# Characterisation of γ-glutamylcysteine synthethase-heavy subunit promoter: a critical role for AP-1

Irfan Rahman, Christopher A.D. Smith, Frank Antonicelli, William MacNee*

*Rayne Laboratory, Respiratory Medicine Unit, Department of Medicine, University of Edinburgh, Royal Infirmary, Lauriston Place, Edinburgh EH3 9YW, UK*

Received 16 March 1998; revised version received 1 April 1998

**Abstract The 5′-flanking region of human γ-glutamylcysteine synthetase-heavy subunit (γ-GCS-HS) was characterised by creating a series of chloramphenicol acetyl transferase (CAT) reporter deletion constructs. Analysis of various deleted CAT constructs revealed that a putative AP-1 consensus sequence is required to direct the constitutive and oxidant-mediated promoter activity. Gel mobility shift and mutation analysis of the sequence (−269 to −263 bp), showed binding of AP-1 is involved in the oxidant-mediated regulation of γ-GCS-HS promoter activity.**



*Key words:* Glutathione; γ-Glutamylcysteine synthetase; Promoter analysis AP-1; Oxidant; A549

## 1. Introduction

The tripeptide glutathione (GSH) or L-γ-glutamyl-cysteinyl-glycine is a ubiquitous cellular non-protein sulfhydryl which has an important role in maintaining intracellular redox balance and is involved in the detoxification of peroxides, free radicals, heavy metals and xenobiotics [1]. Decreased levels of GSH in epithelial lining fluid (ELF) have been demonstrated in various inflammatory diseases such as HIV infection [2], idiopathic pulmonary fibrosis [3], adult respiratory distress syndrome [4] and cystic fibrosis [5].

Glutathione is synthesised by two enzymes, γ-glutamylcysteine synthetase (γ-GCS) which is the rate limiting enzyme, and glutathione synthetase [6]. The γ-GCS holoenzyme exists as a dimer, composed of heavy (γ-GCS-HS; 73 kDa) and light (γ-GCS-LS; 28 kDa) subunits [7]. The heavy subunit possesses all of the catalytic activity [8].

The 5′-flanking region of the human γ-GCS-HS has been cloned and sequenced. Various putative transcription factors such as AP-1 (c-fos/c-jun)/TRE, AP-1-like, NF-κB, AP-2, SP-1, metal response element (MRE) and antioxidant response (ARE)/electrophile response (EpRE) elements have been identified [9–11]. Recently we have shown that exposure to menadione (MQ), hydrogen peroxide ($H_2O_2$) or cigarette smoke induced chloramphenicol acetyl transferase (CAT) activity in human alveolar epithelial cells (A549) transfected with −1050 to +82 bp of the 5′-flanking region of the γ-GCS-HS promoter, subcloned into a CAT reporter system [12,13]. We and others have recently shown that AP-1 binding may be required for the transcriptional activation of γ-GCS-HS in response to cigarette smoke, cisplatin, okadaic acid or tumour necrosis factor (TNF)-α [10,12,14–16]. Both c-jun, and Jun B can participate in transcriptional transactivation of ARE/EpRE elements in addition to TRE/AP-1 activity [17]. Expression of γ-GCS has clearly been shown to be regulated by the AP-1 transcription factor in yeasts [18]. Deletion and mutational analysis of the γ-GCS-HS promoter (−3802: +465), cloned into a reporter system has also revealed that a functional distal TRE/ARE element, located between −3802 and −2752 bp, is important for the constitutive and β-napthoflavone inducible expression [11]. However, there is no information so far available on the characteristics of the γ-GCS-HS promoter region under conditions of oxidative stress.

Menadione (2-methyl-1,4-napthoquinone, MQ) is a quinone which imposes oxidative stress by generating reactive oxygen species (ROS) due to redox cycling. Recent evidence indicates that quinone compounds and hydrogen peroxide can induce γ-GSH-HS in various cell lines [13,19,20]. However, the exact molecular mechanism of induction of γ-GCS-HS is not known. Therefore, in the present study, we have utilised a deletion analysis to identify the sequences that modulate the constitutive and oxidant-induced expression of the human γ-GCS-HS gene 5′-flanking region.

## 2. Materials and methods

Unless otherwise stated, all of the biochemical reagents used in this study were purchased from the Sigma Chemical Co, Poole, England; cell culture media and molecular biology reagents from GIBCO-BRL, Paisley, Scotland.

### 2.1. Promoter deletion constructs

The γ-GCS-HS promoter was isolated by PCR from human genomic DNA using the upstream oligonucleotide 5′-(+82)GGCGACATCCAATATGAAGGCTGTG-3′ and downstream oligonucleotide 5′-(−1050)TTCCTACTTGTGACCAAAACCTGCG-3′. The resulting promoter fragment (−1050 to +82 bp) was confirmed by DNA-sequencing. The promoter fragment was cloned into pCRII cloning vector (Invitrogen, USA) and a *Hin*dIII and *Sph*I fragment (1138 bp) containing the promoter was isolated and subcloned into polylinker of the promoterless plasmid pCAT Basic vector (Promega, USA). This construct was denoted pCBGCS. Deletion fragments of the γ-GCS-HS promoter were generated using *Kpn*I restriction site present within the γ-GCS-HS promoter which resulted in a short fragment containing the ARE (−1050 to −818 bp) (pCBGCSA) and a large fragment (−817 to +82 bp) (pCBGCSΔK) containing AP-1 and AP-1-like sites. Further deletion of γ-GCS-HS promoter was carried out using a *Bal*I restriction site present within the pCBGCSΔK, which resulted in a fragment (−511 to +82 bp) (pCBGCSΔB) containing one of each of the AP-1 and AP-1-like sites. pCBGCSΔB was subjected to digestion with *Dra*II to generate a deletion mutant, pCBGCSΔD (−305 to +82 bp) containing AP-1 at −269 to −263 bp, CAAT and TATA boxes. To create a further deletion mutant lacking the putative proximal AP-1 site, pCBGCSΔK was digested using *Nsi*I which produced a large fragment −817 to −204 bp and a small fragment −201 to +82 bp. The small fragment was denoted by pCBGCSΔN. All of the fragments were subcloned into a pCAT Basic vector.

*Corresponding author. Fax: +44 (131) 536 3255.
E-mail: wmacnee@ed.ac.uk


*PII* S0014-5793(98)00410-4

*2.2. A549 epithelial cells*

The type II alveolar epithelial cell line, A549 (ECACC No. 86012804), which was mycoplasma free, was maintained in continuous culture at 37°C, 5% $CO_2$ in Dulbecco's modified minimum essential medium (DMEM, GIBCO) containing penicillin/streptomycin mixture, L-glutamine, sodium bicarbonate, and 10% foetal bovine serum (FBS) (GIBCO, Paisley, Scotland).

*2.3. Transient transfection and CAT assay*

A549 cells ($0.8\times10^6$) per well were seeded into 6-well tissue culture plates and cultured at 37°C until 70–80% confluent. Plasmid DNA transfections were performed using lipofecAMINE reagent (GIBCO). After $H_2O_2$ (100 μM) or MQ (100 μM) treatments, cell extracts were prepared and assayed for protein content using BCA reagent (Pierce, Rockford, IL, USA). Chloramphenicol acetyl transferase (CAT) activity was quantitated by the CAT enzyme-linked immunosorbent assay (ELISA). The β-galactosidase expression plasmid (PSV-Gal, Promega) was co-transfected as an internal control to normalise transfection efficiency. In all transfection experiments, pCAT-Basic, pCAT-Control were used as negative and positive controls, respectively.

*2.4. Preparation of nuclear extracts and the electrophoretic mobility shift assay (EMSA)*

Nuclear extracts were prepared by the method of Staal et al. [21]. The oligonucleotides for the AP-1 site (−269 to −263 bp) present in native γ-GCS-HS-AP-1 (5′-GAGTTCGTCAT**TGATTCA**AATAAT-3′, 3′-CTCAAGCAGTAACTAAGTTTATTA-5′) and its mutant consensus sequences (5′-GAGTTCGTCAT**GATCCTG**AATAAT-3′, 3′-CTCAAGCAGTACTAGGACTTATTA-5′) were synthesised by MWG-BIOTECH GMbH, Ebersberg, Germany. The consensus sequence of AP-1 is indicated by bold letters and its mutated sequence by the underlined letters. Both the oligonucleotides were end-labelled with (γ-$^{32}$P)-ATP using T4 polynucleotide kinase and (γ-$^{32}$P)-ATP, according to the manufacturer's instructions (Promega). Binding reactions were carried out using 25 μg of nuclear extract protein, 0.25 mg/ml poly(dI-dC)·poly(dI-dC), binding buffer (Promega) and γ-GCS-HS AP-1 and its mutant labelled probes as described above. Incubation was carried out at room temperature for 20 min. The protein-DNA complexes were resolved on non-denaturating 5% polyacrylamide gels at 100 mV for 3–4 h. The gels were then vacuum dried and autoradiographed overnight with an intensifying screen at −80°C. For the competition experiment, a 100-fold molar excess of unlabelled AP-1 (5′-CGCTTGA**TGAGTCA**GCCGGAA-3′, 3′-CGCAACTAC-TCAGTCGGCCTT-5′) and NF-κB (5′-AGTTGAGGGGACTTT-CCCAGGC′-3, 3′-TCAACTCCCCTGAAAGGGTCCG-5′) oligonucleotides (Promega) were incubated in the reaction mixture.

*2.5. Statistical analysis*

Results were expressed as mean ± S.E.M. Differences between values were compared by Duncan's multiple range test.

## 3. Results

*3.1. Regulation of constitutive expression of the γ-GCS-HS 5′-flanking region*

We examined the involvement of various transcription factor binding sites in the regulation of constitutive expression of γ-GCS-HS 5′-flanking region. Various deletion strategies described above were used to produce several deletion mutants, which were then cloned into the upstream region of the CAT reporter plasmid (Fig. 1A). Transcriptional activity of the reporter constructs in A549 epithelial cells was examined. The transcriptional activity of the longest fragment, spanning −1050 to +82 bp of the γ-GCS-HS gene (pCBGCS) was taken as 100% (Fig. 1B). Deletion of the fragment from −1050 to −818 bp did not increase the constitutive transcriptional activity of pCBGCSΔK (97 ± 20%, $n=4$), compared to that of pCBGCS. This deleted fragment contained multiple putative *cis*-acting DNA elements including an ARE and an AP-2 site. However, a further deletion from −817 to −511 bp, which contained an EpRE and AP-1-like sites, increased the CAT activity of pCBGCSΔB to 152 ± 22%, $n=4$, $P<0.01$, compared to that of pCBGCS. The increased activity was not affected by deletion of fragment from −511 to −305 bp, which contained an AP-1-like site pCBGCSΔD 147 ± 17%, $n=4$, $P<0.01$, compared to pCBGCS. However, the activity was significantly decreased after deletion of the proximal putative AP-1 site present between −269 to −263 bp, pCBGCSΔN 26 ± 10%, $n=4$, $P<0.001$, compared to pCBGCS (Fig. 1B).

*3.2. Regulation of oxidant-induced expression of the γ-GCS-HS 5′-flanking region*

We have previously demonstrated a significant increase in the CAT activity in A549 epithelial cells transfected with the full promoter and pCBGCSΔK, linked to the CAT reporter system (pCBGCS) after exposure to $H_2O_2$ (100 μM) and MQ (100 μM) [13]. In the present study, we made further deletion constructs of γ-GCS-HS 5′-flanking region and studied the effects of $H_2O_2$ (100 μM) and MQ (100 μM) on the regulation of CAT activity in A549 epithelial cells (Fig. 1A, B). CAT activity increased significantly in pCBGCSΔB ($H_2O_2$ 325 ± 31%, MQ 277 ± 38%, $P<0.001$, $n=4$), compared to that of pCBGCS in response to the oxidants. The CAT activity was more pronounced in pCBGCSΔD ($H_2O_2$ 342 ± 33%, MQ 293 ± 35%, $P<0.001$, $n=4$) compared to pCBGCS. However, the CAT activity was significantly reduced in pCBGCSΔN ($H_2O_2$ 33 ± 9%, MQ 22 ± 11%, $P<0.001$, $n=4$), compared to pCBGCS.

*3.3. Effect of $H_2O_2$ and MQ on the activation of proximal AP-1*

The use of successive deletion constructs showed that sequence from −303 to −201 bp, which contained a putative AP-1 transcription factor binding site, may be required for the transcriptional regulation of γ-GCS-HS in response to oxidants. Therefore, to determine if the increase in the pCBGCSΔD CAT activity was associated with the activation of the transcription factor AP-1 DNA binding (−269 to −263 bp), we investigated the effects of $H_2O_2$ and MQ on the proximal γ-GCS-HS AP-1, oligonucleotide DNA binding in A549 alveolar epithelial cells by EMSA. We showed an increase in the γ-GCS-HS AP-1 binding activity in response to $H_2O_2$ and MQ ($P<0.001$) (Fig. 2A, B). The binding was not significantly changed when 50-fold excess mutant γ-GCS-HS AP-1 was used in the binding reaction. The specificity of the AP-1 binding was checked using 100-fold excess unlabelled AP-1 oligonucleotides (Promega) and non-specific oligonucleotides for NF-κB. The band obtained by oxidant treatments was completely abolished using labelled AP-1 oligonucleotides (Promega), whereas non-competitor NF-κB oligonucleotides did not interfere in the DNA protein binding confirming the involvement of AP-1.

## 4. Discussion

The 5′-flanking nucleotide sequence of the human γ-GCS-HS gene contains ARE, NF-κB, AP-1/TRE, AP-1-like, and AP-2 binding sites [9–11]. We have previously demonstrated that $H_2O_2$ and MQ induced transcriptional activity of the 5′-flanking sequence of the γ-GCS-HS using a CAT reporter system [12,13]. However, the critical transcription factor(s) involved in the regulation of γ-GCS-HS promoter is not

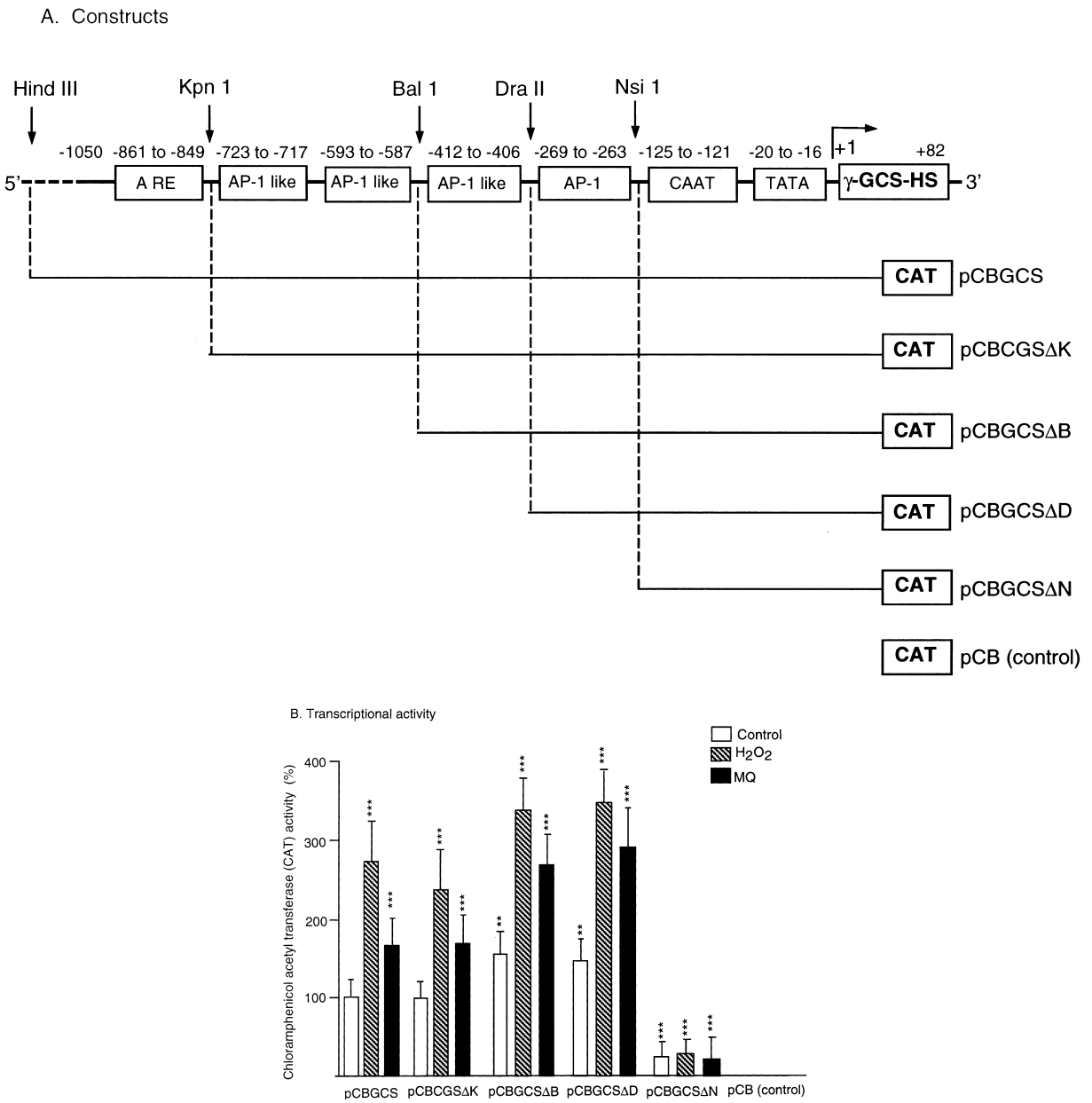


Fig. 1. Constitutive and inducible regulation of the γ-GCS-HS 5′-flanking region in a CAT reporter system. The 5′-flanking successive deletion sequence of the γ-GCS-HS gene from oligonucleotides −1050, −818, −511, −305, −201 to +82 bp were cloned into pCAT-Basic and co-transfected into A549 alveolar epithelial cells along with the control plasmid PSV-β-Gal. Panel A shows the relative positions of the *cis*-acting DNA elements and a restriction map of the promoter region cloned in pCRII vector. The numbers in the figure represent the nucleotide positions from the transcriptional start site of the γ-GCS-HS gene, which is indicated by the bent arrow on the right. The dotted line on the left indicates an additional 50 bp from multiple cloning sites of the pCRII vector. The structure of the γ-GCS-HS CAT plasmids are shown below on the right. Deletion mutants were ligated to upstream of the CAT gene in pCAT Basic vector (pCB) and its constitutive transcriptional CAT activity was measured 36–48 h post-transfection. Various deleted constructs were transfected into A549 cells and exposed to $H_2O_2$ (100 μM) and MQ (100 μM). After 24 h incubation, the cells were harvested and assayed for CAT activity. Panel B: Transcriptional activity among different constructs was standardised by the amount of CAT activity relative to β-Gal activity. The results are shown as percentages of the CAT concentration compared to that of pCBGCS. Each histogram represents the mean and the bars the S.E.M. of four independent transfections, each performed in duplicate with the activity pCBGCS set at 100%. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to pCBGCS.

known so far. In this study, using deletion constructs for γ-GCS-HS 5′-flanking region, we showed that the −511 to +82 bp is involved in the constitutive expression of the γ-GCS-HS promoter activity. Furthermore, deletions of the 5′-flanking region revealed that a proximal sequence of the γ-GCS-HS gene, specifically −303 to −201 bp, was critical in the transcriptional up-regulation in oxidant-exposed alveolar epithelial cells. This is confirmed by deletion of fragment containing the proximal AP-1 site, which produced a dramatic fall in the promoter activity, and the abolition of the oxidant-mediated effect. Thus the 5′-flanking region of the γ-GCS-HS contains a putative AP-1 binding site at −269 to −263, which appears to

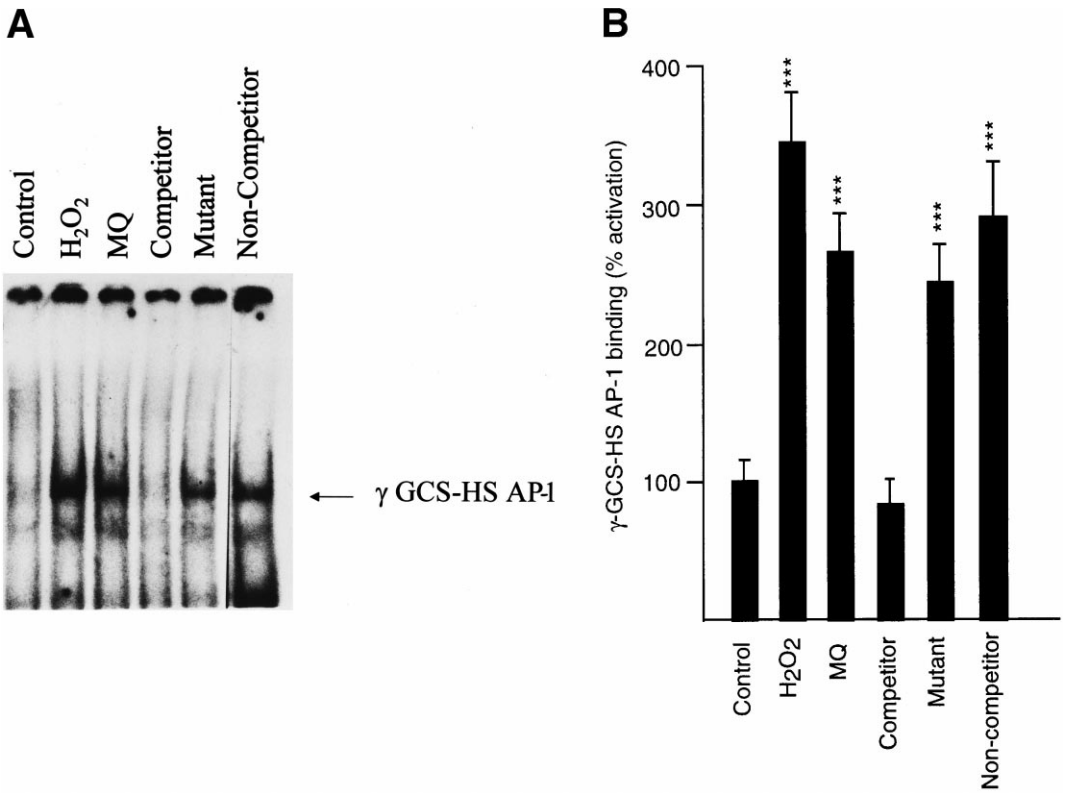


Fig. 2. Effects of $H_2O_2$ (100 μM) and MQ (100 μM) exposure on the induction of γ-GCS-HS AP-1 binding activities in A549 cells. A: Nuclear extracts were prepared and 25 μg of protein was analysed by the electrophoretic mobility shift assay, performed in triplicate, using $^{32}$P-labelled proximal γ-GCS-HS AP-1 sequence as probe. Cold mutant γ-GCS-HS AP-1 and AP-1 (Promega) were used as competitors and NF-κB oligonucleotide was used as non-competitor. The DNA-protein complexes formed are indicated (arrow). Nuclear extracts from HeLa cells were used as a positive control (data not shown). B: The gel was scanned using a GS 2600 gel documentation system, UVP (Orme Technologies, Cambridge, England). The numeric estimates of DNA binding levels were compared with the control value set at 100%. Data are expressed as mean ± S.E.M. of relative intensity of bands of three experiments. ***$P < 0.001$ compared to control.

be involved in the regulation of γ-GCS-HS expression in response to oxidative stress.

Yao and co-workers previously showed that the nuclear factors of the Jun family bind to this AP-1 site [10]. They also showed that expression of the γ-GCS-HS gene increased in cisplatin-resistant human ovarian cells. Similarly, Tomonari and colleagues [14] showed that an AP-1 regulatory site present in the proximal 5′-flanking sequence of the human γ-GCS-HS was involved in cisplatin-induced transcriptional up-regulation in a SBC-3 cancer cell line. These results are supported by the recent demonstration of the participation of AP-1 in the regulation of glutathione metabolism in human hepatoma (HepG2) cells [15]. All of these studies used cisplatin to assess the regulation of the γ-GCS-HS promoter. However, our interest is focused on the role of the oxidant/antioxidant imbalance, in particular GSH and its redox system, which is known to be altered in various inflammatory lung diseases [3–5]. Therefore, in this study using $H_2O_2$ and MQ, we were able to show activation of an AP-1 present in pCBGCSΔD in human alveolar epithelial cells. Thus our data suggest that a putative AP-1 site, present in the proximal region of the 5′-flanking region, is involved in the regulation of γ-GCS-HS expression in response to oxidants in alveolar epithelial cells. Further analysis of γ-GCS-HS AP-1 binding in response to oxidants by the gel shift assay revealed that AP-1 is specifically able to bind with the proximal AP-1 site present in the γ-GCS-HS promoter. This is confirmed by mutant γ-GCS-HS AP-1, which did not inhibit AP-1 binding in A549 cells in response to oxidants, whereas the γ-GCS-HS AP-1 binding was completely abolished by commercially available AP-1 oligonucleotides. Our data are in agreement with the recent observation of Morale et al. [16] who suggested that an AP-1 binding site may be required for the induction of γ-GCS-HS gene by TNF-α in HepG2 cells. However, Mulcahy and co-workers [11] recently reported that an ARE, present at −3147 to −3137 bp played a critical role in the transcriptional up-regulation of the γ-GCS-HS gene by β-napthoflavone in HepG2 cells. The apparently conflicting results of that study to those of our own may be a reflection of the fact that multiple putative transcription factors are involved in response to different stimuli in different cells.

In conclusion, we have shown that the proximal 5′-flanking sequence of the γ-GCS-HS gene, in particular from −303 to−201 bp, is involved in oxidant-induced transcriptional up-regulation in alveolar epithelial cells. Studies using the gel shift assay of the proximal γ-GCS-HS AP-1 oligonucleotides showed that the AP-1 site present in the −269 to −263 bp is involved in the transcriptional up-regulation of γ-GCS-HS gene under oxidative stress.

*Acknowledgements:* This work was supported by the British Lung Foundation.

## References


[1] Meister, A. and Anderson, M.E. (1983) Ann. Rev. Biochem. 52, 711–760.
[2] Staal, F.J.T., Ela, S.W., Roederer, M., Anderson, M.T. and Herzenberg, L.A. (1992) Lancet 339, 909–912.
[3] Cantin, A.M., Hubbard, R.C. and Crystal, R.G. (1989) Am. Rev. Respir. Dis. 139, 370–372.
[4] Bunnel, E. and Pacht, E.R. (1993) Am. Rev. Respir. Dis. 148, 1174–1178.
[5] Roum, J.H., Behld, R., McElvancy, N.G., Borok, Z. and Crystal, R.G. (1993) J. Appl. Physiol. 75, 2419–2424.
[6] Seelig, G.F. and Meister, A. (1984) J. Biol. Chem. 259, 3534–3538.
[7] Seelig, G.F., Simondsen, R.P. and Meister, A. (1984) J. Biol. Chem. 259, 9345–9347.
[8] Huang, C.S., Chang, L.S., Anderson, M.E. and Meister, A. (1993) J. Biol. Chem. 268, 19675–19680.
[9] Mulcahy, R.T. and Gipp, J.J. (1995) Biochem. Biophys. Res. Commun. 209, 227–233.
[10] Yao, K.S., Godwin, A.K., Johnson, W.W., Ozols, R.F., O'Dwyer, P.J. and Hamilton, T.C. (1995) Cancer Res. 55, 4367–4374.
[11] Mulcahy, R.T., Wartman, M.A., Bailey, H.H. and Gipp, J.J. (1997) J. Biol. Chem. 272, 7445–7454.
[12] Rahman, I., Smith, C.A.D., Lawson, M.F., Harrison, D.J. and MacNee, W. (1996) FEBS Lett. 396, 21–25.
[13] Rahman, I., Bel, A., Mulier, B., Lawson, M.F., Harrison, D.J., MacNee, W. and Smith, C.A.D. (1996) Biochem. Biophys. Res. Commun. 229, 832–837.
[14] Tomonari, A., Nishio, K., Kurokawa, H., Fukumoto, H., Fukuoka, K., Iwamoto, Y., Usuda, J., Suzuki, T., Itakura, M. and Saijo, N. (1997) Biochem. Biophys. Res. Commun. 236, 616–621.
[15] Sekhar, K.R., Meredith, M.J., Kerr, L.D., Soltaninassab, S.R., Spitz, D.R., Xu, Z.Q. and Freeman, M.L. (1997) Biochem. Biophys. Res. Commun. 234, 488–493.
[16] Morales, A., Garcia-Ruiz, C., Miranda, M., Montserrat, M., Colell, A., Ardite, E. and Fernandez-Checa, C. (1997) J. Biol. Chem. 272, 30371–30379.
[17] Bergelson, S. and Daniel, V. (1994) Biochem. Biophys. Res. Commun. 200, 290–297.
[18] Wu, A.L. and Moye-Rowley, W.S. (1994) Mol. Cell. Biol. 14, 5832–5839.
[19] Shi, M.M., Kugelman, A., Takeo, I., Tian, L. and Forman, H.J. (1994) J. Biol. Chem. 269, 26512–26517.
[20] Galloway, D.C., Blake, D.G., Shepherd, A.G. and McLellan, L.I. (1997) Biochem. J. 328, 99–104.
[21] Staal, F.J.T., Roederer, M., Herzenberg, L.A. and Herzenberg, L.A. (1990) Proc. Natl. Acad. Sci. USA 87, 9943–9947.