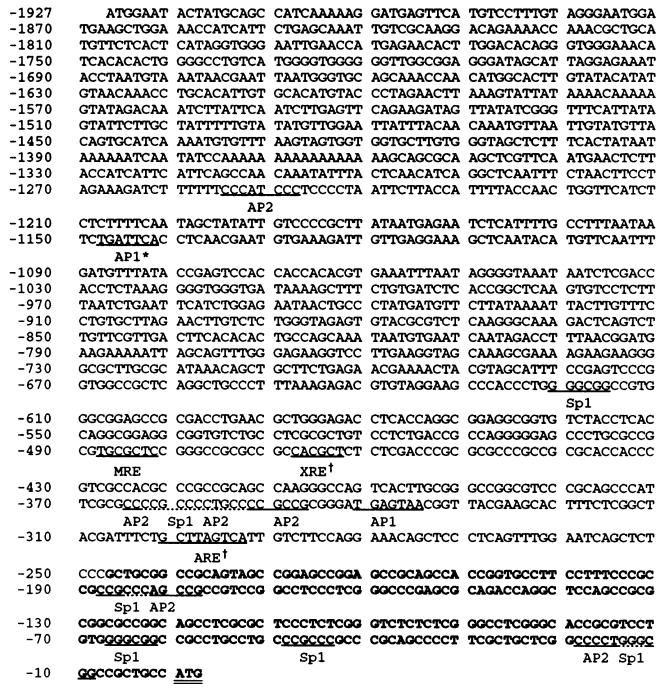

Figure 4 Sequence of the *GLCLR* promoter region

The promoter region of *GLCLR* contained in pDGL5 (nt −1927 to +3) was sequenced. Nucleotides are numbered relative to the GCS_l initiation codon (doubly underlined). The sequence corresponding to the published GCS_l cDNA [27] is shown in bold. Potential regulatory elements are underlined, with dotted lines indicating points of overlap. The symbol † is used to denote the reverse complement of a potential regulatory element. AP1* indicates an AP-1-like site as described by Yao et al. [17].

decrease in the degree of induction in pDGCAT3 and pDGCAT4 coincides with the increase in basal expression observed with these plasmids.

Nucleotide sequence analysis of the promoter region of human *GLCLR*

Both basal and inducible promoter activity were observed with *GLCLR* fragments containing nt −205 to −1927. Therefore the *GLCLR* insert of pDGL5 was sequenced (Figure 4). Analysis of the sequence shows the presence of a number of putative transcription factor-binding elements including AP-1, AP-2 and Sp-1 sites, a metal-responsive element, a reversed ARE and a reversed XRE. An AP-1-like site, as described by Yao et al. [17] in their analysis of the *GLCLC* gene was also found to be present.

DISCUSSION

Mechanisms by which cells regulate glutathione homeostasis are complex, but GCS activity is likely to be of principal importance [7–10]. GCS was first purified from rat kidney over 20 years ago; these early studies demonstrated that GCS is subject to feedback inhibition by glutathione at concentrations at which this is likely to be of physiological importance [43–45]. Rat kidney GCS is composed of two distinct subunits of 73 and 31 kDa, respectively GCS_h and GCS_l. GCS_h was reported to be responsible for all of the catalytic activity of the heterodimer, and from comparative studies with the recombinant subunits and the holoenzyme it was demonstrated that GCS_l is an important mediator of the catalytic efficiency of GCS [12–14]. GCS_l was shown to have the capacity to lower the K_m of GCS_h for glutamate, as well as having a profound effect on the sensitivity to feedback inhibition by glutathione. The regulatory properties of GCS_l have been proposed to be mediated by a disulphide bridge between the subunits that would allow conformational changes in the active site depending on the oxidation state [13]. Thus the potential for increasing the rate of glutathione synthesis exists under conditions of glutathione depletion.

In addition to the regulation of glutathione synthesis at a post-translational level, recent studies have demonstrated that GCS_h can be transcriptionally activated in mammalian cell lines by compounds that have the potential to impose an oxidative stress [19,20,22,23]. Little attention, however, has been paid to the regulation of GCS_l at the transcriptional level. In view of the critical regulatory properties of GCS_l, and its potential to make a significant impact on glutathione homeostasis, it is important to determine whether it is also subject to transcriptional control in response to oxidative stress.

During the present study we found that both GCS_h and GCS_l polypeptides were induced in HepG2 cells in response to treatment with tBHQ. This induction was concomitant with 4-fold and 5-fold increases in GCS_l and GCS_h mRNA respectively, as well as a 2.5-fold increase in intracellular glutathione levels. To study the transcriptional regulation of *GLCLR* we isolated a *GLCLR* genomic clone and subcloned a 6.7 kb fragment of the upstream region of *GLCLR*. Analysis of *GLCLR*/CAT reporter constructs demonstrated that this region contains the *GLCLR* promoter. Reporter constructs pDGCAT1, 2, 3 and 4 were all found to exhibit basal promoter activity, unlike pDGCAT6, which contained the *GLCLR* sequence upstream of nt −1007. This demonstrates that the region between nt −1007 and −205 is necessary for promoter activity. pDGCAT3 and pDGCAT4 exhibited greater basal promoter activity than pDGCAT1 and pDGCAT2, indicating that elements upstream of nt −1927 can cause repression of transcription from the *GLCLR* promoter. Transcriptional activity of pDGCAT1, pDGCAT2, pDGCAT3 and pDGCAT4 was induced by treatment with tBHQ. An increase in CAT activity of approx. 3-fold was shown for pDGCAT1 and pDGCAT2; however, less induction (between 1.5-fold and 2-fold) was observed with pDGCAT3 and pDGCAT4. We have therefore demonstrated that the promoter region of *GLCLR* is inducible by tBHQ, which suggests that transcriptional regulation of this subunit is contributing to the induction of the GCS_l subunit in response to tBHQ in HepG2 cells.

The nucleotide sequence of the promoter region of *GLCLR* was determined and analysed for putative regulatory elements. Comparison of this region with the 5′ flanking region of *GLCLC* reveals several putative enhancer elements in common [17,21], including ARE, XRE, AP-1 and AP-1-like sites, each of which has the potential to be involved in transcriptional regulation in response to chemical and oxidative stress. This observation is of potential relevance to the hypothesis that the GCS_h and GCS_l subunits can be co-ordinately induced as an adaptive response to chemical or oxidative stress.

The ARE was first identified by Pickett and co-workers as an enhancer element involved in the induction of the rat glutathione S-transferase gene *GSTA2* by monofunctional inducing agents such as tBHQ in HepG2 cells ([46,47]; reviewed in [6]). Mulcahy et al. [21] reported the existence of an ARE consensus sequence in the reverse orientation in the human GCS_h gene. The functional importance of this sequence in the GCS_h gene has not been defined, but studies with human alveolar epithelial cells have demonstrated that the ARE is unlikely to be important in the

induction of GCS_h in response to cigarette smoke condensate, menadione or H_2O_2 [22,23]. Rather, these experiments implicated the involvement of the AP-1 or AP-1-like site in induction of GCS_h. The observation that BHA and related compounds can cause the induction of c-*fos* and c-*jun* gene expression in HepG2 cells, as well as an increase in AP-1-binding activity [48], is consistent with a role for these proteins in the regulation of gene expression by phenolic antioxidants.

The involvement of an AP-1-like site in the overexpression of GCS_h in cisplatin-resistant ovarian carcinoma cell lines has been proposed by Yao et al. [17]. The AP-1-like site (5′-TGATTCA-3′) differs from the AP-1 consensus sequence (5′-TGACTCA-3′) [49,50] but was shown to bind Jun homodimers [17]. It is of particular interest that, in addition to an AP-1 consensus sequence, the nucleotide sequence of the human *GLCLR* promoter region also contains this AP-1-like site. The functional importance of this putative enhancer is not yet understood, but it is notable that Yao et al. [17] did not find co-ordinate regulation of GCS_h and GCS_l mRNA in cisplatin-resistant cell lines. Previous studies of the cisplatin-resistant ovarian cancer cell line C200 by Western blotting showed an increase in levels of both the GCS_h and GCS_l polypeptides [4], but the level of GCS_l mRNA was not found to be elevated [17]. These results imply that additional regulatory mechanisms exist, and other studies where the tissue-specific regulation of the GCS_h and GCS_l mRNA have been examined also suggest that the regulation of the two subunits might not be co-ordinated [27]. The biological significance of the differential or co-ordinate regulation of the two subunits is unknown, nor is the molar ratio of the subunits in different tissues or cell types yet known.

Induction of the GCS_l subunit via transcriptional activation clearly represents an additional level of control of glutathione biosynthesis and warrants further investigation. These studies are currently under way in our laboratory.

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