Cloning and characterization of the 5′-flanking region of the rat glutamate-cysteine ligase catalytic subunit

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Glutamate-cysteine ligase (GCL), the rate-limiting enzyme in glutathione synthesis, is made up of two subunits, a catalytic (heavy) subunit (GCLC) and a modifier (light) subunit (GCLM), which are differentially regulated. Increased hepatic GCLC expression occurs during rapid growth, oxidative stress and after ethanol treatment. To facilitate studies of GCLC transcriptional regulation, we have cloned and characterized a 1.8 kb 5′-flanking region of the rat GCLC (GenBank accession number AF218362). A consensus TATA box and one transcriptional start site are located at 302 and 197 nucleotides upstream of the translational start site, respectively. The promoter contains consensus binding sites for many transcription factors including nuclear factor κB (NF-κB) and activator protein 1 (AP-1). The rat GCLC promoter was able to efficiently drive luciferase expression in H4IIE cells. Sequential deletion analysis revealed that three DNA regions, -595 to -111, -1108 to -705 and -705 to -595, are involved in positive (the first two regions) and negative (the latter region) gene regulation. Specific protein binding to these regions was confirmed by DNase I footprinting and electrophoretic mobility-shift assays (EMSAs). Ethanol-fed livers exhibit increased protein binding to region -416 to -336 on DNase I footprinting analysis, which was found to be NF-κB and AP-1 on EMSA and supershift analysis. Acetaldehyde treatment of H4IIE cells led to a time- and dose-dependent increase in GCLC mRNA levels, binding of NF-κB and AP-1 to the GCLC promoter, and luciferase activity driven by the GCLC promoter fragment containing these binding sites.

Key words: acetaldehyde, glutathione, H4IIE cell.

INTRODUCTION

Glutathione (GSH) is the main non-protein thiol in mammalian cells that participates in many critical cellular functions, including antioxidant defence and cell growth [1–3]. GSH is synthesized in the cytosol of all mammalian cells via two ATP-requiring enzymic steps: the formation of γ-glutamylcysteine from glutamate and cysteine, and formation of GSH from γ-glutamylcysteine and glycine. The first step of GSH biosynthesis is generally regarded as rate-limiting and catalysed by glutamate-cysteine ligase (GCL, also known as γ-glutamylcysteine synthetase), which is regulated physiologically by feedback competitive inhibition by GSH and the availability of cysteine [1,4]. The GCL enzyme is composed of a catalytic or heavy subunit (GCLC, $M_r \approx 73000$) and a modifier or light subunit (GCLM, $M_r \approx 30000$), which are encoded for by different genes and dissociate under reducing conditions [5–7]. The heavy subunit exhibits all of the catalytic activity of the isolated enzyme as well as feedback inhibition by GSH [7]. The light subunit is enzymically inactive but plays an important regulatory function by lowering the K_m of GCL for glutamate and raising the K_i for GSH [6,8]. Since GCL is a major determinant of the overall GSH synthesis capacity, regulation of GCL subunits has been a topic of extensive research [1]. Changes in GCL activity can result from regulation at multiple levels, affecting only the heavy or light subunit or both. The 5′-flanking regions of the human GCL subunits have been cloned [9–11]. Antioxidant-response element (ARE), activator protein 1 (AP-1) and nuclear factor κB (NF-κB) are three *cis*-acting elements present in the promoter of the GCLC that have been implicated in its transcriptional regulation, based largely on studies in transfected cell lines [1,9,12–15]. Our laboratory has described regulation of rat hepatic GCLC expression using both *in vitro* and *in vivo* treatments. GCLC expression increased during periods of rapid hepatocyte growth, after treatment of hepatocytes with hormones such as insulin or glucocorticoids, or agents that induce oxidative stress, and after treatment of rats with thioacetamide or ethanol [16–22]. In order to better understand the molecular mechanism(s) responsible for these changes, we have cloned and characterized the 5′-flanking region of the rat GCLC.

MATERIALS AND METHODS

Materials

Cell-culture media and fetal bovine serum were obtained from Gibco-BRL Life Technologies (Grand Island, NY, U.S.A.). The Luciferase Assay System and the β-Galactosidase Enzyme Assay System were obtained from Promega (Madison, WI, U.S.A.). All restriction enzymes were obtained from either Promega or Gibco-BRL. [^{32}P]dCTP (3000 Ci/mmol) was purchased from New England Nuclear (DuPont, Boston, MA, U.S.A.). Total RNA isolation kit was obtained from Promega. All other reagents were of analytical grade and were obtained from commercial sources.

Abbreviations used: AP-1, activator protein 1; ARE, antioxidant-response element; C/EBP, CAAT-enhancer-binding protein; EMSA, electrophoretic mobility-shift assay; GCL, glutamate-cysteine ligase; GCLC, GCL catalytic or heavy subunit; GCLM, GCL modifier or light subunit; HSF, heat-shock transcription factor; MZF1, myeloid zinc finger 1; NF1, nuclear factor 1; NF-κB, nuclear factor κB; SRY, sex-determining region of the Y chromosome; VBP, vitellogenin gene-binding protein.

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Animal model of alcoholic liver disease

The rat model of alcoholic liver disease was detailed previously [22]. Animals were killed after 9 weeks of intragastric infusion of a high-fat diet plus isocaloric dextrose or ethanol. Liver specimens were snap-frozen in liquid nitrogen for subsequent extraction of nuclear protein as described in [22].

Effect of acetaldehyde on GCLC expression in H4IIE cells

H4IIE cells were grown according to instructions provided by the ATCC (ATCC no. CRL-1548). Cells were treated with 25–200 μM acetaldehyde for 16 h or 100 μM acetaldehyde for 30 min to 16 h. At the end of the treatment, total RNA was extracted and Northern hybridization analysis was performed using specific rat GCLC cDNA probe as described in [22]. To ensure equal loading of RNA samples and transfer in each of the lanes, prior to hybridization, membranes were rinsed with ethidium bromide and photographed, and the same membranes were also rehybridized with a ^{32}P-labelled β-actin cDNA probe as described in [20]. Autoradiography and densitometry (Gel Documentation System, Scientific Technologies, Carlsbad, CA, U.S.A., and NIH Image 1.60 software program) were used to quantitate relative RNA. Results of the Northern-blot analysis were normalized to β-actin.

Cloning of the 5′-flanking region of the rat GCLC gene

A oligonucleotide probe corresponding to positions -35 to $+2$ of the rat GCLC cDNA [5] was used to screen the rat genomic library EMBL 3 (Clontech, Palo Alto, CA, U.S.A.). Five positive plaques were selected, and DNA was isolated and digested with *Eco*RI. The insert fragment was subcloned into pGL-3 enhancer vector (Promega) and sequenced in both directions using the automated ABI Prism dRhodamine Terminator Cycle Sequencer performed by the Sequencing and Genetic Analysis Core Facility, Department of Cell and Neurobiology, USC School of Medicine, Los Angeles, CA, U.S.A. The initial primers were universal primers for the pGL-3 enhancer vector, and all subsequent primers were nested primers designed using the available sequence information and the MacVector program. The nucleotide sequence was verified by multiple bi-directional sequencing reactions. Sequences were aligned and a consensus sequence generated using the ASSEMBLIGN program. A 1.76 kb 5′-flanking region of the rat GCLC was cloned into the *Sma*I site of promoterless pGL-3 enhancer vector creating the recombinant plasmid $-1758/+2$ GCLC-luc.

RNase protection assay

RNase protection assay was done according to instructions provided in the Multi-NPA™ manual (Ambion). The probes were synthesized by linear amplification, the primers were 5′-CCGGTGTCTCCGCACGTGGTCGGC-3′ and 5′-GTGTCTCCGCACGTGGTCGGCGTG-3′, which are reverse and complementary to positions $+52$ to $+76$ and $+49$ to $+73$ of the rat GCLC [5]. The templates were *Acc*III- and *Afl*II-digested fragments (301 bp and 577 bp) from the 1.76 kb 5′-flanking region.

Primer-extension analysis

Primer-extension analysis was done as described in [23]. One antisense oligonucleotide primer complementary to -24 to $+2$ nt relative to the translational start site of the rat GCLC [5] was end-labelled with [γ-^{32}P]ATP using T_4 polynucleotide kinase. Poly(A$^+$) RNA (2.5 μg) from rat liver, isolated as described in [24], was annealed to 10^6 c.p.m. of the primer and extended with 200 units of Moloney murine leukaemia virus reverse transcriptase (Superscript II, Life Technologies). The primer-extended product was analysed on 7 M urea/6 % polyacrylamide gels.

Construction of 5′-deletion constructs

The 1.76 kb fragment in the sense orientation upstream of the luciferase coding sequence of the pGL-3 enhancer vector is the construct that contains the longest 5′-flanking sequence (-1758 to $+2$) employed in the transfection assay. To prepare 5′-deletion constructs, this plasmid was subjected to digestion with additional restriction enzymes to generate a series of deletion mutants. The enhancer/reporter transgene $-1108/+2$ GCLC-luc was created by cloning an *Acc*65I fragment, $-705/+2$ GCLC-luc was created by cloning a *Nhe*I fragment, $-595/+2$ GCLC-luc was created by cloning an *Age*I fragment and $-111/+2$ GCLC-luc was created by cloning a *Sma*I fragment.

Analysis of promoter constructs in cell culture

To study the relative transcriptional activities of the GCLC promoter fragments, H4IIE cells (1×10^6 cells in 4 ml of medium) were transiently transfected with 8 μg of GCLC-promoter/luciferase gene construct or promoterless pGL3 enhancer vector (as negative control) and 2 μg of a β-galactosidase expression plasmid (as an internal standard for transfection efficiency) using the calcium phosphate precipitation method [25]. After 20 h, cells were harvested and lysed in 1 ml of reporter lysis buffer (Luciferase Assay System, Promega). The luciferase assay was performed on 20 μl of the cleared lysate and 100 μl of luciferase assay reagent using a TD-20/20 Luminometer (Promega). The β-galactosidase assay was done according to the supplier's instructions (β-Galactosidase Enzyme Assay System, Promega) using 150 μl of the cell lysate. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). The luciferase activity of each transfection was normalized to β-galactosidase activity/protein concentration.

The effect of acetaldehyde on GCLC promoter activity was examined by measuring luciferase activity driven by GCLC-promoter/luciferase gene constructs in transfected H4IIE cells treated with acetaldehyde (100 μM) during the last 16 h of the transfection.

DNase I footprinting analysis

^{32}P-End-labelled fragments of the 5′-flanking region of rat GCLC implicated in positive and negative regulation were generated by digestion with restriction endonucleases and PCR. DNase I footprinting analysis was performed using double-stranded fragments corresponding to nucleotides -1108 to -918, -917 to -706, -705 to -590, and -566 to 290 of the rat GCLC gene. Singly end-labelled fragments were generated by filling 5′-protruding ends with [α-^{32}P]dCTP (3000 Ci/mmol) using the exo-Klenow enzyme or end-labelled with [γ-^{32}P]ATP using T_4 polynucleotide kinase. Labelled probes were purified by electrophoresis with 2 % agarose gel. Approx. 5×10^4 c.p.m. of end-labelled DNA fragments were incubated with 0–20 μg of nuclear protein from H4IIE cells. After 30 min of incubation on ice, $CaCl_2$ and $MgCl_2$ were added to give final concentrations of 0.5 mM and 1 mM, respectively. DNase I digestions were performed at room temperature for 1 min. Upon phenol extraction and ethanol precipitation, DNA fragments were resolved by electrophoresis in a denaturing 8 % acrylamide sequencing gel.

DNase I footprinting analysis of the region −566 to 290 was also performed using liver nuclear protein obtained from rats fed the intragastric ethanol plus high-fat diet for 9 weeks or high-fat controls.

Electrophoretic mobility-shift assay (EMSA) and supershift analysis

EMSAs for different regions of the rat GCLC promoter were done as described in [23]. Nuclear protein (20–40 μg) from H4IIE cells was preincubated with 2 μg of poly(dI-dC) in a buffer containing 10 mM Hepes (pH 7.6), 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$ and 10 % glycerol for 10 min on ice. ^{32}P-End-labelled double-stranded DNA fragments (positions −1086 to −998, −990 to −965, −707 to −597, −538 to −479, −478 to −419, −418 to −359, and −358 to −303) were then added with or without a 100-fold excess of unlabelled specific probe or oligonucleotides containing sequences for binding of potential transcription factors. Mixtures were incubated for 20 min on ice, loaded on to a 4 % non-denaturing polyacrylamide gel and subjected to electrophoresis in 50 mM Tris, 45 mM borate and 0.5 mM EDTA (pH 8.0). Gels were dried and subjected to autoradiography.

To see whether ethanol feeding of rats and acetaldehyde treatment of H4IIE cells resulted in increased NF-κB and AP-1 binding to the GCLC promoter, EMSA for the NF-κB site (shown underlined, 5′-TTGCTAACACCCGGGAACACCC-ACGGCCTC-3′, −390 to −361 of GCLC) and the AP-1 site (shown underlined, 5′-GGCCTCAACCCCTGACGGCCCCG-3′, −366 to −344 of GCLC) was done using 20 μg of nuclear protein from 9-week ethanol-fed livers, pair-fed control livers, and acetaldehyde- (100 μM for 16 h) or vehicle-treated H4IIE cells as above. Further confirmation of the identity of the binding proteins was done by antibody supershift assays with anti-c-Jun, anti-c-Fos and anti-p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) as described in [21].

Statistical analysis

Data are given as means ± S.E.M. Statistical analysis was performed using ANOVA followed by Fisher's test for multiple comparisons. For changes in mRNA levels, ratios of GCLC to β-actin densitometric values were compared by two-tailed paired Student's t test. Significance was defined by $P < 0.05$.

RESULTS

Cloning and sequencing of the 5′-flanking region of the rat GCLC

The sequence of the 1.76 kb product is shown in Figure 1. A canonical TATA box is located at position 302–296 upstream of the translational start site. Analysis of the transcription-factor-binding site was done using Transcription Factor Search (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html) and MatInspector V2.2 (http://www.gsf.de/cgi-bin/matsearch.pl). The 5′-flanking region of the rat GCLC contains several consensus binding sites for CAAT-enhancer-binding protein (C/EBP), AP-1 and myeloid zinc finger 1 (MZF1), and one binding site for NF-κB. In addition, consensus binding sites for heat-shock transcription factors (HSFs) 1 and 2, transcription factor encoded by the sex determining region of the Y chromosome (SRY), upstream stimulatory factor (USF), nuclear factor 1 (NF1), vitellogenin gene-binding protein (VBP) and c-Myc are also present.

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                                    SRY
-1758 CTGGAGAATC  TCCAGCATCC  AGAAACAAAG  AAAGATCAGA  ACATAGTTAA  GGACCTAGAA
            MZF1                                                      MZF1
-1698 AGGTTGGTGG  GGAGGGCATA  GTAAGGTAGG  AGGCAGTACG  TAGCAAAGGT  TGAGTGGGGA
                  AP1
-1638 GGGTAGGCTG  ACTTTTTTTA  AAAGCATTAC  TCCAGCTATG  TGCTAGAGCT  AGATGGCATC
       c-Myc
-1578 ACCTGTCTCC  TCGGTTTAAG  ATGTCATCTA  ACCCATGTAC  ACATCTTATG  CATTCCACTG
                                    IK2
-1518 TGGAAACCAC  AGCCATACCA  TCAGTGTCCG  TGGTCCCAAG  CTCTTTGGTG  GACACATTCA
                                                              SRY
-1458 ATATGAAGGC  AGTGAGGAAA  TATTCCTATC  TTGCATTCAT  CCATTAGTGT  GTTTATTGTA
                                    CdxA
-1398 TGTTGTGATT  GGTGTTCGTT  AAATATTAAA  TCTAAGGCAA  ATGTGGAAGT  CCATGATTCT
            MZF1
-1338 AATCCCAGCA  CTGGGGAAGC  TGAGGCAAGA  CGGCTGCCTG  GAGTTCAAGG  CCAGATTGGG

-1278 CTACATAGTG  AGATCTTGTC  TCTAAATAAC  TACATATTAA  GAATGTAGTC  ACAGTGTGGG
            CdxA
-1218 GGAAGGGTAT  AATAAATGTT  TTCTGCCCTC  TGTACATGAA  TGGATCGTCT  AGTTACTGCA
                        C/EBP                             Sp1
-1158 TATATTTATC  CAGTTGCAAA  ACATTAATCG  AACAACTACT  CAGGGGCAAG  GTACCTTCCC
                                          AP4                 RFX1
-1098 TTCGTTCCCA  CGGGATCCAG  TTCCTAGAGC  CTTCAGCACT  CAGGGTAAAG  CGCTCCTGAG
                  AP1                     E2F                 HSF1&2
-1038 CAACAGAATG  ACCACTGCTG  GACGAATCTT  TGGCTCTGAA  GGTGGGGAAA  CTTCTGAAGA
      HSF1&2                                                  C/EBP
 -978 AATTTCTCAC  ACCACACACT  TGGAGACAGT  CACACAACCT  CTTTTGCACA  ATGAATGATG
      AP1
 -918 TCTGTGTCAA  CCAGAGATCT  TATTGGGAAG  TGGGATTTAC  AATGATTAGA  CACACGTATG

 -858 TCTAATCTCC  GTAGATACAT  GAACGCATGT  AAATAACCAG  CAATCAACTC  TATGCACATG
                        CdxA
 -798 TGGAGCCACA  CAACATTCAT  TAAAAAGTAC  AACTCAGCCA  GGCATGGTGG  CACACACCTT
            USF
 -738 TGATCCCGGC  ACATGGGAAG  CAGGAGTTCC  AGGCTAGCTT  GGTCTACAAA  GCCAGTCCGG
       NF1                              C/EBP                  MZF1
 -678 GACTGCCAAG  GCTACACAGA  GAAACTCTGT  CTTGAAAAAC  CAAGAAGAAA  AAAGGGGGGA
            VBP
 -618 AAAAGTGTTA  CTTTAACTAT  ATACCGGTAC  AAACAGTTGA  GAAGATACTT  AAGCTGCCTT
                        C/EBP
 -558 CGGAGTTAAT  TATTTTTCAC  AACACATTTT  TATCTCTCAT  CGTTTTGTAA  GCATGAGGCT
       MZF1                                    CdxA        Sox-5
 -498 CCCTCCCCAT  CTAAAAAGTA  ATCTGGTCCG  CTCTTCATTA  ATTTAAACAA  TCTCTTTTTT
                              IK1/IK2                         C/EBP
 -438 TTCCTTGGCC  CAGTATTCTC  TTGGGAACCA  AGATTTTTCA  GTCCTTATTT  GCTAACACCC
      NFkB                    AP1                         AP4
 -378 GGGAACACCC  ACGGCCTCAA  CCCCTGACGG  CCCCGCCCAC  GACTCAGCCG  CTTCGTTCCC
      Sp1
 -318 CGCCCCCGCC  GTAGCCTATA  AAATCCGGAC  GCGGCGCCCG  GAGCGGCAGG  CGAGGAACTG

 -258 GGCATGCTCG  GTGTCGCCGC  AGCCTTGGGT  CGCAAGTAGG  AAGCCCGTGC  ACGACACCGC

 -198 GGCGCCGAGA  GGACGTTACC  GCGGCGGCCT  GGACCGCAGG  GCCGGCGTCC  AAGCGCGAGG

 -138 CGGGGCGGCG  GCGAGAGACC  GCGGCCCGGG  CCGTCAACGC  GCGGCGGGGT  CCGAGCCGGA

  -78 GCGGGAACGG  ACGGGACGCC  GCCGCGCGGC  CCGAGGGCGC  CCCCAGAGCG  GAGCTGCGGC
                  Translational start site
  -18 CAGGAGGAGG  ACGCGGCCAT
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Figure 1 Nucleotide sequence of the 5′-flanking region of the rat GCLC gene

The sequence is numbered relative to the translational start site. The consensus TATA box is shown in italic and underlined. The putative regulatory elements are indicated in bold letters above the underlined sequences. USF, upstream stimulatory factor; RFX1, regulatory factor X-1. For other abbreviations, see the text.

Transcriptional start site

RNase protection assay was used to determine the transcriptional start site. Two antisense oligonucleotide primers complementary to nt +52 to +76 (primer 1) and +49 to +73 (primer 2) relative to the translational start site of the rat GCLC [5] were annealed to poly(A^+) RNA from rat liver and extended towards the 5′ end of the mRNA by reverse transcription. Figure 2 shows that the primer-extension reaction yielded products of approx. 270 nt long using both primers. These products were not detected when the assay was carried out using tRNA (results not shown). These results are consistent with the transcriptional start site being located approx. 99–103 nt downstream of the concensus TATA box or about 194–197 nt upstream of the translational start site. To further delineate and confirm the transcriptional start site, primer-extension analysis was carried out. Using a primer that is reverse and complementary to −24 to +2, a product of about 200 nucleotides was obtained. The sequencing

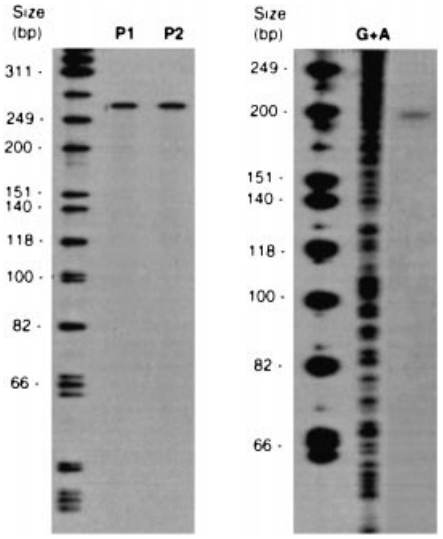

Figure 2 Determination of the transcriptional start site of the rat GCLC gene by RNase protection assay (left-hand panel) and primer-extension analysis (right-hand panel)

In RNase protection assay, the primers are reverse and complementary to +52 to +76 (primer 1 or P1) and +49 to +73 (primer 2 or P2) of the rat GCLC. In primer extension, the primer is reverse and complementary to −24 to +2 of the rat GCLC. See the Materials and methods section for details. Lanes G+A represent a Maxam–Gilbert sequencing reaction in the same fragment. Size markers correspond to ØX174 digested with *Hin*f1.

gel confirmed that the transcriptional start site is at 197 nucleotides upstream of the translational start site (Figure 2).

Functional analysis of the 5′-flanking region of rat GCLC

To delineate sequences that drive the expression of the rat GCLC, five 5′-terminal nested deletion mutants ranging from −1758/+2 to −111/+2 were cloned into the promoterless luciferase reporter-gene vector pGL3 enhancer. The promoterless construct pGL3 enhancer served as the background control. Luciferase activity was measured after transient transfection of H4IIE cells with these constructs. Figure 3 shows that the rat GCLC promoter was able to drive efficiently luciferase expression in H4IIE cells. The construct −111/+2 produced almost no activity, as it is downstream from the transcriptional start site. The construct −595/+2 produced maximal promoter activity whereas the construct −705/+2 produced about half-maximal activity, indicating the presence of important elements between −595 and −111 and −705 and −595 that positively or negatively regulated the promoter activity, respectively. Presence of positive regulatory element is also suggested in the region of −1108 and −705 as the construct −1108/+2 produced nearly maximal activity. Inclusion of an additional 650 bp upstream had no significant influence on promoter activity.

DNase I footprinting analysis of rat GCLC 5′-flanking region

To further characterize the regulatory regions, DNase I footprinting analysis was carried out. Figure 4 shows footprinting results using probes consisting of different promoter regions. Three nuclear-protein-dependent DNase I-protected areas are present in each of the regions −1108 to −918 (−1058 to −1039, −1032 to −1006, −988 to −966), −705 to −590 (−685 to −663, −654 to −628, −612 to −600) and −566 to −290

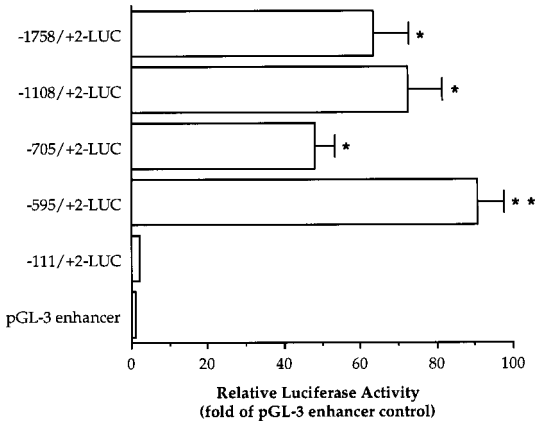

Figure 3 Transient-transfection analysis of the rat GCLC-promoter/luciferase constructs in H4IIE cells

Progressive 5′ deletions of the GCLC promoter extending from −1758 to +2 bp were generated and fused to the promoterless luciferase pGL-3 enhancer vector as described in the Materials and methods section. Numbering is defined relative to the translational start site. Results represent means ± S.E.M. from four independent experiments performed in triplicate. Data are expressed as relative luciferase activity to that of pGL-3 enhancer vector control, which was assigned a value of 1.0. $^{*}P < 0.05$ versus the pGL-3 enhancer control; $^{**}P < 0.05$ versus the pGL-3 enhancer control and the construct −705/+2-LUC (ANOVA followed by Fisher's test).

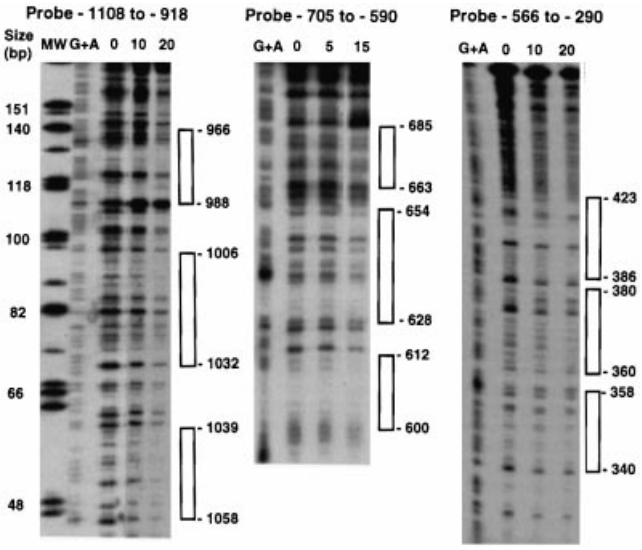

Figure 4 DNase I footprinting analysis of the −1108 to −918, −705 to −590 and −566 to −290 regions of the rat GCLC promoter

DNA fragment containing the −1108 to −918 (upper strand), −705 to −590 (lower strand) and −566 to −290 (lower strand) regions of the rat GCLC promoter were end-labelled and digested with DNase I in the absence (0) or presence of 5–20 μg of nuclear-protein extracts from H4IIE cells. Positions of the protected regions are indicated on the right of the panels. Lanes G+A represent a Maxam–Gilbert sequencing reaction in the same fragments. Size markers correspond to ØX174 digested with *Hin*f1.

(−423 to −386, −380 to −360, −358 to −340) of the rat GCLC promoter. No DNase I-protected areas were seen in the region −917 to −704 (results not shown).

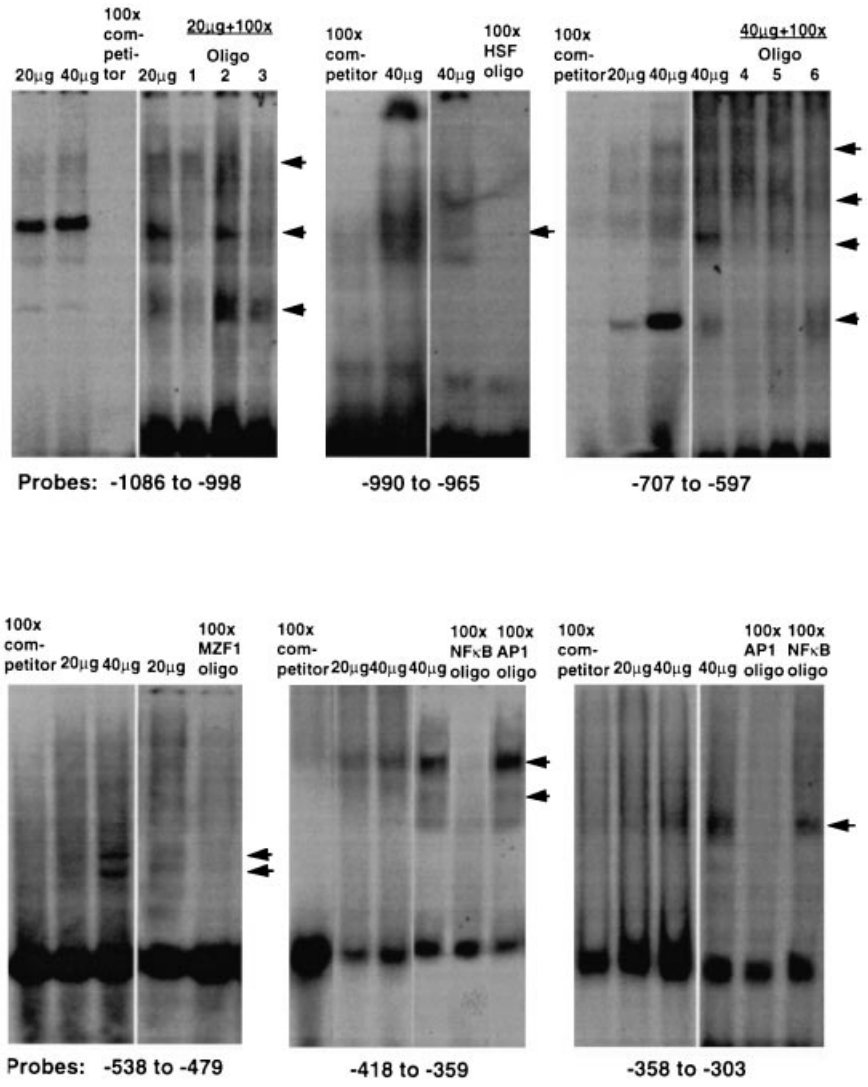

Figure 5 EMSAs for different regions of the rat GCLC promoter

Nuclear-protein extracts (20–40 μg) were obtained from H4IIE cells and EMSA was performed as described in the Materials and methods section using probes that span different regions of the rat GCLC promoter. The arrows point to specific complexes that were competitively blocked when 40 μg of nuclear protein was incubated with radiolabelled probes in the presence of 100 × unlabelled specific probes or 100 × unlabelled oligonucleotides containing specific sequences for binding of transcription factors (see Table 1).

Table 1 Oligonucleotide probes used for EMSA

Consensus binding sites for transcription factors (in parentheses unless indicated by name) are underlined. RFX1, regulatory factor X-1.

Name of probe	Sequence (5′ → 3′)	Position
Oligo 1 (AP-1)	CAACAGAATGACCACTGCTGGA	−1038
Oligo 2 (RFX1)	TAAAGCGCTCCTGAGCAACAG	−1053
Oligo 3 (AP-4)	TTCCTAGAGCCTTCAGCACTCAGG	−1078
HSF oligo	GAAGAAATTTCTCACACCACACACT	−983
Oligo 4 (NF1)	GCCAGTCCGGGACTGCCAAGGCTA	−688
Oligo 5 (C/EBP)	GAAACTCTGTCTTGAAAAACCAA	−658
Oligo 6 (VBP)	AAGTGTTACTTTAACTATATACCGG	−615
MZF1 oligo	AGGCTCCCTCCCCATCTAAAAA	−503
NF-κB oligo	TTGCTAACACCCGGGAACACCCACGGCCTC	−390
AP-1 oligo	GGCCTCAACCCCTGACGGCCCCG	−366

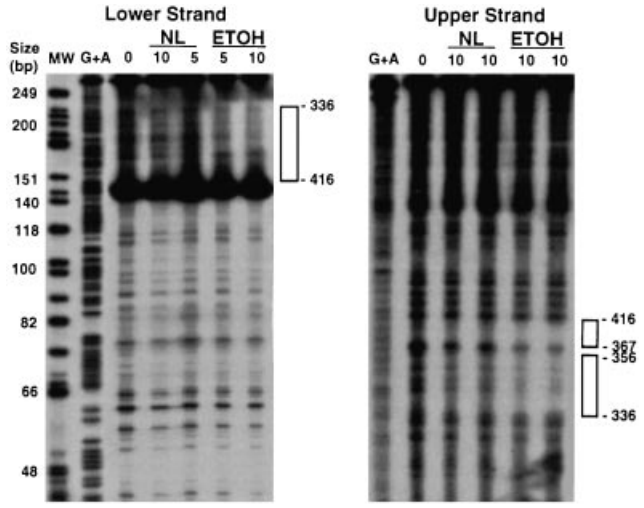

Figure 6 DNase I footprinting analysis of the −566 to −290 region of the rat GCLC promoter

DNA fragment was end-labelled on either strand and digested with DNase I in the absence (0) or presence of 5–10 μg of nuclear-protein extracts from ethanol-fed (ETOH) or paired-fed normal liver (NL). Positions of the protected regions are indicated on the right. Lanes G+A represent a Maxam–Gilbert sequencing reaction in the same fragments. Size markers correspond to ØX174 digested with *Hin*f1.

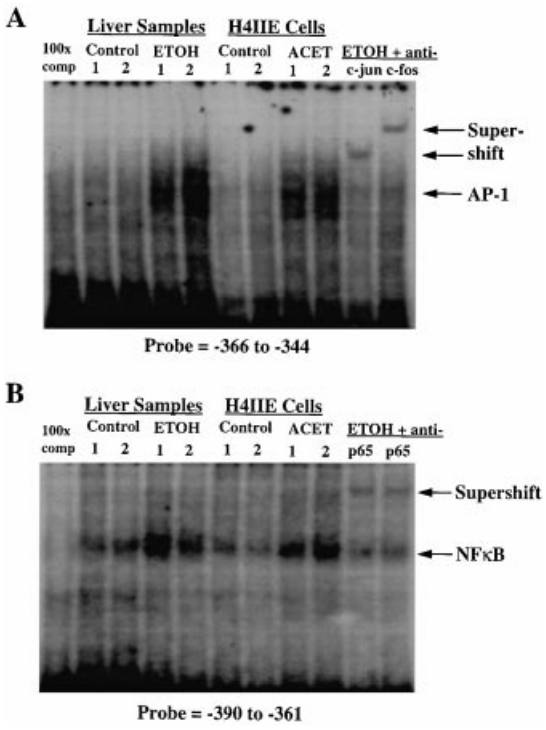

Figure 7 EMSA and supershift assay for the probes that span −366 to −344 (A) or −390 to −361 (B) of the rat GCLC gene

EMSA and supershift were done as described in the Materials and methods section. There is increased binding to both probes in ethanol-fed livers (ETOH) and acetaldehyde-treated (ACET) H4IIE cells as compared with their respective controls. Binding of ethanol-fed liver nuclear extracts to the probes disappeared in the presence of 100 × unlabelled probe. Supershift analysis showed that the increased binding to probes −366 to −344 and −390 to −361 is due to AP-1 complex containing both c-Jun and c-Fos (**A**) and NF-κB (**B**), respectively.

Analysis of regulatory regions using EMSA

Based on the results of the deletion analysis and DNase I footprinting, EMSAs were carried out using probes that span regions implicated in positive or negative regulation of the rat GCLC. Figure 5 shows that there is specific protein binding to regions −1086 to −998 (three distinct bands), −990 to −965 (one broad band), −707 to −597 (four distinct bands), −538 to −479 (two distinct bands), −418 to −359 (two distinct bands) and −358 to −303 (one distinct band). No specific binding was seen in the region −478 to −419 (results not shown). In each case, specificity was assured by the disappearance of the bands in the presence of 100-fold specific competitor.

To further identify specific transcription factors that may be bound, competition by oligonucleotides containing specific binding sequences was carried out for each of the fragments. Table 1 describes the oligonucleotide probes which were designed to span potential binding sites for specific transcription factors in these regions. In the region −1086 to −998, the lower two bands decreased in intensity in the presence of oligonucleotide 1, which spans the AP-1 site, and the upper two bands decreased in intensity in the presence of oligonucleotide 3, which spans the AP-4 site, whereas oligonucleotide 2, which spans the RFX1 (regulatory factor X-1) site had no effect. In the region −990 to −965, binding was completely prevented in the presence of the HSF oligonucleotide. In the region −707 to −597, the lower two bands disappeared or decreased in intensity in the presence of oligonucleotide 4, which spans the NF1 site, the lower band also decreased in the presence of oligonucleotide 5, which spans the C/EBP site, whereas the top three bands decreased in the presence of oligonucleotide 6, which spans the VBP site. In the region −538 to −479, binding was prevented with the MZF1 oligonucleotide. In the region −418 to −359, both bands disappeared in the presence of the NF-κB but not the AP-1 oligonucleotide. Finally, in the region −358 to −303, the specific band disappeared in the presence of the AP-1 but not the NF-κB oligonucleotide.

Molecular mechanism of increased GCLC expression in alcoholic rat liver

We showed previously that the steady-state GCLC mRNA level more than doubled in response to ethanol feeding [21]. To elucidate the molecular mechanism, we examined DNase I footprinting analysis of the region −566 to −290 in control and ethanol-fed livers. Figure 6 shows results of the DNase I footprinting analysis using double-stranded fragment corresponding to nucleotides −566 to −290 of the rat GCLC gene. The region −416 to −336 is protected from DNase I digestion in the presence of nuclear proteins from ethanol-fed livers but not from control livers on both strands. Since we had identified NF-κB and AP-1 as possible transcription factors that bind to this region (Figure 5), we next performed EMSA with supershift analysis using probes that span these sites in the ethanol-fed livers. Figure 7 shows that in ethanol-fed livers there is increased AP-1 and NF-κB binding to the GCLC promoter fragments, as confirmed by supershift analysis.

Effect of acetaldehyde on GCLC expression in H4IIE cells

Acetaldehyde is a major metabolite of ethanol and is considered as a critical mediator of many of ethanol's effects [26]. To develop a convenient *in vitro* model for the studies of ethanol's effect, we examined the effect of acetaldehyde treatment on

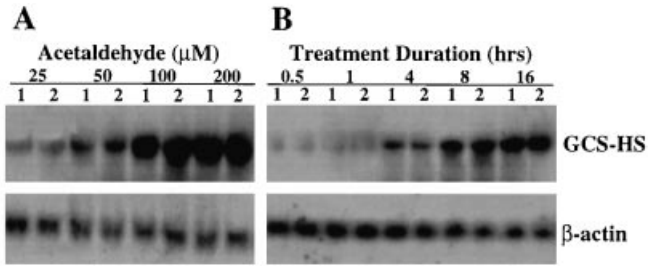

Figure 8 Effect of acetaldehyde on GCLC expression in H4IIE cells

RNA (30 μg/lane) samples from H4IIE cells treated with various doses (25–200 μM) of acetaldehyde for 16 h (**A**), or 100 μM acetaldehyde for 0.5–16 h (**B**) were analysed by Northern-blot analysis with a ^{32}P-labelled GCLC cDNA probe (GCL-HS) as described in the Materials and methods section. The same membrane was then rehybridized with a ^{32}P-labelled β-actin cDNA probe. Representative Northern blots are shown.

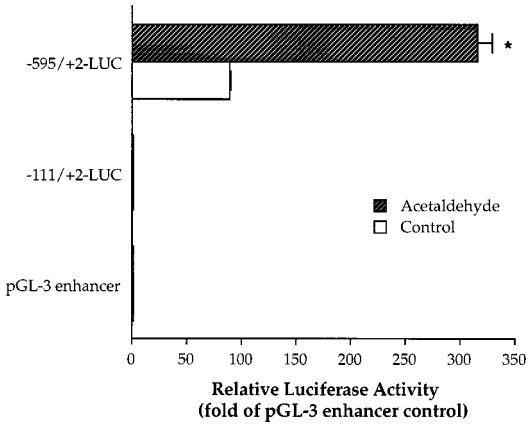

Figure 9 Effect of acetaldehyde treatment on luciferase expression driven by the rat GCLC promoter

H4IIE cells were transfected with rat GCLC promoter/luciferase constructs $-111/+2$-LUC, $-595/+2$-LUC or promoterless pGL-3 enhancer vector and treated with acetaldehyde (100 μM for 16 h) or vehicle (control). Results represent means $\pm$ S.E.M. from three independent experiments performed in duplicate. Data are expressed as relative luciferase activity to that of the pGL-3 enhancer vector, which was assigned a value of 1.0. $^*P < 0.05$ versus the vehicle control.

GCLC expression in H4IIE cells. Figure 8 shows that acetaldehyde caused a dose- and time-dependent increase in GCLC mRNA level. Maximum effect was seen with a 100 μM dose and treatment for 16 h. There was no cell lysis with this treatment regimen, as measured by release of lactate dehydrogenase [20] (results not shown). Similar to ethanol-fed livers, acetaldehyde-treated H4IIE cells also exhibited increased AP-1 and NF-κB binding to the GCLC promoter fragments (Figure 7). Finally, to see if increased transcription-factor binding results in increased promoter activity, the effect of acetaldehyde on luciferase activity driven by GCLC promoter constructs was examined. Acetaldehyde treatment resulted in a 3.6-fold increase in reporter-gene activity driven by the GCLC promoter fragment that contains both AP-1 and NF-κB binding sites (Figure 9).

DISCUSSION

GSH is an important intracellular peptide with multiple functions ranging from antioxidant defence to modulation of cell proliferation [1]. One of the major determinants of the synthesis of GSH is the activity of GCL. Because of its importance, regulation of GCL has been a topic of extensive research. Regulation can occur transcriptionally or post-transcriptionally, affecting only the heavy or light subunit, or both [1,27]. We showed previously that oxidative stress, hormones and rapid growth all transcriptionally activated the heavy subunit of GCL in rat liver or hepatocytes [18–20]. The light subunit is also transcriptionally activated by oxidative stress but not by hormones or rapid growth [18,20]. This led us to speculate that in rat liver there is more light subunit than heavy subunit, so that regulation of the heavy subunit alone resulted in a change in GCL activity. Although the 5′-flanking regions of the human GCL subunits have been cloned [9–11], studies of transcriptional regulation in the rat model can best be accomplished with rat GCL subunit promoters. Cloning of the rat GCL promoters would also facilitate comparative studies using both *in vitro* and *in vivo* models, which is more difficult to accomplish with the human GCL promoters. Indeed, the current literature regarding human GCL promoter regulation is largely based on data derived from transfected cell lines [9–15]. Based on these published works, ARE, AP-1 and NF-κB are three *cis*-acting elements implicated in the transcriptional regulation of human GCLC [1,9,12–15]. In the current work, we describe cloning and characterization of the 5′-flanking region of the rat GCLC.

The sequence of the 5′-flanking region of the rat GCLC shares little similarity with the 5′-flanking region of the human GCLC [9]. RNase protection and primer-extension analyses revealed a single transcriptional start site located 99 nt downstream of a putative TATA box or 197 nt upstream of the translational start site. Although the sequence of the rat GCLC promoter shares little similarity with the human GCLC promoter, both contain several consensus binding sites for AP-1 and one binding site for NF-κB. The rat GCLC promoter also contains several consensus binding sites for C/EBP, MZF1, SRY and one or more sites for HSF and c-Myc. AREs (5′-TGACNNNGC-3′), present in the human GCLC promoter, are not found in the 1.76 kb 5′-flanking region of the rat GCLC. However, although the human GCLC promoter contains several AREs, the functional element (ARE4) that mediates the effect of β-naphthoflavone is approx. 3.1 kb upstream of the transcriptional start site [9]. Thus it is possible that functional AREs may be present upstream of the 1.8 kb portion of the rat GCLC promoter cloned.

Transfection studies showed that the 5′-flanking sequence of the rat GCLC gene contains a functional promoter that was able to drive luciferase expression in H4IIE cells efficiently. Three regions in the rat GCLC promoter are important for the overall activity. They are positions -595 to -111, -705 to -595 and -1108 to -705 relative to the translational start site. The first and third regions are involved in positive regulation whereas the second region is involved in negative regulation. We next examined protein binding to these regions important for promoter activity. DNase I footprinting assay of the region from -1108 to -918 revealed three protected areas, -1058 to -1039, -1032 to -1006 and -988 to -966. Consensus binding sites in these protected areas include AP-1, E2F (adenoviral E2 factor) and HSF. Interestingly, HSF1 nuclear-binding activity is modulated by oxidative stress and GSH level [28]. Treatment of a neuroblastoma cell line with hydrogen peroxide increased HSF1 DNA-binding activity, an effect that was potentiated by GSH depletion and blocked by GSH supplementation [28]. We showed that GCLC is transcriptionally induced when GSH is profoundly depleted [20]. It would be of interest to see whether HSF1 might be involved in mediating this effect. DNase I footprinting of the region -705 to -590 also revealed three protected areas, -685

to −663, −654 to −628 and −612 to −600. Consensus binding sites in these areas include NF1, C/EBP, MZF1 and VBP. NF1 has been shown to be a transcriptional activator for some genes and a transcriptional silencer for others [29]. MZF1 has been shown to be a bi-functional transcriptional regulator, repressing transcription in non-haematopoietic cells and activating transcription in haematopoietic cells [30]. Both of these would be of interest for further investigation. Finally, DNase I footprinting of the region −566 to −290 also revealed three protected areas, −423 to −386, −380 to −360 and −358 to −340. Potential consensus binding sites in these areas include IK1/IK2 (Ikaros 1 and 2), C/EBP, NF-κB and AP-1. NF-κB and AP-1 are of major interest since they have been implicated in the transcriptional regulation of the human GCLC [1,12–15].

To see if specific protein binding can be confirmed, we performed EMSA using probes consisting of different regions of the rat GCLC promoter. In general, results from the EMSA corroborated those from the DNase I footprinting analysis, with one exception. Specific protein binding was observed in the region −538 to −479 on EMSA. This part is near the origin of the gel on DNase I footprinting (see Figure 4, right-hand panel) and it is difficult to be certain of the presence or absence of DNase I protection. To further delineate the identity of the transcription factors that bind to the GCLC promoter, we performed competition analysis in the presence of oligonucleotide probes that span binding sites for specific transcription factors. Using this strategy, we have identified: AP-1, AP-4 and HSF as potential factors that bind to the region −1086 to −965, which may be involved in positive regulation; NF1, C/EBP and VBP as potential transcription factors that bind to the region −707 to −597, which is involved in negative regulation; and MZF1, NF-κB and AP-1 as potential factors that bind to the region −538 to −303, which is involved in positive regulation. Further work will be necessary to confirm the functionality of these *cis*-acting elements and transcription factors.

We next used the rat GCLC promoter to examine the molecular mechanism of increased GCLC expression in ethanol-fed livers. We chose to examine the region −566 to −290 because sequential deletion analysis suggests that this region is likely to contain important enhancer elements. Using DNase I footprinting, EMSA and supershift analyses, there was increased AP-1 and NF-κB binding to this region of the GCLC promoter in ethanol-fed livers. We cannot exclude involvement of other *cis*-acting elements upstream of this region at the present time.

To confirm functional involvement of these two *cis*-acting elements, we examined whether an important mediator of ethanol's effect, namely acetaldehyde, can also increase GCLC expression in our cell line. An *in vitro* model would facilitate studies of the signalling pathways involved. As expected, acetaldehyde increased the steady-state GCLC mRNA level dramatically in H4IIE cells. Similar to ethanol-fed livers, acetaldehyde-treated H4IIE cells also exhibited increased AP-1 and NF-κB binding to the GCLC promoter. Finally, acetaldehyde treatment of H4IIE cells transfected with GCLC-luc gene constructs increased luciferase activity driven by the promoter fragment that contains binding sites for both AP-1 and NF-κB. The relative contribution of these two transcription factors to the overall GCLC promoter activity in response to acetaldehyde is unknown at present and will require further study to delineate.

In summary, we have cloned and analysed the 5′-flanking region of the rat GCLC gene. The rat GCLC promoter contains both positive and negative regulatory regions. Candidate transcription factors that bind to the promoter have been identified. Finally, we have identified NF-κB and AP-1 as two *trans*-activating factors that may be largely responsible for the increased GCLC expression in alcoholic rat liver and after acetaldehyde treatment.

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