

Expression of the Aflatoxin B₁-8,9-Epoxy-Metabolizing Murine Glutathione S-Transferase A3 Subunit Is Regulated by the Nrf2 Transcription Factor through an Antioxidant Response Element

IAN R. JOWSEY, QING JIANG, KEN ITOH, MASAYUKI YAMAMOTO, and JOHN D. HAYES

Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Dundee, United Kingdom (I.R.J., Q.J., J.D.H.); and Centre for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan (K.I., M.Y.)

Received May 5, 2003; accepted July 18, 2003

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

High expression of the aflatoxin B₁ (AFB₁)-8,9-epoxide-conjugating glutathione S-transferase A3 (mGSTA3) subunit in mouse liver confers intrinsic resistance to AFB₁ hepatocarcinogenesis. It is not known how the gene encoding this protein is regulated. The murine *mGSTA3* gene has been identified using bioinformatics. It localizes to mouse chromosome 1 (A3-4), spans approximately 24.6 kilobases (kb) of DNA, and comprises seven exons. High levels of mGSTA3 mRNA are present in organs associated with detoxification. Expression of mGSTA3 in Hepa1c1c7 mouse hepatoma cells was found to be inducible by sulforaphane, an organic isothiocyanate that can transcriptionally activate genes through the antioxidant response element (ARE). Sulforaphane also induced transcription of a luciferase reporter containing a 1.5 kb fragment of the

mGSTA3 5'-upstream region. A putative ARE, with sequence 5'-TGACATTGC-3', was identified within this fragment, approximately 150 base pairs upstream of exon 1. Mutation of this sequence abrogated both basal and sulforaphane-inducible reporter activity. Overexpression of the basic-region leucine zipper Nrf2 transcription factor augmented activity of the *mGSTA3*-luciferase reporter through this ARE. Electrophoretic mobility shift assays demonstrated that Nrf2 binds the *mGSTA3* ARE. Measurement of mGSTA3 mRNA levels in tissues isolated from both wild-type and *nrf2*-null mice revealed that loss of the Nrf2 transcription factor is associated with a reduction in basal expression of mGSTA3. Collectively, these data demonstrate a role for Nrf2 and the ARE in regulating transcription of *mGSTA3*.

Aflatoxin B₁ (AFB₁), a difuranocoumarin mycotoxin produced by the mold *Aspergillus flavus*, is a potent naturally occurring carcinogen (Eaton and Gallagher, 1994). Its harmful effects arise because of biotransformation to AFB₁-8,9-*exo*-epoxide (Fig. 1), a metabolite that can react readily with the N⁷ atom of guanine residues in DNA. Consistent with this observation, AFB₁-initiated liver tumors in the rat often contain G→T and G→A mutations in codon 12 of *ras* oncogenes (McMahon et al., 1990), whereas in human hepatocytes, exposure to AFB₁ produces mutations within the *p53* tumor suppressor gene (Aguilar et al., 1993).

The toxicity of AFB₁ varies significantly between species. Whereas rats are sensitive to AFB₁ hepatocarcinogenesis, many mouse strains can tolerate exposure to the mycotoxin

(Eaton and Gallagher, 1994; Hengstler et al., 1999). This selective toxicity reflects a differential ability of mouse and rat to detoxify AFB₁-8,9-epoxide through glutathione (GSH) conjugation. The species differences in hepatic AFB₁-GSH conjugating activity is caused by the constitutive expression in mouse liver of a class α glutathione S-transferase subunit (mGSTA3 or Yc) that exhibits high catalytic activity toward the mycotoxin (Buetler et al., 1992; Hayes et al., 1992). Support for this hypothesis was provided by the finding that ectopic expression of mGSTA3 in hamster V79 cells confers resistance to AFB₁ cytotoxicity and causes a reduction in formation of DNA adducts (Fields et al., 1999). A rat class α GST subunit (rGSTA5 or Yc₂) exhibiting significant AFB₁-GSH conjugating activity has also been identified (Hayes et al., 1991, 1994). Although this subunit is constitutively present only at low levels, expression of rGSTA5 is highly

This work was supported by the Association for International Cancer Research (grant 01-096) and the Medical Research Council (grant G0000281).

ABBREVIATIONS: AFB₁, aflatoxin B₁; GSH, glutathione; GST, glutathione S-transferase; ARE, antioxidant response element; NQO1, NAD(P)H:quinone oxidoreductase 1; NF-E2, nuclear factor-erythroid 2; bZIP, basic-region leucine zipper; CNC, cap 'n' collar; Nrf, nuclear factor-erythroid 2 p45-related factor; Sul, sulforaphane; MTC, multiple tissue cDNA; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; FISH, fluorescence in situ hybridization; kb, kilobase(s); bp, base pair(s); SSC, standard saline citrate; DAPI, 4,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription-polymerase chain reaction.

inducible by a number of cancer chemopreventive agents that provide protection against AFB₁ hepatocarcinogenesis (Hayes et al., 1998; Kelly et al., 2000). Although there is a strong association between either constitutive or induced expression of GST capable of metabolizing AFB₁-8,9-epoxide and resistance to the carcinogenic effects of the mycotoxin, little is known about the mechanisms that regulate the genes encoding these enzymes.

Genes encoding certain detoxification enzymes are transcriptionally regulated through an enhancer known as the antioxidant response element, or ARE (Hayes and McMahon, 2001). This enhancer was first identified as a regulatory element within the rat (*rGSTA2*) 5'-upstream region that conferred transcriptional responsiveness to β -naphthoflavone (Rushmore et al., 1990). Subsequently, the ARE has been shown to mediate a transcriptional response to a broad spectrum of structurally diverse xenobiotics (Nguyen et al., 2003). Mutational analysis of the 5'-upstream region of *rGSTA2* led to the identification of the ARE sequence required for both basal and/or inducible activity as 5'-TGAC-nnnGC-3' (Rushmore et al., 1991). Other genes known to be regulated through an ARE include those encoding mouse GSTA1, mouse, human and rat NAD(P)H:quinone oxidoreductase (NQO1), human glutamate cysteine ligase catalytic and regulatory subunits, and mouse heme oxygenase-1 (Nguyen et al., 2003; Nioi et al., 2003).

The identity of the transcription factors that mediate regulation of ARE-driven genes has stimulated much interest. The similarity between the ARE and the binding motif for nuclear factor-erythroid 2 (NF-E2) lead to the supposition that this transcription factor may regulate the expression of ARE-containing genes. NF-E2 is a dimeric protein composed of 45-kDa (p45) and 18-kDa (p18) subunits, each belonging to the basic-region leucine zipper (bZIP) transcription factor superfamily (Motohashi et al., 2002). The restricted expression of the p45 subunit to hematopoietic tissues was not consistent with it being involved in regulating the transcrip-

tion of hepatic genes. However, the related cap 'n' collar (CNC) bZIP family members, NF-E2 p45-related factors 1 and 2 (Nrf1 and Nrf2) exhibit a more ubiquitous tissue-specific expression profile (McMahon et al., 2001). Numerous studies since have shown that these transcription factors activate target gene expression through the ARE (for review, see Nguyen et al., 2003). Of particular relevance to the selective toxicity of AFB₁ is the observation that expression of mGSTA3 is reduced in mouse lines with targeted disruption of CNC bZIP genes. Specifically, red blood cells from *NF-E2 p45*-null mice show a marked reduction in mGSTA3 (Chan et al., 2001). Also, liver and small intestine from *nrf2*^{-/-} mice express less mGSTA3 than *nrf2*^{+/+} mice (McMahon et al., 2001; Chanas et al., 2002; Thimmulappa et al., 2002). Although CNC bZIP proteins are clearly involved in regulating the expression of the transferase, it is unclear whether these transcription factors act directly through an ARE present in the *mGSTA3* 5'-upstream sequence. Functional characterization of the *mGSTA3* regulatory region has been undertaken as a first step to establish the molecular genetic basis for the intrinsic resistance of the mouse to AFB₁.

Materials and Methods

Chemicals and Reagents. Unless stated otherwise, all chemicals were obtained from Sigma Chemical (Poole, Dorset, UK). Sulforaphane (Sul) (>98% pure) was purchased from LKT Laboratories (St. Paul, MN). Oligonucleotide primers were supplied by MWG Biotech (Ebersberg, Germany) and are listed in Table 1. Restriction endonucleases were obtained from Roche Diagnostics Ltd. (Lewes, E. Sussex, UK).

Tissue-Specific Expression of mGSTA3. The tissue-specific expression of *mGSTA3* and mouse *GAPDH* (*mGAPDH*) was examined using a multiple tissue cDNA (MTC) panel (BD Biosciences Clontech, Palo Alto, CA). This panel comprised pooled first-strand cDNA preparations isolated from eight mouse tissues. The panel is supplied after normalization to four different housekeeping genes. The relative abundance of mGSTA3 cDNA in each tissue sample was determined by PCR using the MA3MTC-A/MA3MTC-B primer pair (Table 1). The relative expression of mGAPDH in the tissue samples was examined using primers supplied with the panel. The PCR reaction was carried out in a 50- μ l volume, containing reaction buffer (5 μ l), 200 μ M each of dATP, dCTP, dGTP, and dTTP, 0.4 μ M of each primer, 1 ng of cDNA from each tissue, and TITANIUM *Taq*DNA polymerase (1 μ l). The template was denatured initially through incubation at 94°C for 30 s; thereafter, amplification was achieved by denaturation at 94°C for 30 s followed by primer annealing/extension at 68°C for 30 s. Agarose-gel electrophoresis was used to examine PCR products after successive rounds of amplification to ensure linearity of the reaction.

Cell Culture. Unless stated otherwise, all cell culture reagents were obtained from Invitrogen (Paisley, Renfrewshire, UK). Mouse hepatoma Hepa1c1c7 cells were supplied by the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK) and were maintained in minimal essential medium Eagle, α modification (Sigma Chemical) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 50 units/ml penicillin/streptomycin, and 2 mM L-glutamine. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

SDS-PAGE and Western Blot Analysis. Discontinuous SDS-PAGE was performed using 12% (w/v) polyacrylamide resolving gels. For Western blot analyses, proteins resolved by SDS-PAGE were transferred to Immobilon-P as described previously (Ellis et al., 1996). Membranes were incubated with primary antibodies specific for mGSTA3 (McLellan et al., 1991) or rat lactate dehydrogenase (Ellis et al., 1996). Specific binding of immobilized proteins was

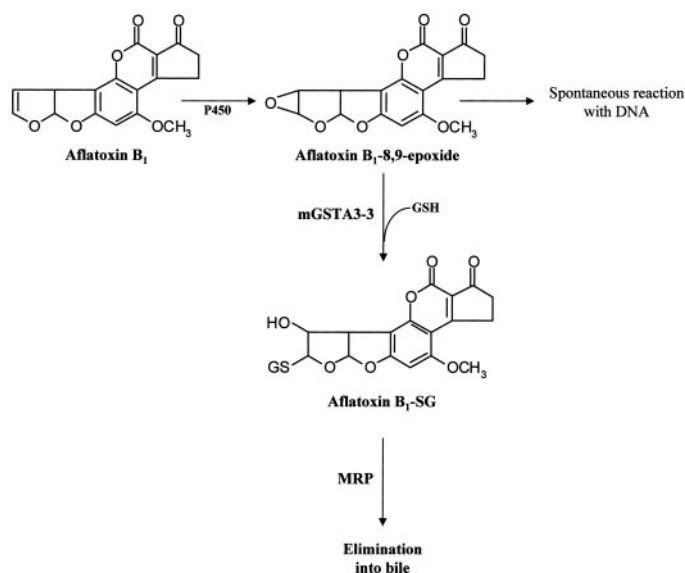


Fig. 1. Metabolism of aflatoxin B₁. The initial 8,9-epoxidation of AFB₁ is catalyzed by cytochrome P450. Mouse GSTA3-3 catalyzes the conjugation of GSH to AFB₁-8,9-epoxide, thus targeting this genotoxic metabolite for elimination from the cell via the multidrug resistance protein (Loe et al., 1997). MRP, multidrug resistance protein.

detected with goat anti-(rabbit IgG) secondary antibodies conjugated with horseradish peroxidase (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). Secondary antibody complexes were visualized using enhanced chemiluminescence and autoradiography.

DNA Sequence Analysis. Sequence analysis was carried out by the DNA Sequencing Laboratory (Department of Molecular and Cellular Pathology, Ninewells Hospital, University of Dundee, Scotland, UK).

5'-Rapid Amplification of cDNA Ends (5'-RACE). The 5' sequence of the *mGSTA3* transcript was determined using the SMART RACE cDNA amplification kit (BD Biosciences Clontech, Hampshire, UK) in accordance with the manufacturer's instructions. Total RNA isolated from Hepa1c17 cells was used as the template for reverse transcription using the gene-specific primer MA3RACE (Table 1). Reaction products were subcloned into the pCR2.1 vector (Invitrogen) and sequenced from T7 and M13 (reverse) priming sites. The sequence of eight individual clones was determined.

Identification of the *mGSTA3* Gene. The *mGSTA3* gene was identified using the BLAT search facility available through the University of California Santa Cruz (Genome) World Wide Web site (<http://www.genome.ucsc.edu>). The *mGSTA3* mRNA sequence derived from the 5'-RACE experiments and previous studies (Hayes et al., 1992) was used to search against the February 2002 mouse genome data assembly.

Chromosomal Localization of the *mGSTA3* Gene. The chromosomal localization of the *mGSTA3* gene was confirmed by fluorescence in situ hybridization (FISH) analysis of mouse metaphase chromosomes. A 9.7-kb fragment of the *mGSTA3* gene was first amplified as a probe from mouse genomic DNA by long-range PCR using primers MA3CL-A and MA3CL-B. The reaction was performed in a 50- μ l volume, containing reaction buffer (5 μ l), 200 ng of each primer, 400 μ M each of dATP, dCTP, dGTP, and dTTP, 5 units of Herculanase DNA polymerase (Stratagene, Amsterdam, The Netherlands), and 1 μ g of mouse genomic DNA as template. Amplification was carried out using a ThermoHybaid PCR Sprint temperature cycling system (ThermoHybaid, Ashford, Middlesex, UK). Samples were heated initially to 92°C for 2 min. Preliminary amplification was achieved over 10 cycles comprising denaturation at 92°C for 10 s, primer annealing at 58°C for 30 s, and extension at 68°C for 11 min. This was followed immediately by 20 cycles involving denaturation at 92°C for 10 s, primer annealing at 58°C for 30 s, and extension at 68°C for 11 min 10 s. The reaction was completed with a single incubation at 68°C for 10 min. The reaction product was subcloned into the pBluescript KS⁻ vector (Stratagene). Successful amplification of the desired target was confirmed using a combination of restriction analysis and DNA sequence analysis. FISH analysis was

performed by ChromBios GmbH (Raubling, Germany). Probe DNA (1.5 μ g) consisting of the 9.7-kb *mGSTA3* genomic fragment in pBluescript KS⁻ was labeled by standard nick translation. Digoxigenin (Roche Diagnostics Ltd.) was used as labeled dUTP at a concentration of 40 μ M. An optimal average probe length of approximately 300 bp was confirmed by agarose-gel electrophoresis.

Chromosomes were prepared according to standard cytogenetic techniques (further details available at www.chrombios.com). Excess cytoplasm was removed from slides by treating with pepsin (0.5 mg/ml in 0.01 M HCl, pH 2.0) at 37°C for 30 min and washing at room temperature with phosphate-buffered saline (2 \times 10 min) followed by phosphate-buffered saline containing 50 mM MgCl₂ (1 \times 10 min). Chromosome denaturation was achieved by submerging slides in 2 \times SSC containing 70% (v/v) formamide for 1 min at 70°C. Thereafter, slides were dehydrated through successive exposure to 70% (v/v), 90% (v/v), or absolute ethanol for 5 min, followed by air drying. Approximately 50 ng of the labeled DNA probe was resuspended in 15 μ l of hybridization solution [2 \times SSC containing 50% (v/v) formamide] and denatured at 75°C for 5 min. The probe was then preannealed at 37°C for 1 h and applied to the denaturing chromosomes. The site of hybridization was covered with a 22 \times 22-mm coverslip and sealed with rubber cement. Slides were incubated at 37°C for 24 h. The coverslip was removed and the slide washed for 5 min at 45°C in 2 \times SSC containing 50% (v/v) formamide and then for a further 5 min at 60°C in 0.1 \times SSC. Signal detection was performed with an anti-digoxigenin antibody labeled with rhodamine (Roche Diagnostics Ltd.) diluted 1:500 in 4 \times SSC containing 5% (w/v) bovine serum albumin. Slides were incubated in the antibody solution for 30 min and washed twice in 4 \times SSC containing 0.1% (v/v) Tween for 10 min. Before microscopy, the slides were mounted in antifade solution containing DAPI (Citifluor Ltd., London, UK). Images of metaphase spreads were captured with a cooled charge-coupled device camera (Photometrics Sensys camera equipped with a Kodak KAF1400 chip) coupled to a Zeiss Axioscope 2 microscope (Welwyn Garden City, Hertfordshire, UK). Camera control and digital image acquisition was achieved using SmartCapture software (Digital Scientific, Cambridge, UK). Chromosomes were identified by computer-enhanced DAPI banding. Merging of chromosome images was performed using SmartCapture software.

Isolation of *mGSTA3* 5'-Upstream Sequence. Approximately 1.5 kb of sequence upstream of exon 1 was amplified by PCR from mouse genomic DNA using primers MA3PROM-A and MA3PROM-B (Table 1). Reactions were carried out in a 50- μ l volume, containing reaction buffer (5 μ l), 1 mM MgSO₄, 200 ng of each primer, 400 μ M each of dATP, dCTP, dGTP, and dTTP, 500 ng of mouse genomic DNA, and 2.5 units of Platinum Pfx DNA polymerase (Invitrogen).

TABLE 1
Oligonucleotide sequences

Oligonucleotide	Sequence (5' \rightarrow 3')
Cloning and PCR-Based Analyses	
MA3MTC-A	CTGGCAAGGTTACGAAGTGATGGAGTCTG
MA3MTC-B	GTTGTGACTCTGCTTCTCAGCGCTTTCAG
MA3RACE	CGGGTCCAGCTCTTCCACATGGTAGAGG
MA3CL-A	CTTCAACACCTGAGGAATTCAAGTTG
MA3CL-B	GCAACCAAGAATTCTAGGAATGTCAG
MA3PROM-A	CACATCTCTGCTTCTATTGAGGTG
MA3PROM-B	GCTAGCTTTTACCATATGCAAGAG
MA3MUT1-A	CCAACCTCTTAGCAACTCAGGCAGGCCCTGGAATTTTCTTCTAATCC
MA3MUT1-B	GGATTAGAAGAAAAATTCAGGGCCTGCCTGAGTTGCTAAAGAGTTGG
MA3MUT2-A	CCAACCTCTTAGCAACTCAGGCAGGCATTCATTTTCTTCTAATCC
MA3MUT2-B	GGATTAGAAGAAAAATGCAATGTCTGCCTGAGTTGCTAAAGAGTTGG
EMSA Analyses	
A3-WT 1 (top)	GGAAGAAACATATTAACCAACTCTTTAGCAACTCAGGCATGACATTGCTTTTCTTCTAAT
A3-WT 2 (bottom)	GGATTAGAAGAAAAATGCAATGTCTGCCTGAGTTGCTAAAGAGTTGGTTAATATGTTTCTT
ARE-MUT 1 (top)	GGAAGAAACATATTAACCAACTCTTTAGCAACTCAGGCAGGCCCTGGAATTTTCTTCTAAT
ARE-MUT 2 (bottom)	GGATTAGAAGAAAAATTCAGGGCCTGCCTGAGTTGCTAAAGAGTTGGTTAATATGTTTCTT
T-MUT 1 (top)	GGAAGAAACATATTAACCAACTCTTTAGCAACTCAGGCAGGCATTCATTTTCTTCTAAT
T-MUT 2 (bottom)	GGATTAGAAGAAAAATGCAATGTCTGCCTGAGTTGCTAAAGAGTTGGTTAATATGTTTCTT

The amplification reaction was carried out in a ThermoHybrid PCR Sprint temperature cycling system. The template DNA was denatured initially through incubation at 94°C for 2 min. Thereafter, amplification was performed over 30 cycles through denaturation at 94°C for 30 s, primer annealing at 55°C for 2 min, followed by extension at 68°C for 2 min. The reaction was completed with a single incubation at 68°C for 5 min. The reaction product was subcloned into the pCR-Blunt vector (Invitrogen), and the fidelity of the PCR reaction was confirmed by DNA sequence analysis. The *mGSTA3* fragment was excised from this holding vector as a *KpnI/XhoI* fragment and subcloned into the corresponding restriction sites in pGL3-Basic (Promega, Southampton, UK) to give the construct A3-WT.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using the QuikChange kit (Stratagene), in accordance with the supplier's instructions. The A3-WT construct was used as a template in all reactions. Mutation of alternate nucleotides throughout the core ARE motif, 5'-TGACATTGC-3', was achieved using primers MA3MUT1-A and MA3MUT1-B to give the construct ARE-MUT. Mutation of the first thymine nucleotide in this core sequence was achieved using primers MA3MUT2-A and MA3MUT2-B to give the construct T-MUT. The entire sequence of the *mGSTA3* promoter region in each construct was confirmed after mutagenesis.

DNA Transfection and Reporter Gene Assays. Cells were transfected at 70% confluence using Lipofectin reagent (Invitrogen), in accordance with the manufacturer's instructions. The pRL-TK *Renilla reniformis* luciferase reporter vector was included in all samples to provide an internal control for transfection efficiency. In some instances, cells were treated 18 h after transfection with either 5 μ M Sul or 0.1% (v/v) DMSO. In all experiments, cells were lysed 48 h after transfection and *R. reniformis* and Firefly luciferase activities determined using the Dual-Luciferase reporter assay system (Promega) and a TD-20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA). Firefly luciferase values derived from the *mGSTA3* reporter constructs were normalized to the corresponding *R. reniformis* luciferase activities.

Electrophoretic Mobility Shift Assays (EMSAs). Binding reactions comprising 100 ng each of recombinant mouse Nrf2 and recombinant mouse MafG were incubated at 20°C for 20 min in a buffer containing 20 mM HEPES, pH 7.9, 1 mM EDTA, 50 mM KCl, 5 mM MgCl₂, 10% (v/v) glycerol, 1 mM dithiothreitol, and 3 μ g of poly(dI:dC). Reactions were supplemented with an internally-labeled double-stranded oligonucleotide probe containing the *mGSTA3* ARE (Table 1) and incubated at 20°C for a further 20 min. For competition experiments, a 25- or 50-fold molar excess of unlabeled double-stranded oligonucleotides containing either the wild-type (A3-WT) or mutant (ARE-MUT and T-MUT) ARE sequences were added to the binding reaction before the addition of the labeled probe. Complexes were resolved at 4°C through a 4.2% (w/v) polyacrylamide gel in 0.5× Tris borate-EDTA and visualized using autoradiography.

TaqMan Semiquantitative RT-PCR. Total RNA was prepared from tissues isolated from either wild-type or *nrf2*^{-/-} mice using RNeasy kits (QIAGEN, Dorking, Surrey, UK). Relative quantification of *GSTA3* mRNA transcripts was performed using the PCR-based 5'-nuclease assay with a gene-specific TaqMan oligonucleotide probe, according to methods described previously (Chanas et al., 2002). The TaqMan probe was labeled at the 5' end with a fluorescent reporter dye (6-carboxyfluorescein), and at the 3' end with a quencher dye (6-carboxytetramethylrhodamine). Thermal cycling and real-time fluorescence detection were performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). The level of 18S ribosomal RNA was determined as an internal control using reagents purchased from PerkinElmer.

Results

Tissue-Specific Expression of mGSTA3. Insight into gene regulation can be gained by determining whether a

given transcription factor is coexpressed with the target gene. Thus, expression of *mGSTA3* mRNA was examined in mouse tissues and compared with data documented previously for Nrf2 (McMahon et al., 2001). For these investigations, a panel of normalized cDNA samples isolated from a range of mouse organs was analyzed by semiquantitative PCR (Fig. 2). Reactions specific for either *mGSTA3* (i and ii) or *mGAPDH* (iii) yielded a single amplicon of the predicted size. Consistent with the role of *mGSTA3*-3 in hepatic detoxification processes, the mRNA encoding this transferase is expressed most abundantly in liver. Relatively high levels of mRNA are also present in lung. After further amplification, robust expression could be detected in kidney and testis. By contrast, *mGSTA3* mRNA is represented at relatively low or undetectable levels in heart, spleen, brain, and skeletal muscle. Northern blot analysis of mouse tissue RNA has revealed that the Nrf2 transcript is also expressed abundantly in kidney, liver, and testis, with moderate levels present in heart and spleen. Relatively low levels of Nrf2 mRNA were observed in brain, lung, and skeletal muscle. This compari-

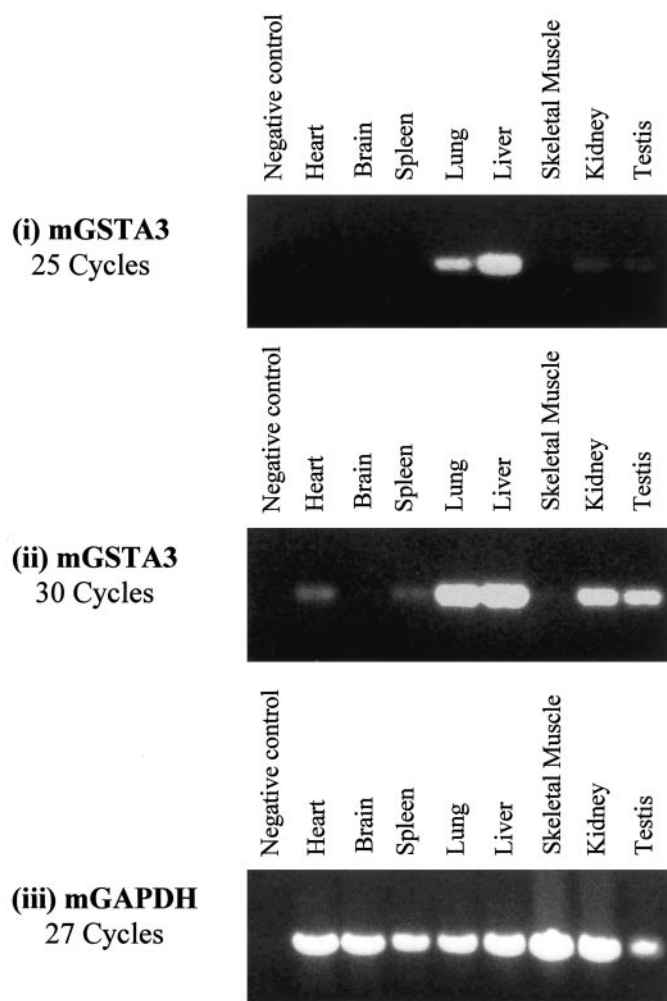


Fig. 2. Tissue-specific expression of *mGSTA3* mRNA. A normalized panel of cDNAs isolated from various mouse tissues was analyzed for *mGSTA3* (i and ii) and *mGAPDH* (iii) expression by PCR. Amplification of *mGSTA3* was performed over 25 and 30 cycles. The expression of *mGAPDH* mRNA was examined as a control. Amplification of *GAPDH* was performed over 27 cycles. Reaction products were analyzed by agarose-gel electrophoresis.

son demonstrates that the Nrf2 transcription factor and mGSTA3 are coexpressed in a number of tissues.

Expression of mGSTA3 Is Induced by Sulforaphane in Mouse Hepatoma Cells. A wide range of structurally diverse compounds can activate gene expression through the ARE (Nguyen et al., 2003). One such compound is Sul, a naturally occurring isothiocyanate generated as a hydrolysis product of glucosinolates from cruciferous vegetables. The effect of Sul on mGSTA3 expression was thus examined in the mouse Hepa1c1c7 hepatoma cell line (Fig. 3). As a positive control, the ability of the isothiocyanate to influence the expression of a validated ARE-regulated gene was confirmed (i). Consistent with previous observations, expression of mouse NQO1 (mNQO1) was potently induced by Sul in the Hepa1c1c7 cell line (Nioi et al., 2003). Significantly, mGSTA3 expression was also found to be inducible by Sul, albeit to a lesser extent (ii). These data demonstrate that mGSTA3 expression is inducible by a compound known to stimulate ARE-driven gene transcription.

Identification of the mGSTA3 Gene. To establish the mechanism of mGSTA3 induction by Sul, a direct sequence analysis of the gene promoter was required. It was first necessary to determine the organization of the *mGSTA3* gene. The sequence of the mGSTA3 transcript was derived using a combination of 5'-RACE and previously published data (Hayes et al., 1992), and it was used in a bioinformatics analysis of the mouse genome using the University of Cali-

fornia Santa Cruz (Genome) worldwide Web site. This analysis revealed that the mGSTA3 transcript is encoded by seven exons, spanning approximately 24.6 kb of genomic DNA (Fig. 4a). The translation initiation and termination codons are located within exons 2 and 7, respectively. As such, exon 1 encodes only 5'-untranslated sequence. These gross aspects of genomic organization are conserved in genes encoding other class α GST (Daniel et al., 1987; Rohrdanz et al., 1992; Rozen et al., 1992; Fotouhi-Ardakani and Batist, 1999). A more detailed comparison was made between the *mGSTA3* gene and the previously described rat *GSTA3* (*rGSTA3*) gene (Fig. 4b). In addition to this conservation in gross aspects of genomic organization between *mGSTA3* and *rGSTA3*, the positions of the predicted splice junctions are also identical between these genes. Analysis of the sequence flanking these splice junctions reveals that each conforms to the gt-ag rule.

Chromosomal Localization of mGSTA3. The chromosomal localization of murine class α GST has not been examined in great detail. Assembled mouse genome data predicts that the *mGSTA3* gene is located on chromosome 1, region A3-4. Analysis of metaphase mouse chromosomes by FISH confirms this localization (Fig. 5).

Identification of the mGSTA3 5'-Upstream Region. Having determined the organization of the *mGSTA3* gene, the region upstream of exon 1 was next examined (Fig. 6). An A/T-rich TATA box-like sequence was observed approximately 25 nucleotides upstream of exon 1. Other putative promoter elements such as "GC" and "CAAT" boxes were also identified. A detailed search for potential transcription factor binding sites within this 1.5 kb of sequence was performed using the MatInspector version 2.2 software. This sequence-based analysis identified numerous putative regulatory elements. These included a nuclear factor- κ B binding site, a glucocorticoid response element, and two barbiturate response elements. The presence of such regulatory elements in other GST genes has been documented (reviewed in Hayes and Pulford, 1995). Of particular relevance in the context of the current study is the presence of a possible ARE located approximately 150 bp upstream of exon 1. The sequence of this putative ARE (5'-TGACATTGC-3') is identical to the ARE consensus (5'-TGACnnnGC-3') previously documented by Rushmore et al. (1991). The identification of this ARE-like sequence within the *mGSTA3* 5'-flanking region may provide a molecular basis for the Sul-inducible expression of the transferase observed in Hepa1c1c7 cells.

Identification of a Functional ARE in the mGSTA3 5'-Upstream Region. Functional assays were designed to determine whether the *mGSTA3* gene is indeed regulated through an ARE. Approximately 1.5 kb of sequence upstream of exon 1 was amplified by PCR from a mouse genomic DNA template and subcloned into a luciferase reporter vector lacking any native promoter elements (A3-WT). Similar constructs were designed to assess the functionality of the putative ARE (Fig. 7). In the first instance, alternate nucleotides throughout the ARE core were mutated (ARE-MUT). A second construct was created in which only the first nucleotide of the core sequence was substituted (T-MUT). Previous studies have demonstrated that a thymine nucleotide in this position is essential for transcriptional activation through this regulatory element (Rushmore et al., 1991; Nguyen et al., 2000; Nioi et al., 2003). The activity of these

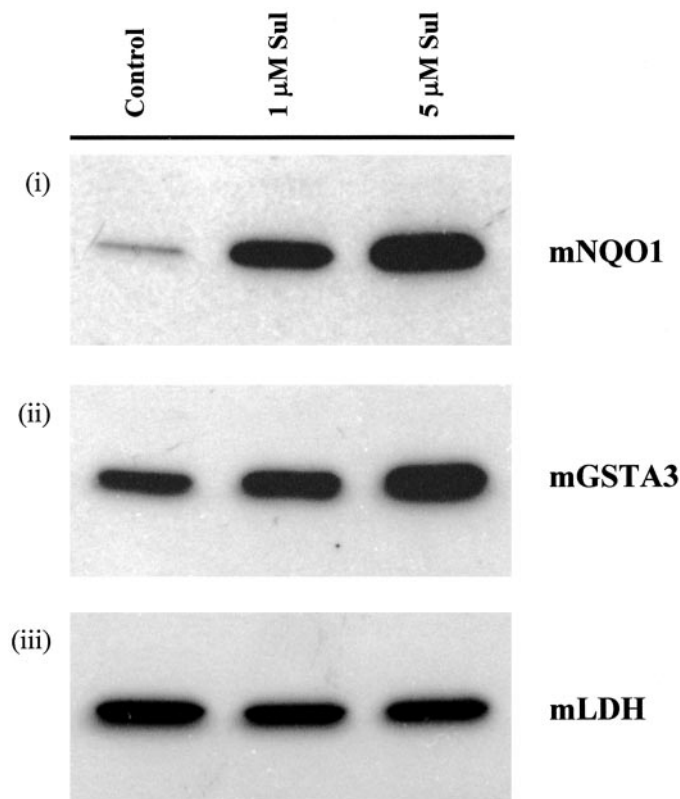


Fig. 3. Effect of sulforaphane on mNQO1 and mGSTA3 expression in Hepa1c1c7 cells. Populations of Hepa1c1c7 cells were treated for 18 h with either Sul (1 μ M or 5 μ M) or 0.1% (v/v) DMSO (Control). Total cellular protein was isolated and 10 μ g of each sample resolved by SDS-PAGE. The expression of mNQO1 (i), mGSTA3 (ii) and mouse lactate dehydrogenase (mLDH) (iii) was examined by Western blot using antibodies described previously (McLellan et al., 1991; Ellis et al., 1996; Kelly et al., 2000).

constructs was examined in Hepa1c1c7 cells, in the presence or absence of Sul (Fig. 8). Consistent with the constitutive expression of mGSTA3 in this cell line (Fig. 3), significant activity was detected from the A3-WT vector under basal conditions. Furthermore, mutations within the ARE-like sequence were found to be associated with a dramatic reduction in basal reporter gene activity. Significantly, Sul was found to potentiate transcription of the reporter driven by the wild-type 5'-upstream region of *mGSTA3* around 2-fold. The magnitude of this increase in activity is broadly consistent with the modest induction in mGSTA3 protein expression ob-

served in Hepa1c1c7 cells after treatment with Sul (Fig. 3). By contrast, the isothiocyanate failed to augment the luciferase activity of either the ARE-MUT or T-MUT constructs. The predicted ARE, therefore, mediates the activation of the *mGSTA3* gene by Sul.

Collectively, these data confirm that the *mGSTA3* 5'-upstream region contains a functional ARE that mediates both basal and Sul-inducible transcription in the mouse Hepa1c1c7 cell line.

The *mGSTA3* Gene Is Activated by Nrf2. Having established that the *mGSTA3* gene is regulated through an

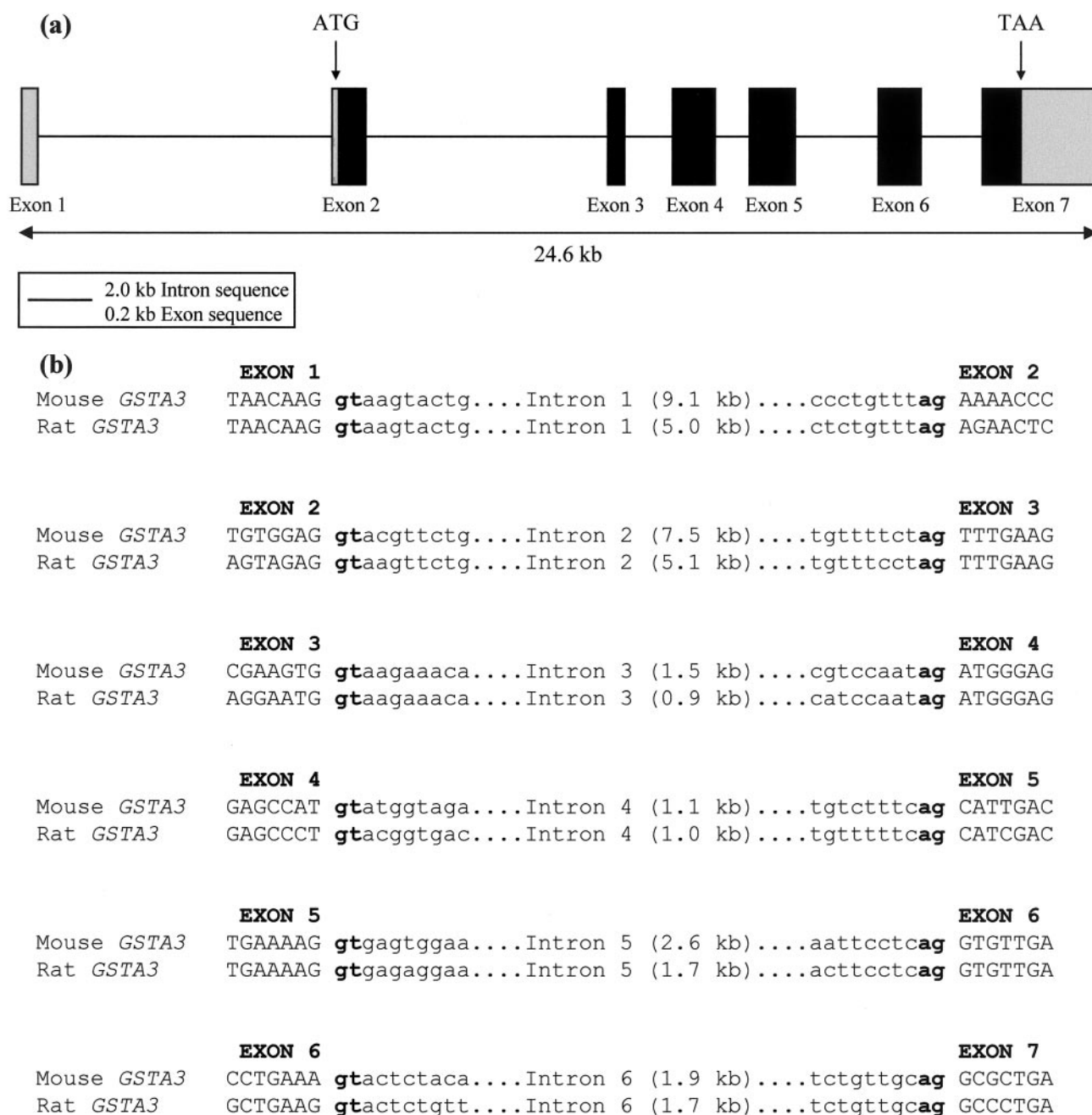


Fig. 4. Organization of the *mGSTA3* gene. The *mGSTA3* gene was identified using the BLAT search facility available through the University of California Santa Cruz (Genome) Web site. The *mGSTA3* mRNA sequence was used as the search template. The gene structure is drawn to scale (a). Exons are represented by boxes, whereas introns are represented by lines. Protein-coding regions of exons are shown in black, whereas noncoding sequence is represented in gray. The sequences flanking each of the exon-intron boundaries in *mGSTA3* and *rGSTA3* have been aligned (b). Sequence located within exons is shown in uppercase, whereas sequence located within introns is shown in lowercase. The gt-ag splice donor-acceptor sites are shown in bold.

ARE present in the 5'-upstream sequence, studies were conducted to determine whether the Nrf2 transcription factor is able to modulate gene expression through this regulatory region. The *mGSTA3* constructs were transfected into Hepal1c7 cells in the presence or absence of an expression vector containing the mouse Nrf2 open reading frame (Fig. 9). Consistent with the data presented in Fig. 8, significant activity from the A3-WT construct was detectable under

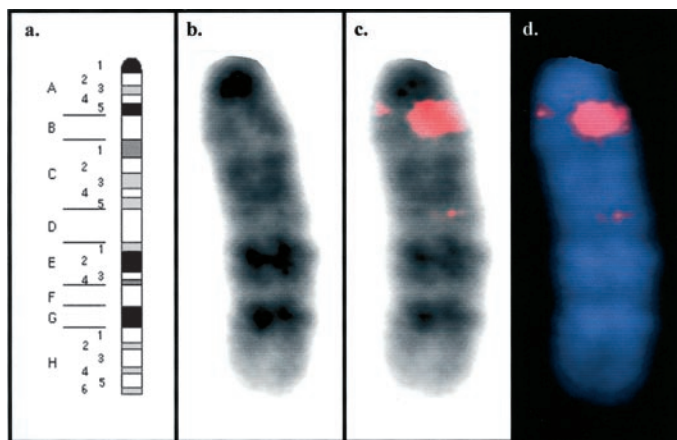


Fig. 5. Chromosomal localization of *mGSTA3*. The chromosomal localization of *mGSTA3* was determined by FISH using a labeled genomic probe. a, ideogram of mouse chromosome 1. b, DAPI-banded chromosome 1. c, DAPI-banded chromosome 1 with the hybridization pattern obtained with the *mGSTA3* probe. d, the same hybridization signal as presented in c showing the original DAPI pattern. More than 40 metaphases were analyzed of which approximately 61% displayed at least one hybridization signal on the four different chromatids, at this region of the chromosome.

```

-1507  CACATCTCTGCTTCTATTGAGGTGCTGCCACCATATTGAAAGTGTCTGTGGGTTATTAAAGATGAAGTCAATCAAGT
                                     BRE
-1427  TCCACAAATTAAGAACATCTGTAAGACCTTTTCCAAATTCATTTCTATTACAAGAGCCTAGGATTTGGACATGGGTC
-1347  TAACTTTGAGGGAAGGCACATGATAAACTTAACATCTTCTACATAATAGTTAACAAAAAAGTGAAGTTATCAATCTGA
-1267  GTGATAGAATGTATTTGGGGTTTTTAAAAATACTATTCCTCTTTTGTGTTTTTGGTAGAAATTATTGCAACCATTCTAAA
-1187  TTAATTTGCTGCTACTATCTATGGTCATATTTTATGCCATTTATAGGACTTGACACATACCACAGCCCATGAGAGCA
-1107  CTTGAAACATATAATGTAAAAATAATTCAGGACACAATAATTGAGAAATGAAATAGTAGTGATAGTCAGGAGTCTAAC
-1027  TGAACACAGGGCAGATGCAAGGCCTTTTCCGGAGAAGGTAACATCTAGGACAGCCATGTCAATGTGAAGTTCAAGATA
-947   ACATCTCTTTTAGCATAAGCAGCATTCCTCAATACTTGTGACATACTAATACTACTACGTGAAACTCAGCTCGTTGTG
-867   GGGTCTGCTTAATCCAACATAATTCAAACAGCAGGTCAATGCAGTTGACAAAAATTTGATTGTAATCAAGCCACTACTG
-787   CTCAATCAGGGCCACAGATCAGACTTGGGAGCTGAAGTAAAGCCCTGAAGGGATGCTATCCATCATATTTATAGTATGAA
-707   ACCGTAAAGAGATGGGATGCTAGGGGGCTATAGCATTGTCTAATCATACTTTGATATAAGGGACTGAGCAAGGAAGTTT
-627   CCATTAATCAAGAGTGGAGTTGATTCCTGAGCCTGGGAAGATGAAGTGTAGGTTGACCTTGCCAGCAAGTTGGAGAAGTTG
                                     GRE
-547   TCCTTGAGATGTCTGCACTCAGACAGCTGTTTCTTACAACAGAAGCTGGTCACTACTGGGAAGTTCCAGCTCCCTAG
                                     NF-κB
-467   GGCTGAGACCTTGAATTTAATTTTTTCTTAGGATTAAATGGAGTCTGAAACCAACTGGTAGTACCTAGAATGAGGTT
-387   CTATTGCTTGTCCCAACAGATTATCTGTTCTGTACCTAACCTGGTGGCCAGTCCACAGAAGTACTCCCTGGTGTCAC
-307   CAGAGTTCTCTTTATAACATCTGCCAGCTGAGATTTCCCACTTGCTTTAGCCCATCCTCATTGACAGCCGAAGAGTTAAC
                                     BRE
-227   TCTGCTTGAACAGGTTCTTCCAGCTTGCCATTGGCATTGAGGAAGAACATATTAAACCAACTCTTTAGCAACTCAGGCA
-147   TGACATTGCAATTTTCTTCTAATCCTTTATCTAAATGTCTGGCCTCTGAACACTGTGGGAGTAGCTTTTCCCTTATTGGA
                                     ARE
-67    TTGTCTCCCTTAAGTGGGAAGGGCGTATTCAAATTTAATAAGTAGCTTACAAACAGCAGAAACTCT
                                     GC          TATA

```

basal conditions. This constitutive transcription was again abrogated upon mutation of the ARE. Significantly, overexpression of Nrf2 resulted in an ~10-fold increase in reporter activity from the A3-WT construct. The ability of Nrf2 to transactivate the *mGSTA3* construct was abolished when mutations were introduced within the core ARE sequence. These data demonstrate that the ARE present in the *mGSTA3* 5'-upstream region can be transactivated by Nrf2.

Nrf2 Binds the *mGSTA3* ARE. EMSA analyses were performed to determine whether Nrf2 binds the *mGSTA3* ARE (Fig. 10). Because the interaction of Nrf2 with the ARE is dependent upon the formation of a heterodimeric complex with small Maf bZIP proteins (for review, see Motohashi et al., 2002), binding reactions were performed in the presence of both recombinant Nrf2 and recombinant MafG. These analyses demonstrated that a labeled oligonucleotide probe containing the wild-type *mGSTA3* ARE was bound efficiently in the presence of Nrf2/MafG (lanes 1 and 2). Competition EMSA experiments were also performed to determine whether the mutations shown to abolish ARE activity in the ARE-MUT and T-MUT constructs also influenced Nrf2/MafG binding. Although association with Nrf2/MafG could be effectively competed with a 25- or 50-fold molar excess of unlabeled A3-WT *mGSTA3* ARE (lanes 3 and 4), neither the ARE-MUT (lanes 5 and 6) nor T-MUT (lanes 7 and 8) mutant forms of the ARE similarly competed for binding. Thus, Nrf2/MafG does not seem to exhibit significant binding activity for these mutant forms of the ARE.

Expression of *mGSTA3* Is Regulated by Nrf2 in Vivo. The data described above demonstrate that Nrf2 and *mGSTA3* are coexpressed in a number of tissues. Further-

Fig. 6. Sequence of the *mGSTA3* 5'-upstream region. The 5'-upstream region of *mGSTA3* was identified using assembled mouse genome data available through the University of California Santa Cruz (Genome) worldwide Web site. The sequence of the 5'-flanking region was confirmed by direct DNA sequence analysis after amplification by PCR from mouse genomic DNA. Putative regulatory elements were identified using MatInspector version 2.2 software. The +1 site was assigned based upon data derived through 5'-RACE showing that the 5'-ends of *mGSTA3* transcripts localized around this region and the presence of the putative TATA box approximately 25 bp upstream. A number of potential promoter and regulatory elements are underlined. NF-κB, nuclear factor-κB binding site; GRE, glucocorticoid response element; BRE, barbiturate response element.

more, it is clear that Nrf2 can bind and transactivate the *mGSTA3* gene through the ARE. It was next necessary to determine whether Nrf2 regulates *mGSTA3* gene expression in vivo. The expression of *mGSTA3* mRNA was examined in a range of tissues, isolated from groups of either wild-type or Nrf2-null mice (Fig. 11). A sensitive TaqMan quantitative RT-PCR assay was used for this study. Analysis of relative mRNA levels revealed that, in all of the tissues examined, loss of the Nrf2 transcription factor is associated with reduced expression of *mGSTA3* mRNA. This effect was most pronounced in small intestine, large intestine and stomach, in which expression levels in *nrf2*^{-/-} mice were less than 6% of the levels observed in wild-type animals. Expression of the transferase in Nrf2-null liver, lung, and kidney was reduced to between 15 and 45% of the level in wild-type tissues.

Discussion

Constitutive expression of *mGSTA3* confers intrinsic resistance to the mouse against AFB₁ hepatocarcinogenesis (Fig. 1). Although data derived from null mice have implicated NF-E2 p45 and Nrf2 in the transcriptional regulation of *mGSTA3*, it was previously unclear whether the effects of

these CNC bZIP factors were mediated directly through an ARE. The present study demonstrates that the *mGSTA3* 5'-flanking sequence contains a functional ARE. The in vivo role of Nrf2 in regulating *mGSTA3* expression in lung and throughout the gastrointestinal tract (targets of AFB₁ toxicity) has been revealed through analysis of tissue samples derived from either wild-type or *nrf2*^{-/-} mice.

Most class α GST genes in human, rat, and mouse have been shown to comprise seven exons, with the first being a noncoding exon (Daniel et al., 1987; Rohrdanz et al., 1992; Rozen et al., 1992; Fotouhi-Ardakani and Batist, 1999). Together, these observations suggest that the ancestral gene structure of class α GST has been conserved. The present study demonstrates that the *mGSTA3* gene also comprises seven exons. However, at 24.6 kb, *mGSTA3* is relatively large, in that other characterized class α GST genes span between 11 and 15 kb. Interestingly, the *rGSTA5* gene is predicted to comprise only six exons (Pulford and Hayes, 1996). It apparently lacks the first exon equivalent to that encoding the 5'-untranslated sequence in other class α GST genes. The organization of the protein-coding region of the *rGSTA5* gene is, nevertheless, comparable with that of other class α GST genes. It is thus possible that *rGSTA5* does indeed comprise seven exons, but the gene may be under the control of two promoters; one upstream of the as yet unidentified exon 1, whereas the previously identified promoter actually resides within the first intron.

The present study demonstrates that in the Hepa1c1c7 mouse hepatoma cell line, the *mGSTA3* ARE mediates both constitutive and Sul-inducible expression of the transferase. The *mGSTA3* 5'-upstream sequence was found to confer marked luciferase reporter activity under basal conditions in this cell line (Figs. 8 and 9). This observation is consistent with the constitutive expression of the transferase observed in Hepa1c1c7 cells (Fig. 3). This relatively high basal level of *mGSTA3* seems to restrict the level of induction that can be achieved after treatment with Sul. Significantly, however, the induction in *mGSTA3* expression observed in this study is associated with a comparable increase in reporter activity.

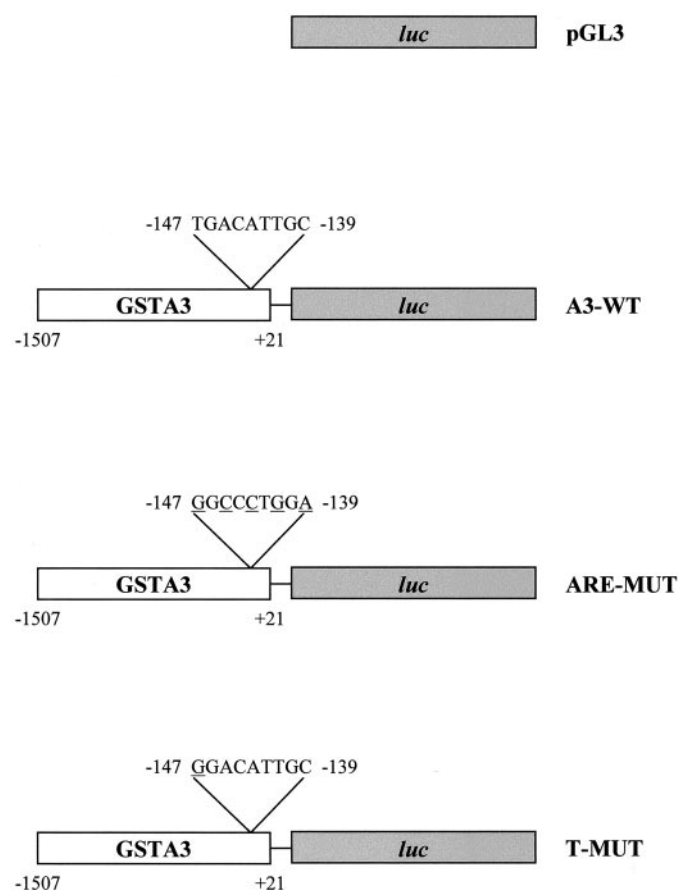


Fig. 7. Design of *mGSTA3* reporter constructs. The functionality of the *mGSTA3* ARE was assessed by generating reporter constructs using the pGL3-Basic luciferase vector (pGL3). Approximately 1.5 kb of sequence from the *mGSTA3* 5'-flanking region was ligated into pGL3-Basic to give the A3-WT construct. A mutant vector (ARE-MUT) was generated in which alternate nucleotides within the ARE core were substituted. A second mutant construct was made in which only the first nucleotide of the core sequence was mutated (T-MUT). The mutations introduced within the ARE core in each construct are underlined.

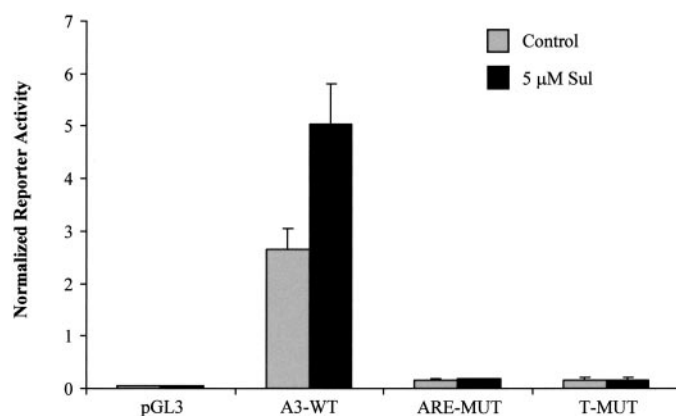


Fig. 8. Effect of sulforaphane on *mGSTA3* reporter activity. Hepa1c1c7 cells were transfected with 600 ng of pGL3, A3-WT, ARE-MUT, or T-MUT *mGSTA3* reporter constructs and 400 ng of pRL-TK *R. reniformis* reporter vector. Approximately 24 h after transfection, cells were treated with either 5 μ M Sul or 0.1% (v/v) DMSO (control). Cells were lysed 18 h later and both Firefly (derived from the pGL3-Basic vector) and *R. reniformis* (derived from the pRL-TK vector) luciferase activities determined. All data were normalized to *R. reniformis* internal control luminescence. Results from three independent experiments are expressed as mean normalized reporter activity \pm S.D.

In addition to the 5'-TGACATTGC-3' motif, nucleotides flanking this core enhancer sequence in *mGSTA3* exhibit similarities to AREs found in other genes (Fig. 12). Particularly obvious is the presence of the A/T-rich sequence immediately 3' of the core "GC" dinucleotide. Despite conservation of the A/T-rich region between AREs in different genes, comprehensive mutational analysis of the *mNQO1* ARE suggests that only the first 5' "A" is essential for function (Nioi et al., 2003). The region 5' of the core in *mGSTA3* contains, in reverse orientation, the sequence 5'-TGAGT-3'. A related sequence, 5'-TGACT-3', is found in a similar position and orientation upstream of AREs in human, rat, and mouse *NQO1* and rat *GSTP1* (*rGSTP1*) and has been shown to be important for both basal and/or inducible expression (Okuda et al., 1990; Favreau and Pickett, 1995; Xie et al., 1995; Nioi et al., 2003). Although there is a single additional nucleotide spacing between this pentanucleotide motif and the ARE core in *mGSTA3* compared with *NQO1* and *rGSTP1*, it is predicted that this flanking sequence in *mGSTA3* is of functional significance. It is unclear which transcription factors bind this upstream 5'-TGAC/GT-3' sequence, but its similarity to motifs recognized by activator protein-1 and activating transcription factor/cAMP response element-binding protein family members suggests that certain of these proteins may be recruited to the 5'-flanking region of *mGSTA3*, *rGSTP1*, and *NQO1*. In this context, it is noteworthy that Nrf2 can interact with c-Jun and ATF4 (Venugopal and Jaiswal, 1998; He et al., 2001). If the sequence upstream of the core ARE is involved in transcription factor binding, the components of the protein complex recruited to the response element are likely to differ between genes that exhibit heterogeneity in this flanking sequence. In this regard, it is important to recognize that the 5'-TGAC/GT-3' motif in *mGSTA3*, *rGSTP1*, and *NQO1* is less well conserved in mouse *GSTA1* (*mGSTA1*) and *rGSTA2* (Fig. 12).

In this study, multiple approaches have been used to demonstrate the importance of Nrf2 in regulating *mGSTA3* expression through the ARE. First, overexpression of Nrf2 in Hepal1c7 cells was found to activate transcription from wild-type, but not mutant, *mGSTA3* reporter constructs. Sec-

ond, EMSA analyses demonstrated association of the *mGSTA3* ARE with Nrf2/MafG. Furthermore, RT-PCR analyses were adopted to show that *mGSTA3* mRNA levels were diminished in tissues isolated from *nrf2*^{-/-} mice, compared with wild-type animals. It is now clear that Nrf1 is also able to stimulate ARE-driven gene expression (Venugopal and Jaiswal, 1996; Hayes and McMahon, 2001; Nguyen et al., 2003). However, the role of this transcription factor in regulating the expression of ARE-dependent genes in vivo has not been established, due to the fact that disruption of *nrf1* in mice confers an embryonically lethal phenotype (Farmer et al., 1997; Chan et al., 1998). Nevertheless, the expression of transcripts encoding GSH-biosynthetic enzymes is reduced in *nrf1*^{-/-} fibroblasts (Kwong et al., 1999). Because certain of these genes are probably regulated through an ARE, these observations are consistent with a role for Nrf1 in regulating ARE-dependent gene expression. Furthermore, loss of NF-E2 p45 is associated with abrogated expression of *mGSTA3* in murine red blood cells (Chan et al., 2001). Figure 11 shows that although loss of Nrf2 is consistently associated with a reduction in *mGSTA3* expression levels, the magnitude of this reduction varies between mouse tissues. These observations may reveal the potential for redundancy between CNC bZIP proteins. Thus, although disruption of the *nrf2* gene may have a dramatic impact on the expression of *mGSTA3* in tissues that express little Nrf1, this effect may be diminished in tissues expressing this transcription factor at higher levels.

Although both mouse and rat possess GST capable of metabolizing AFB₁-8,9-epoxide (*mGSTA3-3* and *rGSTA5-5*, respectively), the contrasting sensitivity of these species to AFB₁ hepatocarcinogenesis has been attributed to differences in the constitutive expression levels of these transferases. Whereas robust hepatic expression of *mGSTA3* affords the mouse inherent resistance to the mycotoxin, marked induction of the *rGSTA5* subunit is necessary to confer protection to the rat. These observations suggest that although *mGSTA3* and *rGSTA5* are closely related in terms of primary structure and catalytic properties, the genes encoding these transferases are regulated differently in mouse

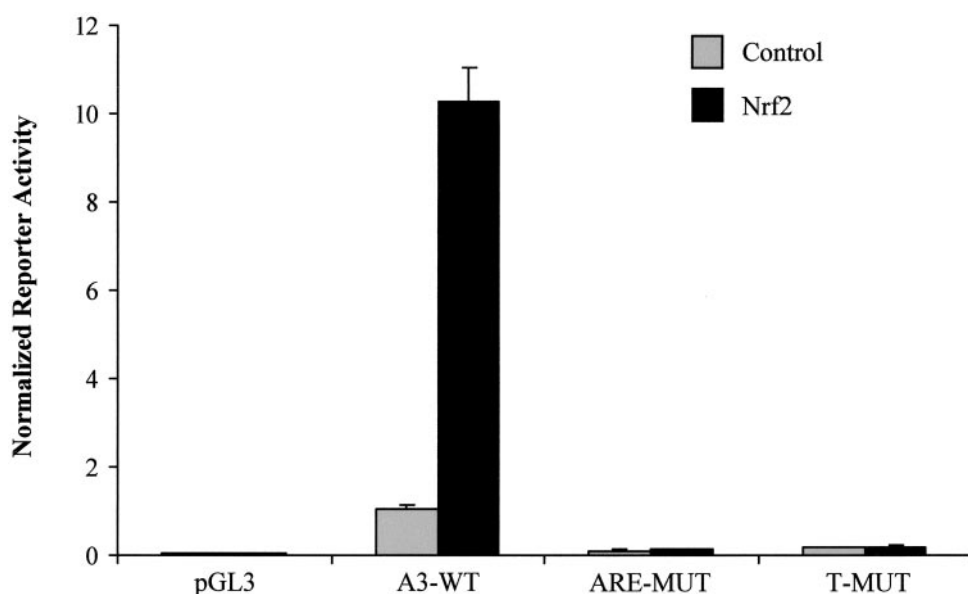


Fig. 9. Effect of Nrf2 overexpression on *mGSTA3* reporter activity. An expression vector encoding V5 epitope-tagged mNrf2 has been described previously (McMahon et al., 2003). Hepal1c7 cells were transfected with either 600 ng mNrf2 expression construct (Nrf2) or empty pCDNA3.1 vector (control), 200 ng of either pGL3, A3-WT, ARE-MUT, or T-MUT, and 200 ng of pRL-TK. Cells were lysed 48 h after transfection and both Firefly (derived from the pGL3-Basic vector) and *R. reniformis* (derived from the pRL-TK vector) luciferase activities determined. All data were normalized to *R. reniformis* internal control luminescence. Results from three independent experiments are expressed as mean normalized reporter activity \pm S.D.

and rat. This may reflect the presence of distinct regulatory elements in the respective 5'-flanking sequences, or a differing ability to drive basal gene expression through similar regulatory elements. The latter could result from quantitative variations in the availability of transcription factors and/or coactivator proteins, or divergent affinities of conserved regulatory elements for their associated transcription factors. As such, further analysis of the *rGSTA5* gene promoter(s) is

required to determine the evolutionary relationship between these transferases and elucidate the molecular basis for their divergent expression levels under basal conditions.

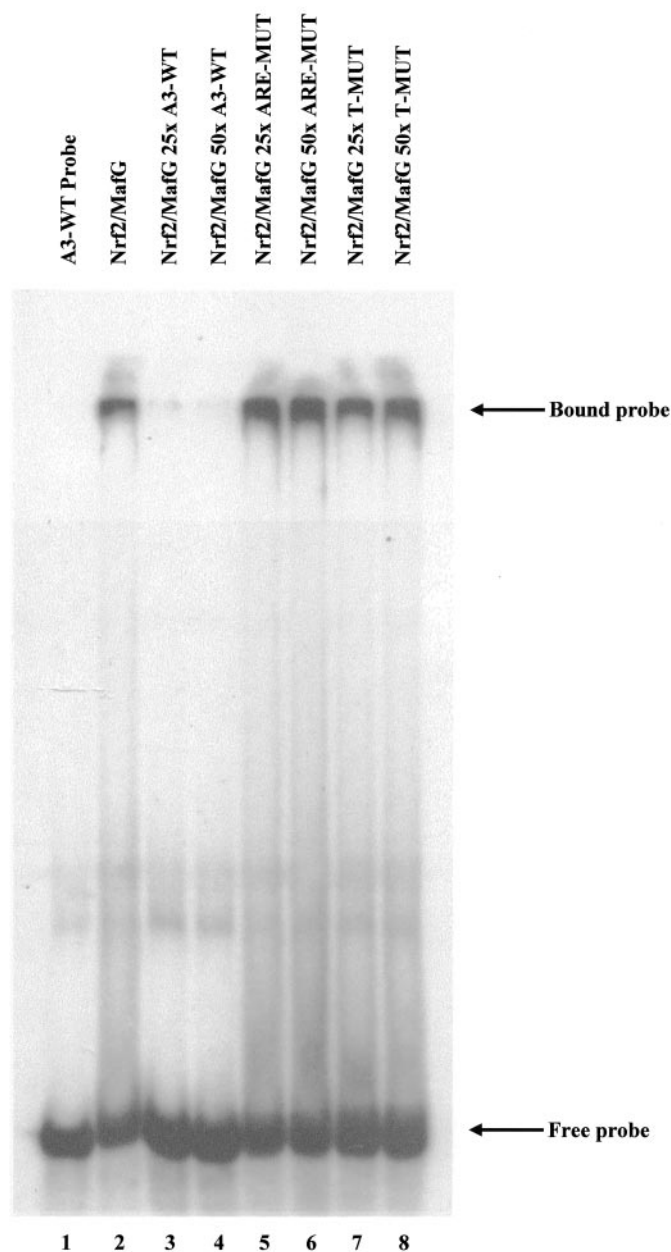


Fig. 10. Association of Nrf2/MafG with the *mGSTA3* ARE. EMSA analyses were performed using recombinant Nrf2 and recombinant MafG in the presence of a radiolabeled double-stranded oligonucleotide probe containing the A3-WT *mGSTA3* ARE (the sequences of the oligonucleotides used in these analyses are presented in Table 1). For competition experiments, reactions were supplemented with a 25- or 50-fold molar excess of competitor DNA. This comprised annealed oligonucleotides containing either the A3-WT ARE, or the mutant sequences shown to abolish enhancer function in the ARE-MUT and T-MUT constructs. Complexes were resolved through a 4.2% (w/v) polyacrylamide gel, run under non-denaturing conditions, and visualized by autoradiography. The positions of bound probe and free probe are indicated by horizontal arrows.

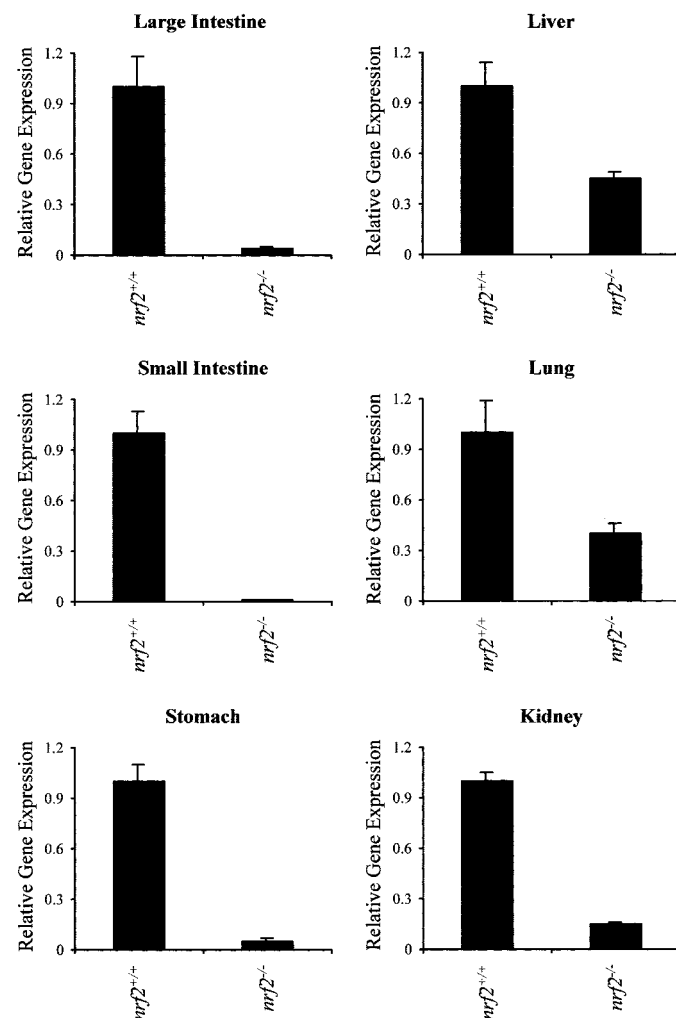


Fig. 11. Nrf2 regulates *mGSTA3* expression in vivo. Total RNA was prepared from various tissues isolated from groups ($n = 3$) of male *Nrf2*^{+/+} or *Nrf2*^{-/-} mice and pooled. Expression of *mGSTA3* mRNA was determined in each tissue sample by TaqMan RT-PCR using 18S RNA as an internal control. Assays were performed in triplicate. For each tissue, the expression of *mGSTA3* mRNA in *Nrf2*-null samples is expressed relative to the corresponding wild-type values (set at 1). Values are expressed as means \pm S.D.

		'Core' ARE	
<i>mGSTA3</i>	ACTCAGGCA	TGACATTGC	ATTTT
<i>hNQO1</i>	AGTCAC-AG	TGACTCAGC	AGAAT
<i>rNQO1</i>	AGTCAC-AG	TGACTTGGC	AAAAT
<i>mNQO1</i>	AGTCAC-AG	TGAGTCGGC	AAAAT
<i>rGSTP1</i>	AGTCAC-TA	TGATTTCAGC	AACAA
<i>mGSTA1</i>	GCTAAT-GG	TGACAAAGC	AACTT
<i>rGSTA2</i>	GCTAAT-GG	TGACAAAGC	AAACT

Fig. 12. Comparison of ARE enhancers. The sequences of various ARE enhancers have been aligned. Data for *hNQO1*, *rNQO1*, *mNQO1*, *rGSTP1*, *mGSTA1*, and *rGSTA2* were taken from Xie et al. (1995), Favreau and Pickett (1995), Nioi et al. (2003), Okuda et al. (1990), Daniel et al. (1987), and Rushmore et al. (1991).

Acknowledgments

We thank Dr. Kerstin Gross-Steinmeyer for helpful discussions about genomic sequence data. We also thank Dr. Clifford R. Elcombe for help in obtaining the TaqMan equipment. It is a pleasure also to acknowledge Michael Mitchell for expert assistance with the bioinformatics analysis.

References

- Aguilar F, Hussain SP, and Cerutti P (1993) Aflatoxin B₁ induces the transversion of G→T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proc Natl Acad Sci USA* **90**:8586–8590.
- Buetler TM, Slone D, and Eaton DL (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.
- Chan JY, Kwong M, Lo M, Emerson R, and Kuypers FA (2001) Reduced oxidative-stress response in red blood cells from p45 NFE2-deficient mice. *Blood* **97**:2151–2158.
- Chan JY, Kwong M, Lu R, Chang J, Wang B, Yen TS, and Kan YW (1998) Targeted disruption of the ubiquitous CNC-bZIP transcription factor, Nrf-1, results in anemia and embryonic lethality in mice. *EMBO (Eur Mol Biol Organ) J* **17**:1779–1787.
- Chanas SA, Jiang Q, McMahon M, McWalter GK, McLellan LI, Elcombe CR, Henderson CJ, Wolf CR, Moffat GJ, Itoh K, et al. (2002) 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. *Biochem J* **365**:405–416.
- Daniel V, Sharon R, Tichauer Y, and Sarid S (1987) Mouse glutathione S-transferase Ya subunit: gene structure and sequence. *DNA* **6**:317–324.
- Eaton DL and Gallagher EP (1994) Mechanisms of aflatoxin carcinogenesis. *Annu Rev Pharmacol Toxicol* **34**:135–172.
- Ellis EM, Judah DJ, Neal GE, O'Connor T, and Hayes JD (1996) Regulation of carbonyl-reducing enzymes in rat liver by chemoprotectors. *Cancer Res* **56**:2758–2766.
- Farmer SC, Sun CW, Winnier GE, Hogan BL, and Townes TM (1997) The bZIP transcription factor LCR-F1 is essential for mesoderm formation in mouse development. *Genes Dev* **11**:786–798.
- Favreau LV and Pickett CB (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.
- Fields WR, Morrow CS, Doehmer J, and Townsend AJ (1999) Expression of stably transfected murine glutathione S-transferase A3–3 protects against nucleic acid alkylation and cytotoxicity by aflatoxin B₁ in hamster V79 cells expressing rat cytochrome P450–2B1. *Carcinogenesis* **20**:1121–1125.
- Fotouhi-Ardakani N and Batist G (1999) Genomic cloning and characterization of the rat glutathione S-transferase-A3-subunit gene. *Biochem J* **339**:685–693.
- Hayes JD, Judah DJ, McLellan LI, Kerr LA, Peacock SD, and Neal GE (1991) Ethoxyquin-induced resistance to aflatoxin B₁ in the rat is associated with the expression of a novel alpha-class glutathione S-transferase subunit, Yc₂, which possesses high catalytic activity for aflatoxin B₁-8,9-epoxide. *Biochem J* **279**:385–398.
- Hayes JD, Judah DJ, Neal GE, 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.
- Hayes JD, Nguyen T, Judah DJ, Petersson DG, and Neal GE (1994) Cloning of cDNAs from fetal rat liver encoding glutathione S-transferase Yc polypeptides. The Yc₂ subunit is expressed in adult rat liver resistant to the hepatocarcinogen aflatoxin B₁. *J Biol Chem* **269**:20707–20717.
- Hayes JD and Pulford DJ (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.
- Hayes JD, Pulford DJ, Ellis EM, McLeod R, James RF, Seidegard J, Mosialou E, Jernstrom B, and Neal GE (1998) Regulation of rat glutathione S-transferase A5 by cancer chemopreventive agents: mechanisms of inducible resistance to aflatoxin B₁. *Chem Biol Interact* **111**:112:51–67.
- Hayes JD and McMahon M (2001) Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. *Cancer Lett* **174**:103–113.
- He CH, Gong P, Hu B, Stewart D, Choi ME, Choi AM, and Alam J (2001) Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation. *J Biol Chem* **276**:20858–20865.
- Hengstler JG, Van der Burg B, Steinberg P, and Oesch F (1999) Interspecies differences in cancer susceptibility and toxicity. *Drug Metab Rev* **31**:917–970.
- Kelly VP, Ellis EM, Manson MM, Chanas SA, Moffat GJ, McLeod R, Judah DJ, Neal GE, and Hayes JD (2000) Chemoprevention of aflatoxin B₁ hepatocarcinogenesis by coumarin, a natural benzopyrone that is a potent inducer of aflatoxin B₁-aldehyde reductase, the glutathione S-transferase A5 and P1 subunits and NAD(P)H:quinone oxidoreductase in rat liver. *Cancer Res* **60**:957–969.
- Kwong M, Kan YW, and Chan JY (1999) The CNC basic leucine zipper factor, Nrf1, is essential for cell survival in response to oxidative stress-inducing agents. Role for Nrf1 in gamma-gcs(l) and gss expression in mouse fibroblasts. *J Biol Chem* **274**:37491–37498.
- Loe DW, Stewart RK, Massey TE, Deeley RG, and Cole SP (1997) ATP-dependent transport of aflatoxin B₁ and its glutathione conjugates by the product of the multidrug resistance protein (MRP) gene. *Mol Pharmacol* **51**:1034–1041.
- McLellan LI, Kerr LA, Cronshaw AD, and Hayes JD (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.
- McMahon G, Davis EF, Huber LJ, Kim Y, and Wogan GN (1990) Characterization of c-Ki-ras and N-ras oncogenes in aflatoxin B₁-induced rat liver tumors. *Proc Natl Acad Sci USA* **87**:1104–1108.
- McMahon M, Itoh K, Yamamoto M, Chanas SA, Henderson CJ, McLellan LI, Wolf CR, Cavin C, and Hayes JD (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.
- McMahon M, Itoh K, Yamamoto M, and Hayes JD (2003) Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem* **278**: 21592–21600.
- Motohashi H, O'Connor T, Katsuoka F, Engel JD, and Yamamoto M (2002) Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. *Gene* **294**:1–12.
- Nguyen T, Huang HC, and Pickett CB (2000) Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK. *J Biol Chem* **275**:15466–15473.
- Nguyen T, Sherratt PJ, and Pickett CB (2003) Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu Rev Pharmacol Toxicol* **43**:233–260.
- Nioi P, McMahon M, Itoh K, Yamamoto M, and Hayes JD (2003) Identification of a novel Nrf2-regulated antioxidant response element in the mouse NAD(P)H:quinone oxidoreductase 1 gene; reassessment of the ARE consensus sequence. *Biochem J* **374**:337–348.
- Okuda A, Imagawa M, Sakai M, and Muramatsu M (1990) Functional cooperativity between two TPA responsive elements in undifferentiated F9 embryonic stem cells. *EMBO (Eur Mol Biol Organ) J* **9**:1131–1135.
- Pulford DJ and Hayes JD (1996) Characterization of the rat glutathione S-transferase Yc₂ subunit gene, GSTA5: identification of a putative antioxidant-responsive element in the 5'-flanking region of rat GSTA5 that may mediate chemoprotection against aflatoxin B₁. *Biochem J* **318**:75–84.
- Rohrdanz E, Nguyen T, and Pickett CB (1992) Isolation and characterization of the human glutathione S-transferase A2 subunit gene. *Arch Biochem Biophys* **298**: 747–752.
- Rozen F, Nguyen T, and Pickett CB (1992) Isolation and characterization of a human glutathione S-transferase Ha1 subunit gene. *Arch Biochem Biophys* **292**:589–593.
- Rushmore TH, King RG, Paulson KE, and Pickett CB (1990) Regulation of glutathione S-transferase Ya subunit gene expression: identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds. *Proc Natl Acad Sci USA* **87**:3826–3830.
- Rushmore TH, Morton MR, and Pickett CB (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.
- Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, and Biswal S (2002) Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res* **62**:5196–5203.
- Venugopal R and Jaiswal AK (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 USA* **93**:14960–14965.
- Venugopal R and Jaiswal AK (1998) Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene* **17**:3145–3156.
- Xie T, Belinsky M, Xu Y, and Jaiswal AK (1995) ARE- and TRE-mediated regulation of gene expression. Response to xenobiotics and antioxidants. *J Biol Chem* **270**: 6894–6900.

Address correspondence to: Dr. Ian R. Jowsey, Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland, UK. E-mail: i.r.jowsey@dundee.ac.uk