Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase *Gsta1*, *Gsta2*, *Gstm1*, *Gstm2*, *Gstm3* and *Gstm4* genes in the livers of male and female mice

Simon A. CHANAS^{*1}, Qing JIANG^{*1}, Michael McMAHON^{*}, Gail K. McWALTER^{*}, Lesley I. McLELLAN^{*}, Clifford R. ELCOMBE^{*}, Colin J. HENDERSON[†], C. Roland WOLF[‡], Graeme J. MOFFAT[‡], Ken ITOH[§], Masayuki YAMAMOTO[§] and John D. HAYES^{*2}

^{*}Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland, U.K., [†]Cancer Research UK, Molecular Pharmacology Unit, Ninewells Hospital, Dundee DD1 9SY, Scotland, U.K., [‡]Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, England, U.K., and [§]Centre for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba 305-8577, Japan

Mice that lack the Nrf2 basic-region leucine-zipper transcription factor are more sensitive than wild-type (WT) animals to the cytotoxic and genotoxic effects of foreign chemicals and oxidants. To determine the basis for the decrease in tolerance of the *Nrf2* homozygous null mice to xenobiotics, enzyme assay, Western blotting and gene-specific real-time PCR (TaqMan[®]) have been used to examine the extent to which hepatic expression of GSH-dependent enzymes is influenced by the transcription factor. The amounts of protein and mRNA for class Alpha, Mu and Pi glutathione S-transferases were compared between WT and *Nrf2* knockout (KO) mice of both sexes under both constitutive and inducible conditions. Among the class Alpha and class Mu transferases, constitutive expression of *Gsta1*, *Gsta2*, *Gstm1*, *Gstm2*, *Gstm3*, *Gstm4* and *Gstm6* subunits was reduced in the livers of *Nrf2* mutant mice to between 3% and 60% of that observed in WT mice. Induction of these subunits by butylated hydroxyanisole (BHA) was more marked in WT female mice than in WT male mice. TaqMan[®] analyses showed the increase in transferase mRNA caused by BHA was attenuated in *Nrf2*^{−/−} mice, with the effect being most apparent in the case of *Gsta1*,

Gstm1 and *Gstm3*. Amongst class Pi transferase subunits, the constitutive hepatic level of mRNA for *Gstp1* and *Gstp2* was not substantially affected in the KO mice, but their induction by BHA was dependent on Nrf2; this was more obvious in female mutant mice than in male mice. *Nrf2* KO mice exhibited reduced constitutive expression of the glutamate cysteine ligase catalytic subunit, and, to a lesser extent, the expression of glutamate cysteine ligase modifier subunit. Little variation was observed in the levels of glutathione synthase in the different mouse lines. Thus the increased sensitivity of *Nrf2*^{−/−} mice to xenobiotics can be partly attributed to a loss in constitutive expression of multiple GSH-dependent enzymes, which causes a reduction in intrinsic detoxification capacity in the KO animal. These data also indicate that attenuated induction of GSH-dependent enzymes in *Nrf2*^{−/−} mice probably accounts for their failure to adapt to chronic exposure to chemical and oxidative stress.

Key words: antioxidant responsive element, cancer chemoprevention, carcinogenesis, detoxification, glutamate cysteine ligase (also called γ -glutamylcysteine synthetase).

INTRODUCTION

The ability to withstand toxic chemicals and oxidative stress is essential for the survival of all organisms. Various mechanisms have evolved to protect cells against foreign compounds and reactive oxygen species, including efflux pumps, antioxidant proteins and GSH, drug-metabolism, sequestration of toxins and DNA repair [1]. A number of genes encoding proteins involved in these processes can be induced by the compounds against which they provide protection, thereby enabling cells to survive exposure to harmful xenobiotics as well as oxidants [1,2].

One of the most versatile mechanisms of transcriptional adaptation to chemical stress involves the antioxidant-responsive element (ARE). This enhancer was first discovered because of its involvement in the inducible regulation of rat glutathione

S-transferase class Alpha 2 (*GSTA2*) and rat NAD(P)H:quinone oxidoreductase 1 genes [3,4]. Deletion and mutation analyses of the promoter of rat *GSTA2* defined the ARE as 5'-(A/G)GTG-ACNNNGC-3' [3]. This element responds to Michael reaction acceptors, hydroquinones, catechols, isothiocyanates, peroxides, trivalent arsenicals and heavy-metal salts [3,5,6]. The prototypic inducer of ARE-driven transcription is t-butylhydroquinone (t-BHQ), a major metabolite of the phenolic antioxidant butylated hydroxyanisole (BHA) [7].

The basic-region leucine-zipper (bZIP) factor Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), in combination with a small Maf protein, mediates transcriptional activation of genes via the ARE [8–11]. Evidence that Nrf2 confers protection against xenobiotics has been provided by studies of mice, or murine fibroblasts and macrophages, 'nulled' for this tran-

Abbreviations used: AGLN, α -angelicalactone; ARE, antioxidant-responsive element; BaP, benzo[a]pyrene; BHA, butylated hydroxyanisole; t-BHQ, t-butylhydroquinone; bZIP, basic-region leucine-zipper; CDNB, 1-chloro-2,4-dinitrobenzene; C+K, cafestol and kahweol palmitate; CMRN, coumarin; 3-OH CMRN, 3-hydroxy-CMRN; 7-OH CMRN, 7-hydroxy-CMRN; ECL, enhanced chemiluminescence; EQ, ethoxyquin; EST, expressed sequence tag; GCL(C), glutamate cysteine ligase (catalytic subunit); GCLM, GCL modifier subunit; GST, glutathione S-transferase; *Gsta1/2*, GST class Alpha 1/2; *Gstm1/2/3/4/5/6*, GST class Mu 1/2/3/4/5/6; *Gstp1/2*, Gst class Pi 1/2; I3C, indole-3-carbinol; KO, knockout; LMTN, limettin; Nrf2, Nuclear factor-erythroid 2 p45-related factor 2; OPZ, oltipraz; RACE, rapid amplification of cDNA ends; SUL, sulforaphane; WT, wild-type.

¹ These individuals contributed equally to this work, and should therefore be regarded as joint first authors.

² To whom correspondence and requests for reprints should be addressed (e-mail john.hayes@cancer.org.uk).

The nucleotide sequence data reported for mouse *Gstm4* will appear in DDBJ, EMBL, GenBank[®] and GSDB Nucleotide Sequence Databases under the accession number AF501320.

scription factor. In mouse experimental models possessing disrupted *Nrf2*, increased sensitivity to 1-chloro-2,4-dinitrobenzene (CDNB) [12], acetaminophen [13,14], butylated hydroxytoluene [15], ethoxyquin (EQ) [16] and coumarin (CMRN) [16] has been observed. The *Nrf2*^{-/-} mice are more susceptible to DNA adduct formation in target tissue when exposed to diesel-exhaust fumes [17], and during benzo[*a*]pyrene (BaP) carcinogenesis they develop more forestomach tumours than wild-type (WT) animals [18]. Loss of *Nrf2* might also influence sensitivity to hyperoxic lung injury [19]. These phenotypic changes presumably occur either through altered expression of ARE-driven genes or failure to adapt to chemical/oxidative stress, or both.

In mice with targeted disruption of *Nrf2*, decreases have been reported in the constitutive and/or inducible expression of class Alpha, class Mu and class Pi glutathione S-transferases (GSTs), and the catalytic subunit of glutamate cysteine ligase (GCLC) [7,9,12–18,20]. These studies raise several important mechanistic questions. First, it is unclear precisely how *Nrf2* exerts its pleiotropic effects *in vivo*, because among mouse genes encoding antioxidant and detoxication enzymes, only the promoter of *Gsta1* has been shown to contain a functional ARE; in *Gsta1*, a 41 bp DNA fragment that contains two tandemly arrayed 'core' ARE sequences has been referred to as an electrophile-responsive element [21]. It is therefore not known whether the effect of *Nrf2* on other detoxication genes is direct, or whether it acts indirectly. It could, for example, induce other transcription factors. Secondly, within some of the superfamilies of drug-metabolizing enzymes the identities of genes regulated by *Nrf2* is uncertain. Investigations into the phenotypic changes caused by disruption of *Nrf2* in the mouse have used Western blotting, Northern blotting and reverse-transcriptase PCR. These techniques cannot discriminate between gene products that share substantial sequence identity. In addition, these methods are at best only semi-quantitative. Thus it is not possible to determine whether the expression of *Gsta1* or another class Alpha transferase (for example, *Gsta2*) is influenced by loss of *Nrf2*. Similar criticism can be levelled at data relating to class Mu GST and class Pi GST.

The lack of certainty about the identity of genes that are regulated by *Nrf2* represents a major impediment to a rigorous understanding of why mice, or cell lines, nulled for the bZIP factor are sensitive to xenobiotics. Although, as described above, this problem is primarily due to the lack of specificity and accuracy in analytical techniques employed, problems with the nomenclature of class Mu GST genes causes a further layer of confusion. Specifically, in 1991, protein purification and amino acid sequencing revealed the presence in mouse hepatic cytosol of a BHA-inducible class Mu transferase subunit that was called Yb₈ [22]. This subunit was re-designated Gstm4* when the unified nomenclature devised for human GST was applied to the murine transferases [23], because it is distinct from the three class Mu transferases that had been described previously [24,25]. Thereafter, between 1995 and 1998 two further class Mu family members were identified in the mouse, both of which were called Gstm5 [26–28]. In the present study, we have used the term Gstm5 to describe the testis/brain-specific subunit reported by Fulcher et al. [26] and Rowe et al. [27], since this was the first of the two most recently characterized class Mu transferases to be cloned. We now refer to the remaining class Mu subunit that was reported by De Bruin et al. [28] as Gstm6.

The objective of the present study was to determine the role *in vivo* of *Nrf2* in the expression of GST isoenzymes and the enzymes involved in GSH biosynthesis in livers of male and female mice. We first undertook cloning of the cDNA for Gstm4* to determine its entire primary structure, thereby allowing the nomenclature for the class Mu GST to be defined

unambiguously. Subsequently, we developed gene-specific TaqMan® real-time PCR assays for all known murine mRNAs encoding class Alpha (*Gsta1*, *Gsta2*, *Gsta3*, *Gsta4*), class Mu (*Gstm1*, *Gstm2*, *Gstm3*, *Gstm4*, *Gstm5*, *Gstm6*) and class Pi (*Gstp1*, *Gstp2*) transferases. Similar assays were also established for GCLC and the glutamate cysteine ligase modifier subunit (GCLM; for a review of the function and regulation of GCL subunits, see [2]). Besides examining constitutive gene expression in *Nrf2*^{-/-} and *Nrf2*^{+/+} mice, induction by BHA has also been studied because, through metabolism to t-BHQ, it stimulates ARE-driven transcription [7].

MATERIALS AND METHODS

Chemicals

These were all of the highest purity that was commercially available, and the sources have been described in previous publications from our laboratories [16].

Animals

Throughout this study, mice were treated as recommended by regulations contained in the Animals and Scientific Procedure Act (1986) of the U.K. Generation of the *Nrf2*^{-/-} mouse and genotyping of progeny has been described elsewhere [9,16].

Mice were housed at a temperature range of 19–23 °C under 12 h light/dark cycles. They were routinely fed *ad lib.* on RM1 laboratory animal feed (SDS Ltd, Witham, Essex, U.K.), and given free access to water. Mice of between 10 and 12 weeks of age were used for feeding experiments. These were each of 14 days' duration, and involved supplementing the RM1 diet as follows: BHA, 0.5 % (w/w) or 0.25 % (w/w); EQ, 0.25 % (w/w); oltipraz (OPZ), 0.075 % (w/w); indole-3-carbinol (I3C), 0.5 % (w/w); cafestol and kahweol palmitate (C + K), 0.025 % (w/w); sulforaphane (SUL), 3 µmol/g; coumarin (CMRN), 0.25 % (w/w); 3-hydroxy-CMRN (3-OH CMRN), 0.25 % (w/w); 7-hydroxy-CMRN (7-OH CMRN), 0.25 % (w/w); limettin (LMTN), 0.25 % (w/w); and α-angelicalactone (AGLN), 0.25 % (w/w).

Five separate feeding experiments were undertaken, each of 14 days' duration. In the first four experiments, male *Nrf2* knockout (KO) and WT mice were fed the following diets: 1, control or BHA [at 0.5 % (w/w)]; 2, control, EQ, OPZ or I3C; 3, control, C + K or SUL; 4, control, CMRN, 3-OH CMRN, 7-OH CMRN, LMTN or AGLN. In experiment 5, both male and female *Nrf2* KO and WT mice were fed either control or BHA [at 0.25 % (w/w)] diets.

Once the test-feeding period was complete, animals were killed by exposure to a rising concentration of CO₂, followed by cervical dislocation. Livers were removed and snap-frozen immediately in liquid N₂, before being stored at –70 °C.

Preparation of hepatic cytosol

Upon removal from the –70 °C freezer, portions (approx. 350 mg) of livers from each mouse were pulverized separately using a pestle and mortar under liquid N₂. The resulting finely ground material from each sample was resuspended in 4 ml of ice-cold 50 mM Hepes buffer, pH 7.5, containing 150 mM NaCl and 1 mM dithiothreitol. This buffer was fortified with Complete EDTA-free protease inhibitor at a dose of one tablet/50 ml buffer, as recommended by Roche Diagnostics Ltd (Lewes, East Sussex, U.K.). The pulverized hepatic material was placed on ice and homogenized mechanically by three separate 30 s pulses using an Omni homogenizer. The resulting extracts were subjected to two centrifugation steps at 4 °C (15000 *g* for 45 min,

Table 1 Oligonucleotide primers and probes for TaqMan® analyses

mRNA	Primers	Probe
Gsta1	forward: 5'-CCCCTTCCCTCTGCTGAAG-3' reverse: 5'-TGCAGCTTCACTGAATCTTGAAAG-3'	5'-TTCCTTGCTTCTTGAATTTGTTTGCATCCAT-3'
Gsta2	forward: 5'-CCCCTTCCCTCTGCTGAAG-3' reverse: 5'-TGCAGCCACACTAAACTTGAAAA-3'	5'-TTCCTTGCTTCTTCAATTTGTTTGCATCCAA-3'
Gsta3	forward: 5'-TGGACAACCTCCCTCTCCTGAA-3' reverse: 5'-AATCTTCTTTGCTGACTCAACACATT-3'	5'-TGAGAAGCAGAGTCAGCAACCTCCCCA-3'
Gsta4	forward: 5'-AACTTGATGGGAAGGACCTGAA-3' reverse: 5'-CCACGGCAATCATCATC-3'	5'-CCATCTGCATACATGTCAATCCTGACTCTC-3'
Gstm1	forward: 5'-CCTATGATACTGGGATACTGGAACG-3' reverse: 5'-GGAGCGTCACCCATGGTG-3'	5'-GCGGGACTGACACACCCGATCC-3'
Gstm2	forward: 5'-CCTATGACACTAGGTACTGGGACA-3' reverse: 5'-CACTGGCTTCGGTCATAGTCA-3'	5'-CCGTGGGTGGCTCAGGCC-3'
Gstm3	forward: 5'-TATGACACTGGGCTATTGGAACAC-3' reverse: 5'-GGGCATCCCCATGACA-3'	5'-GCGGGACTGACTCACTCCATCCG-3'
Gstm4	forward: 5'-AGGCTATGGATGTCTCCAATCAG-3' reverse: 5'-TCCAGGGAGCTGTCTCAA-3'	5'-CCACCTTCAGTTCTCAAAGTCTGGGCTG-3'
Gstm5	forward: 5'-GCTGGACGTGAAATCAAGCTA-3' reverse: 5'-CGTTACTCTGGGTGATCTTGTCTTC-3'	5'-CTGGACTTTCCTAACCTGCCCTACCTCATGG-3'
Gstm6	forward: 5'-CCTCCAGATCAGCTGAACTCTA-3' reverse: 5'-TCAAGGACATAGACGAGGAAGT-3'	5'-TGCAAAGGTGATCTTGTCCCTGCAA-3'
Gstp1	forward: 5'-GCAAATATGTCACCCATCATACACC-3' reverse: 5'-GCAGGGTCTCAAAGGCTTCA-3'	5'-AGGGCCTTCACGTAGTCATTCTTACCATTCTCATAGT-3'
Gstp2	forward: 5'-CAAATATGGCACCATGATCTACAGA-3' reverse: 5'-GCAGGGTCTCAAAGGCTTCA-3'	5'-AGGGCCTTCACGTAGTCATTCTTACCATTCTCATAGT-3'
GCLC	forward: 5'-GGCGATGTTCTTGAGACTCTGC-3' reverse: 5'-TTCCTTGGATCATGTAACCTCCATA-3'	5'-AGGACAAACCCCAACCATCCGACC-3'
GCLM	forward: 5'-CACAGGTAAACCCAATAGTAACCAAGT-3' reverse: 5'-GTGAGTCAGTAGCTGTATGTCAAATGTT-3'	5'-CTGGCCTCCTGCTGTGTGATGCC-3'

followed by 100 000 *g* for 90 min). The final 100 000 *g* supernatants (cytosols) were collected and retained for enzyme analyses.

Analytical biochemistry

Protein concentration was determined as described previously [16]. GST enzyme activity was estimated using CDNB as the substrate [16].

Western blotting

In all experiments, 4 µg of protein from different liver samples, along with a positive standard (usually 0.01 µg of immunogen) was subjected to SDS/PAGE in a Bio-Rad Mini-Protein II Cell apparatus (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.). The electroblotting method used to transfer proteins from the SDS/PAGE-resolving gel to an Immobilon-P membrane, the blocking of free protein-binding sites on Immobilon-P and the various immunochemical reactions have been described elsewhere [16]. The final antibody complexes on Immobilon-P were visualized by enhanced chemiluminescence (ECL) and autoradiography.

To ensure that comparison between different liver samples was valid, SDS/PAGE was always performed in duplicate, with one of the gels being stained with Coomassie Blue to confirm equal loading. Following electroblotting, Immobilon-P membranes were routinely stained with Ponceau S to verify equal transfer of all samples.

Quantification of GST polypeptides

Levels of the various GST subunits in livers of *Nrf2*^{-/-} and WT mice on control and BHA-containing diets were determined using a combination of affinity chromatography and reversed-

phase HPLC. The class Alpha, Mu and Pi GSTs were isolated from hepatic cytosol (approx. 500 mg of protein) by chromatography on 1.6 cm × 8.0 cm columns of GSH-agarose that were equilibrated with 50 mM Tris/HCl, pH 7.5. After extensive washing of the affinity column to remove non-specifically bound protein, the GST isoenzymes were eluted isocratically in a single pool by elution of the affinity column with 20 mM GSH in 100 mM Tris/HCl, pH 9.0. Individual GST subunits were resolved by reversed-phase HPLC on a 0.46 cm × 25.0 cm Brownlee C₁₈ reversed-phase column (7 µm particle size and 30 nm pore size), which was eluted in two stages with a 25–54 % gradient of acetonitrile in aq. 0.1 % (v/v) trifluoroacetic acid: from 0–5 min, the column was eluted with a linear gradient of 25–43 % acetonitrile, and the flow rate was increased from 0.1 ml/min to 1.0 ml/min; from 5–60 min, the column was eluted at 1.0 ml/min with a linear gradient of 43–54 % acetonitrile. The eluate from the HPLC column was monitored for absorbance at 214 nm, and the amount of each subunit was estimated from the peak area using molar absorption coefficients that have been calculated by Johnson et al. [29] for the rat transferases. Collation of results from various groups of workers [22,27,30,31] indicates the murine polypeptides are eluted from reversed-phase HPLC columns in the following order: Gstm3, Gstm1, Gstm2, Gsta3, Gstp1/2, Gstm5, Gsta1, Gsta2, Gstm4* (Yb₅) and Gsta4. Identification of these peaks is facilitated by the fact that the concentration of Gstp1 and Gstp2 in the livers of male mice greatly exceeds that found in livers of female mice [31,32].

Molecular cloning of Gstm4

Both bioinformatics and a nested PCR strategy were employed to clone Gstm4. Sense oligonucleotides specific for the 5'-untranslated region end of the cDNA (GM4F1, 5'-CCGGAG-

CCGGGGACTCA-3'; GM4F2, 5'-AGTCTCAGGCACCAG-CATC-3') were designed following a personal communication from Dr William R. Pearson (University of Virginia, VA, U.S.A.), indicating that an IMAGE clone (GenBank® accession number AA538006) encodes part of Gstm4. Amplification of the cDNA was achieved using the sense oligonucleotides in two 3' RACE (rapid amplification of cDNA ends) reactions. For this purpose, the oligo(dT) anchor primer was 5'-GACCACGCGTATCGA-TGTCGACTTTTTTTTTTTTTTTTTTV-3' (where V is A or G or C), and the anchor primer was 5'-GACCACGCGTATCGAT-GTCGAC-3'.

The cDNA for Gstm4 was amplified in two separate reactions. Total RNA from the liver of a male *Nrf2*^{+/+} mouse on a control diet as template was isolated using an RNeasy kit (Qiagen Ltd., Crawley, West Sussex, U.K.). It was reverse-transcribed in the presence of oligo(dT)-anchor primer using SuperScript™ II (Gibco BRL, Life Technologies Ltd, Paisley, Scotland, U.K.) according to the supplier's instructions. The PCR was performed in 50 µl of reaction buffer containing approx. 100 ng of template DNA, 200 ng of each of the two oligonucleotides, 0.2 mM each of the four dNTPs and 1.25 units of *Pfu* Turbo® DNA polymerase (Stratagene, Amsterdam-Zuidoost, The Netherlands). In the first amplification reaction, the DNA template was subjected to an initial 94 °C denaturation step for 2 min. Thereafter, for the following 35 cycles, the DNA was denatured by heating at 94 °C for 45 s, the GM4F1 oligonucleotide and the oligo(dT)-anchor primer were allowed to anneal at 52 °C for 45 s, and elongation of the primers took place at 72 °C for 2 min. In the final cycle, the DNA was extended at 72 °C for 10 min to yield the first PCR products. An aliquot of this reaction mixture (1 µl) was diluted 1:10 before being subjected to a second nested PCR using GM4F2 and the same anchor primer oligonucleotides used in the first amplification reaction. The conditions employed for the second PCR were identical with those used for the first amplification, with the single exception that the primers were annealed at 55 °C in order to increase specificity.

Measurement of mRNA levels

This was performed using TaqMan® chemistry. The method first entailed cDNA synthesis from hepatic RNA, and this was followed by quantitative real-time PCR using the PerkinElmer/Applied Biosystems Prism Model 7700 Sequence Detector instrument.

Total RNA from livers of male and female *Nrf2*^{-/-} and WT mice was prepared using RNeasy kits (Qiagen Ltd.). Contaminating genomic DNA was removed from 400 µg of RNA by on-column DNase digestion (Qiagen Ltd.). In each sample, single-stranded cDNA was synthesized using 100 units of SuperScript II reverse transcriptase (Gibco BRL) and 0.15 µg of random hexamers (Promega UK, Chilworth, Southampton, U.K.) in a 20 µl solution of 50 mM Tris/HCl, pH 8.0, 75 mM KCl and 3 mM MgCl₂ containing 10 mM dithiothreitol and 1 mM dNTPs. The reaction mixture was left to equilibrate at 25 °C for 15 min before synthesis was allowed to proceed at 42 °C for 50 min. Finally, the reaction was terminated by incubation at 70 °C for 10 min, and the cDNA-containing reaction mixtures were diluted to 200 µl and stored at -20 °C until required.

Matching oligonucleotide primers and fluorescent probes used for real-time PCR were designed using the software Primer Express™ (PerkinElmer Applied Biosystems), and are listed in Table 1. The primers were synthesized by MWG-Biotech UK Ltd. (Milton Keynes, U.K.). The probes, which were labelled with a 5' fluorescent reporter dye (6-carboxyfluorescein) and a

3' quenching dye (6-carboxytetramethylrhodamine), were synthesized by Cruachem Ltd. (Glasgow, Scotland, U.K.). Each PCR mix (15 µl) contained between 10 and 15 ng of cDNA, 900 nM forward and reverse oligonucleotide primers and 200 nM probe in 1 × (final concentration) TaqMan® Master Mix (PerkinElmer). Amplification of cDNA was performed over 41 cycles in the Prism Model 7700 Sequence Detector instrument. The first cycle was performed using 50 °C for 2 min, followed by 95 °C for 10 min. Cycles 2–41 were performed at 95 °C for 15 s, followed by 60 °C for 1 min. Reactions were monitored by measuring fluorescence at 518 nm with excitation at 494 nm. Each assay was performed in triplicate.

The specificity of PCR amplifications from the various sets of oligonucleotide primers was examined routinely by agarose-gel electrophoresis. In the case of highly similar GST mRNAs, the specificity of the TaqMan® reactions was checked by using serial dilutions of several different authentic cDNAs as template.

The TaqMan® software was used to calculate a *C_t* value for each reaction; this value represents the extension phase of PCR, where the fluorescence signal produced by the amplified product can be distinguished from background. The level of 18 S ribosomal RNA was used as an internal standard (using a kit from PerkinElmer).

RESULTS

Identification of the cDNA for Gstm4* and definition of nomenclature for murine class Mu GST

The class Mu Gstm4* subunit, originally called Yb₅, was first purified as a heterodimer with Gstm1 from the livers of mice fed on a BHA-containing diet, and its C-terminal 198–218 residues

A			
	Reference	Primary structure	Identity (%)
Yb ₅	22	MKTSRFLRTPLTYTKVATWGNK	
Gstm1	24, 25	MKSSRYIATPIFSKMAHWSNK	52
Gstm2	25	MKSSRFLSKPIFAKMAFWNPK	52
Gstm3	24	MKSSRFLPRPVPTKIAQWGT	57
Gstm4	AK003418	MKTSRFLRTPLTYTKVATWGNK	100
Gstm5	26	LQSDRPFKMPINNMAKMGWGNK	43
Gstm6	28	MKTSRFLSPVYLKQATWGNK	71

B			
	Reference	Primary structure	Identity (%)
Yb ₅	22	MDDSNQLARVXSPDFEKLKVEYLEQ	
Gstm1	24, 25	MDTRMQLIMLCYNPDFEKLKVEYLEQ	52
Gstm2	25	MDTRIQLAMVCYSPDFEKLKVEYLEQ	72
Gstm3	24	MDTRIQLMIVCCSPDFEKLKVEYLEQ	56
Gstm4	AK003418	MDVSNQLARVXSPDFEKLKVEYLEQ	96
Gstm5	26	MDFRMQLVRLCYNSNHNELKPKQYLEQ	52
Gstm6	28	MDTRIQMGLCYSDFEKLKVEYLEQ	48

Figure 1 Comparison between sequences of CNBr peptides generated from Yb₅ and equivalent regions of other class Mu GST

(A) The sequence of the Yb₅ CNBr-a peptide described by Hayes et al. [22], is shown aligned with residues 198–218 of class Mu transferases; the numbering of the residues includes the initiator methionine, but not the four-amino-acid N-terminal extension that is unique to mouse Gstm5. The reference provided against the murine class Mu transferases represents the first description of the cDNA clone for individual subunits. The entry against Gstm4 represents the RIKEN cDNA 1110004G14 (accession number AK003418). (B) The sequence of the Yb₅ CNBr-d peptide [22] is shown aligned with residues 105–130 of class Mu GST.

M P M T L G Y W D I R G L A H A I R L L	20
atgcctatgacactgggttactgggacatccgtgggctagctcacgctattcggtgctc	60
L E Y T G S S Y E E K R Y T M G D A P D	40
ctagaatacacaggctcaagctatgaagagaagagatacaccatgggagacgctcctgac	120
Y D R S Q W L S E K F K L G L D F P N L	60
tatgaccgaagccagtggtgagtgagaagttcaaattgggcctggactttcccaatttg	180
P Y L I D G S H K I T Q S N A I L R Y I	80
ccttacttgattgatgggtcacacaagatcacgcagagcaatgccatcctgcgctacatt	240
A R K H N L C G E T E E E K I R V D I L	100
gcccgaagcacaacctgtgtggggagacagaggaagagaagattcgcgtggacattttg	300
E N Q A M D V S N Q L A R V C Y S P D F	120
gagaaccaggctatggatgtctccaatcagctggctcagctctgttacagcccagacttt	360
E K L K V E Y L E Q L P G M V K L F S Q	140
gagaaactgaaggtggaatacttggagcagctccctggaatggtgaagctcttctcacag	420
F L G Q R T W F V G E K I T F V D F L A	160
ttcctgggcccagcggacatggtttgggtgaaaagattactttttagatttcctggct	480
Y D I L D L H L I F E P T C L D A F P N	180
tacgatatcctggacctgcaccttatattcgaaccacgtgctggacgccttcccaaac	540
L K D F V A R F E V L K R I S A Y M K T	200
ctgaaggactttgtggcccgctttgaggtactgaagaggatctctgcttacatgaagacc	600
S R F L R T P L Y T K V A T W G N K -	218
agccgcttcctccgaacacccctatatacaaaggtggccacttggggcaataagtagagc	660
cttgactgggcaggaagtgggaaccgggggttctgggaacagttgaacttctgtagccc	720
tagtgctgctttctgtccatcccccttctgacccagagtgtcaaaggctcctcttttctt	780
tcacccagtcctgccccttcaaacctctaaagcctaggtccttagtttctcttagca	840
aaatgccccttctagcatgactgtgtcccgcgcccacttgatggtcttgccctgccagcagc	900
tgtgctgttgtaagagttgggactctccatcagcactcagcctggggctccccatgctt	960
gtctggagaccagagacggctggtgtgtgaatttggctctctgcacagctcttttgggtcc	1020
ctcctgtaaagcctgaaccacactggctctggctgccgactaccagcttttactactat	1080
ctccagtggctgcctagtgaaccgggcagaggctgagttcacagggattttagttggata	1140
ggcaggggtttggcccttctagccccacctgtttgtttctcaggaggcagctgcagaag	1200
gctctgtggagctcaaagggagcttagatctcctttatgctagcagcactgagatttgct	1260
atgcaggctctcagtggtgaggatccaggctgataggagtccccagcagaaagccaggatc	1320
ctctctgcccactgtgctatggctgccttatatctatgtctccaggatcctgtctctg	1380
atgtcttcagagtatcccgtcttggtcaccagggtgggggcatcttggttaatccctc	1440
ctctttgtgagcccccgtaaaataatttcttcatgctttcggaataaaaaa	1491

Figure 2 Nucleotide and deduced primary protein structure of murine Gstm4

Table 2 GST activities in the cytosolic fraction of mouse liver

GST activity is expressed as $\mu\text{mol}/\text{min}$ per mg of GST with CDNB as substrate. The values presented represent mean catalytic activities ($\pm$ S.E.M.) from triplicate measurements of pooled hepatic cytosol from three separate animals. The numbers in parentheses represent the mean activity value as a percentage of the value obtained with *Nrf2*^{+/+} on a control diet within the same experimental group.

Diet	Genotype ...	GST activity	
		<i>Nrf2</i> ^{+/+}	<i>Nrf2</i> ^{-/-}
Control 1		6.7 $\pm$ 0.1 (100)	4.4 $\pm$ 0.1 (66)
BHA*		23.9 $\pm$ 0.2 (357)	4.2 $\pm$ 0.2 (63)
Control 2		5.8 $\pm$ 0.4 (100)	2.8 $\pm$ 0.1 (48)
EQ		21.1 $\pm$ 0.1 (364)	4.8 $\pm$ 0.2 (83)
OPZ		16.1 $\pm$ 0.5 (278)	2.7 $\pm$ 0.1 (47)
I3C		9.9 $\pm$ 0.1 (171)	3.9 $\pm$ 0.1 (67)
Control 3		6.9 $\pm$ 0.1 (100)	3.4 $\pm$ 0.1 (49)
SUL		5.6 $\pm$ 0.1 (81)	2.9 $\pm$ 0.1 (42)
K + C		6.4 $\pm$ 0.1 (93)	3.1 $\pm$ 0.1 (45)
Control 4		5.9 $\pm$ 0.1 (100)	3.3 $\pm$ 0.1 (56)
CMRN		11.1 $\pm$ 0.1 (188)	3.3 $\pm$ 0.1 (56)
3-OH CMRN		5.0 $\pm$ 0.1 (85)	3.1 $\pm$ 0.1 (53)
7-OH CMRN		4.9 $\pm$ 0.1 (83)	3.3 $\pm$ 0.1 (56)
LMTN		12.2 $\pm$ 0.5 (207)	4.4 $\pm$ 0.1 (75)
AGLN		8.7 $\pm$ 0.1 (147)	3.4 $\pm$ 0.1 (58)

* In this experiment, BHA was administered at 0.5% (w/w) in the RM1 animal feed.

were sequenced following cleavage with CNBr [22]. The C-terminal CNBr peptide from *Gstm4** was used for a BLAST search of cDNA databases, because this region of class Mu GST polypeptides is divergent [27]. The CNBr peptide gave a 100% match with a hypothetical mouse protein encoded by a RIKEN cDNA (1110004G14; accession number AK003418) cloned from the mRNA of an 18-day-old C57BL/6J mouse embryo (Figure 1A). By contrast, the CNBr peptide was found to share between 43% and 72% identity with the equivalent C-terminal regions of murine *Gstm1*, *Gstm2*, *Gstm3*, *Gstm5* and *Gstm6*. An additional CNBr peptide representing residues 105–130 of *Gstm4** was also aligned with the protein encoded by the RIKEN cDNA clone. Although this contained one mismatch with the predicted amino acid sequence of the equivalent peptide in *Gstm4*, at least seven differences were observed when the CNBr peptide was compared with the amino acid sequence of the equivalent region in other mouse class Mu GSTs (Figure 1B).

The published RIKEN expressed sequence tag (EST) sequence for the putative *Gstm4** (AK003418) appeared to lack nucleotides encoding the N-terminus of the protein. According to information provided by Dr William R. Pearson, the N-terminal region of *Gstm4* was considered to be likely to be encoded by IMAGE clone 930982 (AA538006). A 3'-RACE approach was therefore adopted to isolate a cDNA containing the entire coding region of *Gstm4**. This was achieved using RNA isolated from male *Nrf2*^{+/+} mice fed on a control diet using an oligo(dT)-anchor primer targeted to the polyadenylated [poly(A)⁺] tail of the message, and sense oligonucleotides found in the partial-length IMAGE clone aa538006, as described above. Figure 2 shows the cDNA sequence of *Gstm4* and the predicted translated product. The determination of the full coding sequence for *Gstm4* obviates the need for the asterisk in the subunit designation, and it is omitted hereafter.

It was noted originally that *Gstm4* cross-reacted with antibodies raised against the rat GSTM5 subunit [22]. Therefore positive immunoblots of mouse tissues that have been generated by probing with anti-rGSTM5 (see [7] and [16]) could be due to

Figure 3 Constitutive and inducible *Gsta1/2* protein levels in the liver of WT and *Nrf2* mutant mice fed on diets containing different xenobiotics

Both *Nrf2*^{+/+} and *Nrf2*^{-/-} mice were fed on diets supplemented with synthetic chemopreventive agents or phytochemicals, as described in the Materials and methods section. Portions (4 μg of protein) of cytosol from liver of WT and KO mice were subjected to Western blotting. Equality in the loading of protein from different samples was verified after electrophoresis by inspecting the intensity of Coomassie Blue staining of polyacrylamide gels, and of Ponceau S staining of Immobilon-P membranes. ECL located immunochemically cross-reacting polypeptides, and the results were visualized by autoradiography. Results from experiments 1 and 2 are shown in (A), whereas results from experiments 3 and 4 are shown in (B) and (C) respectively. The dietary supplements are indicated at the top of each panel. Affinity-purified rat liver GST was included as a standard (Std) in lane 1 of the gel. The genotype of each group of mice, WT and homozygous *Nrf2* mutant (KO) mice, is shown above the appropriate lane. The results presented are typical of those obtained; they were repeated on at least three separate occasions. Closely similar patterns of cross-reacting proteins were produced when the blots were repeated. Con, control.

cross-reactivity with either murine *Gstm4* or *Gstm5*. Thus, in the blots presented below, cross-reactivity with anti-rat GSTM5 is labelled *Gstm4/5*.

BHA represents a potent inducer of class Alpha GST subunits in mouse liver

Before examining the regulation of hepatic detoxification and antioxidant genes in WT and *Nrf2* KO mice of both sexes, an initial investigation was undertaken to identify which chemopreventive agent might serve as the best model inducer of these genes in the liver. For this purpose, the series of livers that was generated during the study of McMahon et al. [16] into induction of detoxication proteins in the small intestine of WT and *Nrf2* KO mice was examined. It should be noted that this series comprised only livers from male mice.

Table 2 shows that increases in GST activity of between 1.7-fold and 3.6-fold were observed in the livers of WT mice fed on diets containing BHA, EQ, OPZ, I3C, CMRN, LMTN or

Table 3 GST activity in male and female mouse hepatic cytosol

GST activity is expressed as $\mu\text{mol/min}$ per mg of protein for GST. The values presented represent mean catalytic activities ($\pm$ S.E.M.) from triplicate measurements of pooled hepatic cytosol from three separate animals. The numbers in parentheses represent the mean activity value as a percentage of the value obtained with *Nrf2*^{+/+} on a control diet within the same experimental group.

Sex/diet	Genotype ...	GST activity	
		<i>Nrf2</i> ^{+/+}	<i>Nrf2</i> ^{-/-}
Male/control 5		3.4 $\pm$ 0.1 (100)	1.2 $\pm$ 0.1 (35)
Male/BHA*		7.0 $\pm$ 1.1 (206)	1.1 $\pm$ 0.2 (32)
Female/control 5		1.5 $\pm$ 0.1 (100)	0.7 $\pm$ 0.01 (46)
Female/BHA*		9.6 $\pm$ 0.5 (640)	1.1 $\pm$ 0.1 (73)

* In this experiment, BHA was included at 0.25% (w/w) in the diet.

AGLN. In *Nrf2*-null mice, inclusion of the xenobiotics in the diet had essentially no effect on hepatic transferase activity.

Immunoblotting of hepatic cytosols from male WT mice revealed that feeding these animals on diets containing BHA, EQ, OPZ, CMRN, LMTN or AGLN resulted in substantial increases in Gsta1/2 subunits (Figure 3). By comparison, Western blotting with antibodies raised against mouse Gstm1-1, rat GSTM5-5 and mouse Gstp1-2 showed a less obvious increase in the transferase subunits in livers of male mice fed on diets containing the various xenobiotics (results not shown).

Dependence on *Nrf2* for constitutive and inducible hepatic GST activities is apparent in both sexes

Having demonstrated that BHA is one of the more potent inducing agents among those readily commercially available, it was decided to examine induction by this antioxidant in both male and female mouse liver. The decision to study induction in male and female mice was taken because the expression of Pi class GST is sexually dimorphic in mouse liver [31,32], and induction of the transferases can vary in male and female mice [33]. The possibility that the hepatic levels of detoxication and antioxidant proteins are dependent on *Nrf2* in a sex-specific fashion was therefore considered.

GST activity was lower in the livers from female WT mice fed on a control diet than in male mice supplied with the same food (Table 3). This sex difference in constitutive hepatic GST activity was still apparent in the KO animals, but in both females and males on a control diet the CDNB-GSH-conjugating activity in the liver was diminished. Transferase activity in the livers of female WT mice treated with BHA was increased to a greater extent than in males. However, only a modest induction of GST activity by BHA was observed in the livers of female *Nrf2* KO mice.

Sex-specific effects of BHA on GST proteins in livers from *Nrf2*^{+/+} and *Nrf2*^{-/-} mice

Constitutive amounts of the class Alpha Gsta1/2 subunits were similar in livers from male and female WT mice. The livers of male *Nrf2*^{-/-} mice, but not female *Nrf2*^{-/-} mice, showed a modest reduction in the constitutive levels of Gsta1/2. In agreement with previous work, these subunits were found to be induced in the livers of WT mice placed on a BHA-containing diet. A comparison between the hepatic levels of Gsta1/2 in male and female *Nrf2*^{-/-} mice fed on a control diet with genetically similar mice fed on a BHA-containing diet suggests that these

Figure 4 *Nrf2* influences the constitutive and inducible expression of detoxication and antioxidant proteins in the livers of both male and female mice

Male and female *Nrf2* WT and male and female *Nrf2* KO mice were fed on either the RM1 control diet or RM1 diet containing 0.25% (w/w) BHA for 14 days. Hepatic extracts were prepared and portions (4 μg of protein) were examined by Western blotting, as described above. Protein loading was checked as described in the legend to Figure 3. The dietary treatments are shown at the top of the Figure. All gels were loaded in a similar fashion, and the identity of the samples applied to each lane is indicated above the autoradiogram for Gsta1/2. The identity of the murine proteins that are known to cross-react with the antiserum used to probe each blot is indicated in the left-hand margin. In lane 1 of each blot, a positive standard (Std) was included. For class Alpha transferases and Gstm1/2, affinity-purified rat liver GST was used as a standard; for Gstm4/5, affinity-purified mouse testis transferases were used as standard; for Gstp1/2, affinity-purified rat lung GST was used as standard. For GCLC, rat kidney cytosol was used as the standard. For GCLM, recombinant protein served as standard.

class Alpha subunits are modestly inducible in the KO animals of both sexes (Figure 4).

Western blotting revealed little difference in the hepatic level of Gsta3 in male and female WT mice placed on either control or BHA diets. In livers of male *Nrf2*^{-/-} mice fed on a control diet, there was evidence that Gsta3 was expressed in lower amounts than in WT control livers. The Gsta4 subunit was found consistently to be present in slightly higher amounts in livers of

Table 4 Quantification of GST subunits in mouse liver by HPLC

The transferases that were purified from mouse liver by affinity chromatography on columns of GSH-agarose were subjected to reversed-phase HPLC on a Brownlee C₁₈ column, and quantified as described in the Materials and methods section. The BHA was supplied at 0.25% (w/w) in the RM1 laboratory animal feed. nd, not determined (insufficient material).

Sex and genotype	Diet	Level of glutathione <i>S</i> -transferase subunits (mg/g cytosolic protein)*						
		Gsta1/2†	Gsta3	Gsta4	Gstm1	Gstm2	Gstm4	Gstp1/2‡
Male, Nrf2 ^{+/+}	Control	n.d.	10.7	0.50	8.10	0.13	0.14	16.5
Male, Nrf2 ^{-/-}	Control	n.d.	2.8§	0.09§	0.83§	0.05	n.d.	17.5
Male, Nrf2 ^{+/+}	BHA	0.40	11.9§	0.62§	40.7§	4.40§	0.62§	31.7§
Male, Nrf2 ^{-/-}	BHA	n.d.	5.2§	0.17§	1.98§	0.10	n.d.	14.5
Female, Nrf2 ^{+/+}	Control	n.d.	11.7	0.54	10.6	0.44	n.d.	3.8
Female, Nrf2 ^{-/-}	Control	n.d.	7.7	0.27	1.19	0.09	n.d.	1.7
Female, Nrf2 ^{+/+}	BHA	1.12	11.1	0.84	70.0	11.40	0.95	15.3
Female, Nrf2 ^{-/-}	BHA	n.d.	8.2	0.45	2.44	0.36	0.07	3.1

* The mean value from three separate HPLC analyses of affinity-purified GST obtained from three different livers. The statistical significance of the data was determined using ANOVA followed by the Newman-Keuls test.

† The Gsta1 and Gsta2 subunits from mouse liver exhibit poor affinity for the GSH-agarose chromatography matrix [34], and therefore the Gsta1/2 subunit levels in the Table are underestimates.

‡ The Gstp1 and Gstp2 subunits cannot be resolved by reversed-phase HPLC. Thus the data in this Table relating to these subunits are combined.

§ Comparison with male WT control gives a *P* value of < 0.001 by Newman-Keuls test.

|| Comparison with female WT control gives a *P* value of < 0.001 by Newman-Keuls test.

female WT mice that those of male WT mice, and again the constitutive level of Gsta4 was found to be lower in the KO mice.

Mu class Gstm1/2 subunit(s) did not demonstrate sex-specific differences in mouse liver, although both the constitutive and inducible levels of Gstm1/2 were dependent on Nrf2 (Figure 4). The Gstm4/5 polypeptide(s) were expressed at higher levels in livers from female WT and female Nrf2 KO mice than in livers from genetically equivalent male animals. In WT mice of both sexes, Gstm4/5 was found to be inducible by BHA, with higher levels of the protein in livers of female animals still being apparent.

Under normal dietary conditions the Gstp1/2 subunit(s) was present at substantially lower amounts in livers from female WT mice than in livers from male mice. In Nrf2^{-/-} mice fed on a control diet, the sex-specific expression of Pi class GST was still obvious. Indeed, constitutive hepatic expression of Gstp1/2 appeared to be diminished in mutant female mice, but not in male mice. Figure 4 shows that, in livers of female WT mice, the relative increase in Gstp1/2 affected by BHA treatment is much greater than is the case in livers from male mice. The increase in protein is sufficient to largely obscure the sexual dimorphism in this GST class. Treatment of Nrf2^{-/-} mice with BHA did not cause a large induction of Pi class GST. Thus livers from female mutant mice fed BHA contained significantly lower levels of Gstp1/2 protein than did livers from male mice.

Effects of Nrf2 on GSH-biosynthetic proteins in mouse liver

Antibodies against enzymes involved in GSH biosynthesis were used to probe liver samples from female and male mice. As has been noted before in the mouse small intestine [16], significant inter-individual variation in the level of GCLC (previously called GCS_h) was also observed in the murine hepatic samples. Livers from female and male mice did not contain major differences in their levels of GCLC. BHA did not induce this subunit significantly in mouse liver. However, the constitutive level of GCLC in livers from both female and male mice was dependent on Nrf2 (Figure 4). The GCLM subunit did not show evidence of sexual dimorphism in mouse liver, nor was it found to be inducible by BHA. Constitutive expression of GCLM was modestly reduced in the Nrf2 KO mice (Figure 4). Neither sex nor Nrf2 or

inducing agent influenced the expression of glutathione synthetase in mouse liver.

Regulation of GST subunits by Nrf2 in mouse liver from both sexes

Affinity chromatography and HPLC were employed to quantify individual transferase subunits in mouse liver. On the basis of protein recovery from GSH-agarose, it was calculated that, in male WT mice fed on a control diet, the GST pool represented 3.6% of the total hepatic cytosolic protein. In female mice fed on a control diet, the GST pool represented 2.7% of the hepatic cytosolic protein. These data are similar to previous calculations of the hepatic GST content in DBA/2, C3H/He and C57BL6 mice [32]. Reversed-phase HPLC resolved seven GST-containing peaks from the pools of affinity-purified hepatic transferases. These were identified by their order of elution as Gstm1, Gstm2, Gsta3, Gstp1/2, Gsta1/2, Gstm4 and Gsta4. In addition, the Gstm3 subunit was eluted at 14 min as a minor (< 0.5% of affinity-purified GST pool) incompletely resolved peak, immediately in front of Gstm1, and it was not quantified. Two other equally minor peaks that are thought to represent Gstm5 and Gstm6 were also observed, but were not quantified: Gstm5 eluted at 39 min between Gstp1/2 and Gsta1/2; Gstm6 eluted at 17 min immediately after Gstm2.

The Gsta1 and Gsta2 homodimers from mouse liver cytosol fail to bind GSH-agarose [34], and, although Table 4 shows the subunits are induced by BHA, the values presented are underestimates, since the majority of these subunits will not have been present in the GST pool analysed by HPLC. A modest reduction in the level of Gsta3 was observed in the livers of male Nrf2^{-/-} mice when compared with male Nrf2^{+/+} mice. Similar differences in hepatic Gsta3 were also apparent when female KO mice were compared with female WT mice, but the influence of Nrf2 on constitutive expression of the transferase is less obvious than in the male.

Major differences were found in the amount of hepatic Gstm1 in WT and KO mice of either sex. The level of Gstm1 in livers of Nrf2^{-/-} mice fed on a control diet was only 10% of that in WT mice (Table 4). Furthermore, whereas BHA increased the levels of Gstm1 approx. 6-fold in male and female WT mice, the

Table 5 Quantification of hepatic mRNA for GST and GCL in WT and *Nrf2*^{-/-} mice fed on either a control diet or one containing BHA

In each experimental group, total RNA from the livers of three different mice was combined and mRNA was quantified by TaqMan® using 18 S RNA as an internal standard. Each assay was performed in triplicate on at least two separate occasions. The values presented represent means $\pm$ S.E.M. mRNA levels, and are expressed as a ratio of the amount measured in the liver of *Nrf2*^{+/+} mice of the same sex that were fed on a control diet. Comparison between the sexes revealed that the level of mRNA for Gsta1, Gstm4, Gstm6, Gstp1 and Gstp2 was 3.4-, 2.6-, 4.2-, 5.4- and 4.3-fold higher respectively in the livers of male WT mice fed on a control diet than in the livers of female WT mice. The control diet was RM1 animal feed, and BHA was provided in the RM1 diet at a dose of 0.25% by weight.

Sex and genotype	Diet	Levels of mRNA encoding ...						
		Gsta1	Gsta2	Gsta3	Gsta4	Gstm1	Gstm2	Gstm3
Male, <i>Nrf2</i> ^{+/+}	Control	1.00 $\pm$ 0.09	1.00 $\pm$ 0.10	1.00 $\pm$ 0.11	1.00 $\pm$ 0.23	1.00 $\pm$ 0.12	1.00 $\pm$ 0.10	1.00 $\pm$ 0.13
Male, <i>Nrf2</i> ^{-/-}	Control	0.03 $\pm$ 0.01	0.06 $\pm$ 0.01	0.32 $\pm$ 0.04	0.42 $\pm$ 0.08	0.12 $\pm$ 0.02	0.35 $\pm$ 0.04	0.11 $\pm$ 0.01
Male, <i>Nrf2</i> ^{+/+}	BHA	15.65 $\pm$ 3.00	4.67 $\pm$ 0.13	0.95 $\pm$ 0.07	1.62 $\pm$ 0.12	3.57 $\pm$ 0.21	2.76 $\pm$ 0.14	12.38 $\pm$ 1.30
Male, <i>Nrf2</i> ^{-/-}	BHA	0.73 $\pm$ 0.07	1.97 $\pm$ 0.13	1.29 $\pm$ 0.15	1.69 $\pm$ 0.18	0.63 $\pm$ 0.08	2.88 $\pm$ 0.27	2.26 $\pm$ 0.26
Female, <i>Nrf2</i> ^{+/+}	Control	1.00 $\pm$ 0.21	1.00 $\pm$ 0.40	1.00 $\pm$ 0.24	1.00 $\pm$ 0.16	1.00 $\pm$ 0.12	1.00 $\pm$ 0.14	1.00 $\pm$ 0.12
Female, <i>Nrf2</i> ^{-/-}	Control	0.13 $\pm$ 0.04	0.16 $\pm$ 0.02	0.78 $\pm$ 0.10	0.72 $\pm$ 0.08	0.17 $\pm$ 0.01	0.45 $\pm$ 0.05	0.10 $\pm$ 0.01
Female, <i>Nrf2</i> ^{+/+}	BHA	124.5 $\pm$ 9.0	4.29 $\pm$ 0.06	2.24 $\pm$ 0.17	1.79 $\pm$ 0.31	7.28 $\pm$ 0.43	7.19 $\pm$ 0.19	61.18 $\pm$ 3.45
Female, <i>Nrf2</i> ^{-/-}	BHA	0.77 $\pm$ 0.15	0.45 $\pm$ 0.05	0.89 $\pm$ 0.10	0.90 $\pm$ 0.15	0.37 $\pm$ 0.05	2.72 $\pm$ 0.32	0.52 $\pm$ 0.06

Sex and genotype	Diet	Levels of mRNA encoding ...						
		Gstm4	Gstm5	Gstm6	Gstp1	Gstp2	GCLC	GCLM
Male, <i>Nrf2</i> ^{+/+}	Control	1.00 $\pm$ 0.21	1.00 $\pm$ 0.11	1.00 $\pm$ 0.10	1.00 $\pm$ 0.01	1.00 $\pm$ 0.09	1.00 $\pm$ 0.04	1.00 $\pm$ 0.14
Male, <i>Nrf2</i> ^{-/-}	Control	0.10 $\pm$ 0.01	0.76 $\pm$ 0.08	0.33 $\pm$ 0.05	0.85 $\pm$ 0.10	0.86 $\pm$ 0.17	0.24 $\pm$ 0.05	0.60 $\pm$ 0.07
Male, <i>Nrf2</i> ^{+/+}	BHA	2.32 $\pm$ 0.43	0.79 $\pm$ 0.02	1.58 $\pm$ 0.09	1.65 $\pm$ 0.10	2.56 $\pm$ 0.37	1.01 $\pm$ 0.17	0.71 $\pm$ 0.12
Male, <i>Nrf2</i> ^{-/-}	BHA	0.95 $\pm$ 0.14	1.54 $\pm$ 0.16	0.64 $\pm$ 0.05	1.16 $\pm$ 0.17	2.10 $\pm$ 0.24	0.15 $\pm$ 0.03	0.38 $\pm$ 0.10
Female, <i>Nrf2</i> ^{+/+}	Control	1.00 $\pm$ 0.16	1.00 $\pm$ 0.17	1.00 $\pm$ 0.12	1.00 $\pm$ 0.12	1.00 $\pm$ 0.16	1.00 $\pm$ 0.22	1.00 $\pm$ 0.16
Female, <i>Nrf2</i> ^{-/-}	Control	0.45 $\pm$ 0.08	1.03 $\pm$ 0.15	0.54 $\pm$ 0.05	0.21 $\pm$ 0.02	0.81 $\pm$ 0.07	0.41 $\pm$ 0.03	0.78 $\pm$ 0.14
Female, <i>Nrf2</i> ^{+/+}	BHA	8.63 $\pm$ 0.83	1.75 $\pm$ 0.28	3.36 $\pm$ 0.28	3.47 $\pm$ 0.38	3.97 $\pm$ 0.42	1.09 $\pm$ 0.18	1.53 $\pm$ 0.40
Female, <i>Nrf2</i> ^{-/-}	BHA	0.91 $\pm$ 0.12	1.13 $\pm$ 0.13	0.90 $\pm$ 0.11	0.43 $\pm$ 0.07	1.18 $\pm$ 0.17	0.12 $\pm$ 0.03	0.88 $\pm$ 0.09

phenolic antioxidant increased Gstm1 in male and female mutant mice only approx. 2.5-fold over the constitutive levels observed in *Nrf2* KO mice.

The HPLC method allowed measurement of Gstm2 in mouse liver, despite the fact it is quantitatively a relatively minor transferase subunit. Table 4 shows that the constitutive level of this subunit is reduced in the absence of Nrf2. Furthermore, dietary BHA affected a > 20-fold increase in the hepatic content of Gstm2, and this induction was lost in the KO animals. The Gstm4 subunit was not abundant, and its constitutive levels could not be quantified with accuracy. Nevertheless, it was clearly inducible by BHA in WT mice, but not *Nrf2*^{-/-} mice.

The expression of class Pi GST in mouse liver is sexually dimorphic, and this is influenced by Nrf2. Livers of female control WT mice contain only approx. 25% of the amount of Gstp1/2 that is found in male control WT mice (Table 4). By comparison with *Nrf2*^{+/+} mice, the content of Gstp1/2 is unchanged in the livers of male mutant mice fed on a control diet. However, in the livers of female *Nrf2*^{-/-} mice the amount of Gstp1/2 is reduced below that observed in female *Nrf2*^{+/+} mice. As a consequence, the level of Gstp1/2 in livers of female mutant mice is only approx. 10% of that in the livers of male mice. BHA induces hepatic class Pi GST approx. 2-fold and 4-fold, respectively, in male and female WT mice. In both male and female *Nrf2*^{-/-} mice, induction of Gstp1/2 by BHA is abolished.

Nrf2 regulates mRNA levels for GST and GCL

TaqMan® real-time PCR assays were established to allow the effect of loss of Nrf2 on mRNAs for a range of GSH-dependent enzymes to be quantified. In the livers of male and/or female *Nrf2* mutant mice, the constitutive levels of mRNA for Gsta1,

Gsta2, Gstm1, Gstm3 and Gstm4 were reduced to between 3% and 20% of that in WT mice (Table 5). In addition, the levels of mRNA encoding Gsta3, Gsta4, Gstm2, Gstm6, Gstp1 and GCLC in the livers of mutant male and/or female mice fed on a control diet were reduced to between 30% and 60% of that in WT mice.

In addition to the PCR analyses described above, TaqMan® assays have been established for Nrf1, Nrf2, MafG, MafK and Keap1. It has been found that the mRNAs for Nrf1, MafG, MafK and Keap1 did not differ significantly in the livers of WT and KO mice fed on a control diet (Q. Jiang, C. R. Elcombe and J. D. Hayes, unpublished work). This is an important result, because it indicates that a general depression of transcription does not occur in the *Nrf2*^{-/-} mice.

In *Nrf2*^{+/+} mice, BHA affected a large induction of the mRNA encoding Gsta1 and Gstm3, with less marked induction in message for Gsta2, Gstm1 and Gstm2. More modest increases in mRNA encoding Gstm4, Gstp1 and Gstp2 were also observed. It was noted that many of these genes were more inducible in female WT mice than in male mice. This was particularly obvious with Gsta1, Gstm1, Gstm2, Gstm3, Gstm4 and Gstp1. A number of mRNAs examined, such as Gsta3 and Gstm5, appeared relatively unaffected by BHA.

Induction of gene expression by BHA in *Nrf2*^{-/-} mice was variable, and could not be predicted by the response in WT mice. When allowance is made for the low constitutive expression, the mRNA species encoding Gsta1, Gsta2, Gstm1 and Gstm2 were induced by BHA in KO mice, although the relative amount of induction caused by BHA was variable. With Gstp1 and Gstp2, induction by BHA was abolished in the KO mice. Interestingly, the continuous feeding of BHA for 14 days suppressed expression of GCLC in *Nrf2*^{-/-} mice.

DISCUSSION

The objective of this paper was to determine the impact disruption of *Nrf2* has on expression of GST and GCL enzymes. Initially, the cDNA for *Gstm4* was cloned in order to be certain that the class Mu transferases were defined unambiguously: *Gstm1*, *Gstm2* and *Gstm3* have been described in [24,25]; *Gstm4*, originally called *Yb₃* [22], is defined in Figure 2 of this paper; *Gstm5* and *Gstm6* have been reported in [26] and [28] respectively. Subsequently, in this study, TaqMan® assays were developed for all class Mu transferases and other GSTs, as well as GCLC and GCLM, to allow the expression of these antioxidant genes to be monitored.

The most striking observation of the present study is that constitutive hepatic expression of *Gsta1*, *Gsta2*, *Gstm1*, *Gstm3*, *Gstm4* and GCLC is markedly reduced in *Nrf2* homozygous null mice. It is not widely appreciated that *Nrf2* regulates both constitutive and inducible expression of GSH-dependent enzymes *in vivo*. Most importantly, our TaqMan® data reveal that *Nrf2* influences the expression of a substantial battery of genes, including class Alpha, class Mu and class Pi GST.

Reduced constitutive expression of GSH-dependent enzymes in *Nrf2* KO mice and increased sensitivity to xenobiotics

Class Alpha *Gsta1* and *Gsta2* subunits are both present in the livers of *Nrf2* mutant mice fed on a control diet at < 20 % of the levels observed in WT mice. The *Gsta1-2* heterodimer (also called GST 9.5) has been isolated from mouse forestomach [35]. It possesses at least 10-fold higher catalytic efficiency than the other transferases towards 7 β ,8 α -dihydroxy-9 α ,10 α -oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, the metabolite of BaP that represents the ultimate carcinogen [36]. *Gsta1-2* also has high activity towards all the stereoisomers of *trans*-3,4-dihydroxy-1,2-oxy-1,2,3,4-tetrahydrobenzo[*c*]phenanthrene [37]. It is predicted that organs of *Nrf2*^{-/-} mice that express *Gsta1/2* subunits at a reduced level will be more sensitive to polycyclic aromatic hydrocarbons than WT mice. Real-time PCR has shown that the mRNAs for *Gsta1* and *Gsta2* are reduced in the stomach of *Nrf2*^{-/-} mice to approx. 40 % and 10 %, respectively, of the level in the stomach of WT mice. This result may explain the observation that the KO mice develop more forestomach tumours than do WT mice when treated with BaP [18]. The fact that the lungs of mutant mice are more sensitive to the genotoxic effects of diesel fumes [17] might also be attributed to the relative lack of class Alpha transferases in such animals. Specifically, TaqMan® assays have shown that the pulmonary level of mRNA for *Gsta2* in the KO mouse is reduced to approx. 60 % of that found in the lung of WT mice (Q. Jiang, C. R. Elcombe and J. D. Hayes, unpublished work).

Mice are intrinsically resistant to aflatoxin B₁ hepatocarcinogenesis. This is attributed to the constitutive expression in the liver of *Gsta3-3*, a transferase with high activity towards aflatoxin B₁-8,9-epoxide [38–40]. Table 4 shows that *Gsta3* subunit levels in the liver of male *Nrf2*^{-/-} fed on either a control or a BHA-containing diet were approx. 40 % of that in WT mice. Kwak et al. [41] reported that male *Nrf2*-null mice accumulate 2.7-fold more aflatoxin B₁-DNA adducts following exposure to the mycotoxin than do WT mice. Collectively, these data are consistent with the hypothesis that *Gsta3-3* represents a major determinant of resistance to this hepatocarcinogen.

Constitutive expression of a number of class Mu transferases was found to be dependent on *Nrf2*. The HPLC method indicated that the livers of *Nrf2*^{-/-} mice contain significantly lower amounts of the *Gstm1* and *Gstm2* subunits than do WT mice. Similarly,

the levels of mRNA species for class Mu GST in the livers of *Nrf2* KO mice was reduced to 10–50 % of the amount in WT mice. The physiological consequences of the reduced levels of class Mu transferases is difficult to predict, since relatively little is known about the catalytic properties of class Mu GST in the mouse. The conjugation of a number of carcinogens, including BaP-4,5-epoxide and 4-nitroquinoline 1-oxide, with GSH is catalysed *in vitro* by *Gstm1-1* [23], and it is possible that *Nrf2* KO mice will be sensitive to these chemicals. Clearly, experiments are required to characterize the catalytic properties of this family of enzymes.

Hepatic expression of GCLC is diminished in *Nrf2*-null mice, and this will compromise their ability to synthesize GSH in response to oxidative stress. It has been proposed that the sensitivity of the mutant mice to acetaminophen hepatotoxicity is due to an impaired ability to regenerate GSH following depletion by *N*-acetyl-*p*-benzoquinone-imine, the reactive metabolite of acetaminophen [13,14]. It is apparent that other compounds that are conjugated with GSH before biliary excretion, and are eliminated ultimately from the body as mercapturic acids, may also be toxic for similar reasons.

Mechanism of induction of the *Gsta1* gene by BHA

A large number of TaqMan® real-time PCR assays have been developed that allow measurement of individual mRNA species in mouse tissues. By means of these assays, it has been shown for the first time that class Alpha *Gsta1* is highly inducible *in vivo* by BHA. Between nt –750 and –715 of *Gsta1*, a direct repeat-type of ARE, 5'-TTGGAAATGACATTGCTAATGGTGACA-AAGCAACTT-3' (with the 'core' ARE sequences underlined), has been identified [21]. It is reasonable to assume that this enhancer is responsible for the marked transcriptional activation of *Gsta1* by BHA in WT mice.

Mechanism of induction of class Mu and class Pi GST genes by BHA

In the present study, we have provided evidence that *Nrf2* regulates the class Mu *Gstm1* subunit in mouse liver. Approximately 0.6 kb flanking *Gstm1* has been cloned, and because this region does not contain a perfect ARE consensus sequence, it cannot be stated with certainty why expression of the gene is reduced in *Nrf2* KO mice. An imperfect ARE sequence exists (5'-CTGACAGAGTT-3') between nt –533 and –523 that can form a 15 bp palindrome (5'-CTGTCAGACTGACAG-3') centred around nt –534 (underlined) [42]. It is not known whether this motif in *Gstm1* can act as an enhancer, but it does invite comparison with the palindromic-type of ARE in the 5'-flanking region of the rat NAD(P)H:quinone oxidoreductase 1 gene [43] that represents a high-affinity binding site for *Nrf2* [44].

The TaqMan® data showed that *Gstm3* is highly inducible by BHA, a finding that confirms previous Northern blotting experiments [42,45]. However, a perfect ARE has not been identified in the promoter of this gene. To date, about 0.9 kb of the 5'-flanking region of *Gstm3* has been cloned, and the imperfect ARE sequence 5'-CTGACCTCGAA-3' can be identified, in reverse orientation, between nucleotides –653 and –643 [42]. Mutation analysis of the core ARE in rat *GSTA2* has shown that a C → A transversion of the 3' cytosine within the *cis*-element causes loss of inducibility, but not basal expression [3]. It therefore seems improbable that this putative enhancer is responsible for

the loss of constitutive expression in the Nrf2 KO mice. It remains to be established whether a functional ARE exists further upstream of the 5'-flanking region that was described by Reinhart and Pearson [42]. The alternative possibility that BHA induction of *Gstm3* is regulated indirectly by Nrf2 also cannot be discounted. According to this hypothesis, Nrf2 would affect induction of transcription factors that are regulated through ARE enhancers. Such examples could include c-Jun, JunB, ATF3 or ATF4, since they have been reported to be induced by tBHQ [46,47]. Further investigations are required to test these possibilities.

Class Pi *Gstp1* and *Gstp2* are modestly inducible in female mice. The sequence 5'-TTGAGTCAGCA-3' is found not only between nt -60 and -50 flanking *Gstp1*, but also between nt -71 and -61 flanking *Gstp2* [48,49]. Given the fact that induction of *Gstp1*, and, to a lesser extent, *Gstp2*, by BHA is abolished in the KO mice, it seems likely that this putative *cis*-element serves as a recognition site for Nrf2, even though it is not a perfect ARE.

Sex-specific induction

The present study has revealed that BHA is a more potent inducer of many GST genes in the livers of female mice than in the livers of male mice. This finding might be due to some type of co-operative interaction between the oestrogen receptor and Nrf2. Alternatively, BHA may be metabolized in a sex-specific fashion. It could be postulated that t-BHQ accumulates to a greater extent in the livers of female mice than their male counterparts. Sex-specific differences may exist in sulphation or glucuronidation of BHA, or the O-demethylation of BHA to t-BHQ, or the oxidase and GST responsible for conversion of t-BHQ into t-butyl-1,4-benzoquinone and subsequent conjugation with GSH (see [50] for details of biotransformation of BHA). These possibilities warrant investigation.

Concluding comments

TaqMan® real-time PCR has been used to examine the effect of the Nrf2 bZIP transcription on expression of individual antioxidant and/or detoxication genes in a gene KO mouse model. Such assays, along with conventional analytical biochemistry methods, have shown that the constitutive hepatic expression of various class Alpha and class Mu GST, along with expression of GCLC, is reduced significantly in *Nrf2*^{-/-} mice. In *Nrf2*^{-/-} mice, the ability of BHA to affect induction of protein/mRNA appeared to be independent of the changes in constitutive expression affected by the bZIP factor. Our results suggest that the innate loss of hepatic detoxification capacity in the *Nrf2*^{-/-} mouse, as much as failure to elicit an adequate adaptive response, is responsible for the mutant mouse being less able to tolerate chemical and oxidative stress than WT mice. This apparent dissociation between constitutive and inducible expression warrants further investigation.

This work was funded by grants from the Association for International Cancer Research (99-041), the World Cancer Research Fund (2000/11), and the Medical Research Council (G0000281). S.A.C. holds a Co-operative Awards in Science and Engineering Medical Research Council Ph.D. studentship. We thank Dr Christophe Cavin (Nestlé Research Center, Lausanne, Switzerland) for his generosity in providing the cafestol and kahweol palmitate used in the feeding experiments. We also thank Dr Bill Pearson for his helpful expert advice about GST nomenclature, and for providing information about the identity of EST clones. Lastly, we are enormously grateful to Dr Irving Listowsky for his critical comments about GST nomenclature, and for pointing out an error in one of our *Gstm4* cDNA clones prior to publication.

REFERENCES

- Hayes, J. D. and McLellan, L. I. (1999) Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radical Res.* **31**, 273–300
- Wild, A. C. and Mulcahy, R. T. (2000) Regulation of γ -glutamylcysteine synthetase subunit gene expression: insights into transcriptional control of antioxidant defences. *Free Radical Res.* **32**, 281–301
- Rushmore, T. H., Morton, M. R. and Pickett, C. B. (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.* **266**, 11632–11639
- Favreau, L. V. and Pickett, C. B. (1991) Transcriptional regulation of the rat NAD(P)H: quinone reductase gene. Identification of regulatory elements controlling basal level expression and inducible expression by planar aromatic compounds and phenolic antioxidants. *J. Biol. Chem.* **266**, 4556–4561
- Prester, T., Holtzclaw, W. D., Zhang, Y. and Talalay, P. (1993) Chemical and molecular regulation of enzymes that detoxify carcinogens. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2965–2969
- Dinkova-Kostova, A. T., Massiah, M. A., Bozak, R. E., Hicks, R. J. and Talalay, P. (2001) Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 3404–3409
- Hayes, J. D., Chanas, S. A., Henderson, C. J., McMahon, M., Sun, C., Moffat, G. J., Wolf, C. R. and Yamamoto, M. (2000) The Nrf2 transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin. *Biochem. Soc. Trans.* **28**, 33–41
- Venugopal, R. and Jaiswal, A. K. (1996) Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase₁ gene. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14960–14965
- Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I. et al. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* **236**, 313–322
- Zhu, M. and Fahl, W. E. (2001) Functional characterization of transcription regulators that interact with the electrophile response element. *Biochem. Biophys. Res. Commun.* **289**, 212–219
- Hayes, J. D. and McMahon, M. (2001) Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. *Cancer Lett.* **174**, 103–113
- Ishii, T., Itoh, K., Takahashi, S., Sato, H., Yanagawa, T., Katoh, Y., Bannai, S. and Yamamoto, M. (2000) Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J. Biol. Chem.* **275**, 16023–16029
- Enomoto, A., Itoh, K., Nagayoshi, E., Haruta, J., Kimura, T., O'Connor, T., Harada, T. and Yamamoto, M. (2001) High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. *Toxicol. Sci.* **59**, 169–177
- Chan, K., Han, X.-D. and Kan, Y. W. (2001) An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4611–4616
- Chan, K. and Kan, Y. W. (1999) Nrf2 is essential for protection against acute pulmonary injury in mice. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 12731–12736
- McMahon, M., Itoh, K., Yamamoto, M., Chanas, S. A., Henderson, C. J., McLellan, L. I., Wolf, C. R., Cavin, C. and Hayes, J. D. (2001) The cap'n'collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. *Cancer Res.* **61**, 3299–3307
- Aoki, Y., Sato, H., Nishimura, N., Takahashi, S., Itoh, K. and Yamamoto, M. (2001) Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. *Toxicol. Appl. Pharmacol.* **173**, 154–160
- Ramos-Gomez, M., Kwak, M.-K., Dolan, P. M., Itoh, K., Yamamoto, M., Talalay, P. and Kensler, T. W. (2001) Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in *nrf2* transcription factor-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 3410–3415
- Cho, H.-Y., Jedlicka, A. E., Reddy, S. P. M., Zhang, L.-Y., Kensler, T. W. and Kleeberger, S. R. (2002) Linkage analysis of susceptibility to hyperoxia: Nrf2 is a candidate gene. *Am. J. Respir. Cell. Mol. Biol.* **26**, 42–51
- Morimitsu, Y., Nakagawa, Y., Hayashi, K., Fujii, H., Kumagai, T., Nakamura, Y., Osawa, T., Horio, F., Itoh, K., Iida, K. et al. (2002) A sulforaphane analogue that potentially activates the Nrf2-dependent detoxification pathway. *J. Biol. Chem.* **277**, 3456–3463
- Friling, R. S., Bensimon, A., Tichauer, Y. and Daniel, V. (1990) Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6258–6262

- 22 Hayes, J. D., Kerr, L. A., Peacock, S. D., Cronshaw, A. D. and McLellan, L. I. (1991) Hepatic glutathione *S*-transferases in mice fed on a diet containing the anticarcinogenic antioxidant butylated hydroxyanisole. Isolation of mouse glutathione *S*-transferase heterodimers by gradient elution of the glutathione-Sepharose affinity matrix. *Biochem. J.* **277**, 501–512
- 23 Hayes, J. D. and Pulford, D. J. (1995) The glutathione *S*-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit. Rev. Biochem. Mol. Biol.* **30**, 445–600
- 24 Pearson, W. R., Reinhart, J., Sisk, S. C., Anderson, K. S. and Adler, P. N. (1988) Tissue-specific induction of murine glutathione transferase mRNAs by butylated hydroxyanisole. *J. Biol. Chem.* **263**, 13324–13332
- 25 Townsend, A. J., Goldsmith, M. E., Pickett, C. B. and Cowan, K. H. (1989) Isolation, characterization, and expression in *Escherichia coli* of two murine Mu class glutathione *S*-transferase cDNAs homologous to the rat subunits 3 (Yb1) and 4 (Yb2). *J. Biol. Chem.* **264**, 21582–21590
- 26 Fulcher, K. D., Welch, J. E., Klapper, D. G., O'Brien, D. A. and Eddy, E. M. (1995) Identification of a unique μ -class glutathione *S*-transferase in mouse spermatogenic cells. *Mol. Reprod. Dev.* **42**, 415–424
- 27 Rowe, J. D., Patskovsky, Y. V., Patskovska, L. N., Novikova, E. and Listowsky, I. (1998) Rationale for reclassification of a distinctive subdivision of mammalian class Mu glutathione *S*-transferases that are primarily expressed in testis. *J. Biol. Chem.* **273**, 9593–9601
- 28 De Bruin, W. C. C., Te Morsche, R. H. M., Wagenmans, M. J. M., Alferink, J. C., Townsend, A. J., Wieringa, B. and Peters, W. H. M. (1998) Identification of a novel murine glutathione *S*-transferase class mu gene. *Biochem. J.* **330**, 623–626
- 29 Johnson, J. A., Finn, K. A. and Siegel, F. L. (1992) Tissue distribution of enzymic methylation of glutathione *S*-transferase and its effects on catalytic activity. Methylation of glutathione *S*-transferase 11-11 inhibits conjugating activity towards 1-chloro-2,4-dinitrobenzene. *Biochem. J.* **282**, 279–289
- 30 McLellan, L. I., Kerr, L. A., Cronshaw, A. D. and Hayes, J. D. (1991) Regulation of mouse glutathione *S*-transferases by chemoprotectors. Molecular evidence for the existence of three distinct Alpha-class glutathione *S*-transferase subunits, Ya₁, Ya₂ and Ya₃, in mouse liver. *Biochem. J.* **276**, 461–469
- 31 Mitchell, A. E., Morin, D., Lakritz, J. and Jones, A. D. (1997) Quantitative profiling of tissue- and gender-related expression of glutathione *S*-transferase isoenzymes in the mouse. *Biochem. J.* **325**, 207–216
- 32 McLellan, L. I. and Hayes, J. D. (1987) Sex-specific constitutive expression of the pre-neoplastic marker glutathione *S*-transferase, YfYf, in mouse liver. *Biochem. J.* **245**, 399–406
- 33 McLellan, L. I., Harrison, D. J. and Hayes, J. D. (1992) Modulation of glutathione *S*-transferases and glutathione peroxidase by the anticarcinogen butylated hydroxyanisole in murine extrahepatic organs. *Carcinogenesis* **13**, 2255–2261
- 34 McLellan, L. I. and Hayes, J. D. (1989) Differential induction of class Alpha glutathione *S*-transferases in mouse liver by the anticarcinogenic antioxidant, butylated hydroxyanisole. *Biochem. J.* **263**, 393–402
- 35 Hu, X., Benson, P. J., Srivastava, S. K., Mack, L. M., Xia, H., Gupta, V., Zaren, H. A. and Singh, S. V. (1996) Glutathione *S*-transferases of female A/J mouse liver and forestomach and their differential induction by anti-carcinogenic organosulfides from garlic. *Arch. Biochem. Biophys.* **336**, 199–214
- 36 Hu, X., Srivastava, S. K., Xia, H., Awasthi, Y. C. and Singh, S. V. (1996) An alpha class mouse glutathione *S*-transferase with exceptional catalytic efficiency in the conjugation of glutathione with 7 β ,8 α -dihydroxy-9 α ,10 α -oxy-7,8,9,10-tetrahydrobenzo(a)pyrene. *J. Biol. Chem.* **271**, 32684–32688
- 37 Hu, X., Seidel, A., Frank, H., Srivastava, S. K., Xia, H., Pal, A., Zheng, S., Oesch, F. and Singh, S. V. (1998) Differential enantioselectivity of murine glutathione *S*-transferase isoenzymes in the glutathione conjugation of *trans*-3,4-dihydroxy-1,2-oxy-1,2,3,4-tetrahydrobenzo[c]phenanthrene stereoisomers. *Arch. Biochem. Biophys.* **358**, 40–48
- 38 Hayes, J. D., Judah, D. J., Neal, G. E. and Nguyen, T. (1992) Molecular cloning and heterologous expression of a cDNA encoding a mouse glutathione *S*-transferase Yc subunit possessing high catalytic activity for aflatoxin B₁-8,9-epoxide. *Biochem. J.* **285**, 173–180
- 39 Buetler, T. M., Slone, D. and Eaton, D. L. (1992) Comparison of the aflatoxin B₁-8,9-epoxide conjugating activities of two bacterially expressed Alpha class glutathione *S*-transferase isozymes from mouse and rat. *Biochem. Biophys. Res. Commun.* **188**, 597–603
- 40 Townsend, A. J., Fields, W. R., Haynes, R. L., Doss, A. J., Li, Y., Doehmer, J. and Morrow, C. S. (1998) Chemoprotective functions of glutathione *S*-transferases in cell lines induced to express specific isoenzymes by stable transfection. *Chem. Biol. Interact.* **112**, 389–407
- 41 Kwak, M.-K., Egner, P. A., Dolan, P. M., Ramos-Gomez, M., Groopman, J. D., Itoh, K., Yamamoto, M. and Kensler, T. W. (2001) Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. *Mutat. Res.* **480**, 305–315
- 42 Reinhart, J. and Pearson, W. R. (1993) The structure of two murine class-Mu glutathione transferase genes coordinately induced by butylated hydroxyanisole. *Arch. Biochem. Biophys.* **303**, 383–393
- 43 Favreau, L. V. and Pickett, C. B. (1995) The rat quinone reductase antioxidant response element. Identification of the nucleotide sequence required for basal and inducible activity and detection of antioxidant response element-binding proteins in hepatoma and non-hepatoma cell lines. *J. Biol. Chem.* **270**, 24468–24474
- 44 Nguyen, T., Huang, H. C. and Pickett, C. B. (2000) Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK. *J. Biol. Chem.* **275**, 15466–15473
- 45 Sisk, S. C. and Pearson, W. R. (1993) Differences in induction by xenobiotics in murine tissues and the Hepa1c1c7 cell line of mRNAs encoding glutathione transferase, quinone reductase, and CYP1A P450s. *Pharmacogenetics* **3**, 167–181
- 46 Yoshioka, K., Deng, T., Cavigelli, M. and Karin, M. (1995) Antitumor promotion by phenolic antioxidants: inhibition of AP-1 activity through induction of Fra expression. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4972–4976
- 47 He, C. H., Gong, P., Hu, B., Stewart, D., Choi, M. E., Choi, A. M. K. and Alam, J. (2001) Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implications for heme oxygenase-1 gene regulation. *J. Biol. Chem.* **276**, 20858–20865
- 48 Bammler, T. K., Smith, C. A. D. and Wolf, C. R. (1994) Isolation and characterization of two mouse Pi-class glutathione *S*-transferase genes. *Biochem. J.* **298**, 385–390
- 49 Xu, X. and Stambrook, P. J. (1994) Two murine GSTpi genes are arranged in tandem and are differently expressed. *J. Biol. Chem.* **269**, 30268–30273
- 50 Peters, M. M. C. G., Lau, S. S., Dulik, D., Murphy, D., van Ommen, B., van Bladeren, P. J. and Monks, T. J. (1996) Metabolism of *tert*-butylhydroquinone to *S*-substituted conjugates in the male Fischer 344 rat. *Chem. Res. Toxicol.* **9**, 133–139

Received 25 February 2002/16 April 2002; accepted 3 May 2002

Published as BJ Immediate Publication 3 May 2002, DOI 10.1042/BJ2002/0320