Role of Nrf2 in the regulation of the *Mrp2* (*ABCC2*) gene

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The Nrf2 (nuclear factor-erythroid 2 p45-related factor 2) transcription factor regulates gene expression of the GCLC (glutamate–cysteine ligase catalytic subunit), which is a key enzyme in glutathione synthesis, and GSTs (glutathione S-transferases) via the ARE (antioxidant-response element). The Mrp2 (multidrug-resistance protein 2) pump mediates the excretion of GSH and GSSG excretion as well as endo- and xeno-biotics that are conjugated with GSH, glucuronate or sulphate. Considering that Mrp2 acts synergistically with these enzymes, we hypothesized that the regulation of *Mrp2* gene expression is also dependent on Nrf2. Using BHA (butylated hydroxyanisole), which is a classical activator of the ARE–Nrf2 pathway, we observed an increase in the transcriptional activity of *Mrp2*, *GCLC* and *Gsta1/Gsta2* genes in the mouse liver. A similar pattern of co-induction of *Mrp2* and *GCLC* genes was also observed in mouse (Hepa 1-6) and human (HepG2) hepatoma cells treated with BHA, β-NF (β-naphthoflavone), 2,4,5-T (trichlorophenoxyacetic acid) or 2AAF (2-acetylaminofluorene), suggesting that these genes share common mechanism(s) of transcriptional activation in response to exposure to xenobiotics. To define the mechanism of *Mrp2* gene induction, the 5′-flanking region of the mouse *Mrp2* gene (2.0 kb) was isolated, and two ARE-like sequences were found: *ARE-2* (-1391 to -1381) and *ARE-1* (-95 to -85). Deletion analyses demonstrated that the proximal region (-185 to $+99$) contains the elements for the basal expression and xenobiotic-mediated induction of the *Mrp2* gene. Gel-shift and supershift assays indicated that Nrf2–protein complexes bind ARE sequences of the *Mrp2* promoter, preferentially to the *ARE-1* sequence. Overexpression of *Nrf2* increased *ARE-1*-mediated *CAT* (chloramphenicol acetyltransferase) gene activity, while overexpression of mutant Nrf2 protein repressed the activity. Thus Nrf2 appears to regulate *Mrp2* gene expression via an ARE element located at the proximal region of its promoter in response to exposure to xenobiotics.

Key words: antioxidant-response element (ARE), glutamate-cysteine ligase (GCL), liver, multidrug-resistance protein 2 (Mrp2), nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), xenobiotics.

INTRODUCTION

Many endogenous and exogenous lipophilic compounds are eliminated from cells by a sequence of oxidation, conjugation to anionic groups and cellular extrusion across the plasma membrane into the extracellular compartment. The last step is mediated by the MRPs (multidrug-resistance-associated proteins) belonging to subfamily C of the ABC (ATP-binding cassette) transporter superfamily (ABCC). These transporters have been functionally characterized as ATP-dependent export pumps for endo- or xeno-biotic conjugates with glutathione, glucuronate or sulphate [1]. In humans, the MRP family comprises several members (MRP1–MRP9), and their expression is widespread throughout many normal tissues, perhaps most notably in the co-localization with Phase II detoxifying enzymes in excretory organs, such as the liver, kidney and intestine [2]. In the liver, which is a crucial organ in endo- and xeno-biotic detoxification, Mrp2/MRP2, functionally known as the cMOAT (canalicular multispecific organic anion transporter), is one of the most important biliary efflux transporters. Mrp2/MRP2 also mediates canalicular excretion of the antioxidant glutathione in both its reduced (GSH) and its oxidized (GSSG) forms, thereby playing an essential role in detoxification and defence against oxidative stress [3–5]. Mrp2-defective rats show impaired biliary secretion of GSH with a markedly reduced bile flow, indicating that Mrp2 not only represents a decisive step in the elimination of multiple conjugated compounds, but also mediates a major driving force for bile-acid-independent bile flow [6–8].

Biosynthesis of GSH involves two enzymatic reactions that are catalysed by the rate-limiting enzyme GCL (glutamate–cysteine ligase), also named γ-GCS (γ-glutamylcysteine synthetase), and GSH synthetase [9]. The GST (glutathione S-transferase) family comprises a group of Phase II detoxifying enzymes that are involved in the conjugation of a variety of electrophilic compounds and of ROS (reactive oxygen species) with GSH [10]. It has been suggested that the Phase II metabolizing enzymes and efflux transporters act synergistically in the process of xenobiotic detoxification [11]. An especially interesting proposal is that the enzymes that are responsible for the formation of GSH-conjugates (GCL and GSTs) and MRPs are co-ordinately induced in normal mouse tissues, untreated tumour cells and drug-resistant cells [12]. Genes encoding a subset of antioxidative and Phase II metabolizing enzymes are induced on exposure to electrophilic compounds and phenolic antioxidants. Induction of these enzymes is mediated by the ARE (antioxidant-response element) [13], a *cis*-acting sequence found in the 5′-flanking region of genes

Abbreviations used: 2AAF, 2-acetylaminofluorene; ARE, antioxidant-response element; BHA, butylated hydroxyanisole; t-BHQ, t-butylhydroquinone; CAR, constitutively active receptor; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT/enhancer-binding protein; cMOAT, canalicular multispecific organic anion transporter; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; ER-8, everted repeat 8; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCL, glutamate–cysteine ligase; GCLC, GCL catalytic subunit; GST, glutathione S-transferase; HNF, hepatocyte nuclear factor; LUC, luciferase; MRP, multidrug-resistance-associated protein; β-NF, β-naphthoflavone; NF-κB, nuclear factor κB; NF-Y, nuclear factor Y; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; PXR, pregnane X receptor; ROS, reactive oxygen species; RT, reverse transcription; SP-1, specificity protein 1; 2,4,5-T, trichlorophenoxyacetic acid.

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The nucleotide sequence data for the 5′-flanking region of the *Mrp2* gene will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY905402.

Table 1 Primer sequences used for RT–PCR

DNA probe	GenBank® accession number	Primer sequences	Amplified size (bp)
Mouse *Mrp2*	AF227274	Forward: 5′-GCTTAGTTCAAGTCTATGGAGT-3′ Reverse: 5′-TCCGGCCGATACCGCACTTGATA-3′	822
Human *MRP2*	E15807	Forward: 5′AGCCTGAAGGAAGACGAAGAACTAG-3′ Reverse: 5′-CCAATGTCACAAGTGATCCCTC-3′	1163
Mouse *GCLC*	U85414	Forward: 5′-GGAGAGGAGAAAAGGTTGTCAT-3′	995
Human *GCLC*	M90656	Reverse: 5′-CTTCCCATTGATGATGGTGTCT-3′	
Mouse *Gsta1*/*Gsta2*	NM_008181 (*Gsta1*) NM_008182 (*Gsta2*)	Forward: 5′-ACCACAGTTGCTGCAATGGC-3′ Reverse: 5′-ATCCAAGGGAGGCTTTCTCTGG-3′	643
Mouse *Gsta3*	NM_010356	Forward: 5′-TCGACGGGATGAAACTGGTG-3′ Reverse: 5′-CTACCTGAATTGACACAGACGCC-3′	626
Mouse *GAPDH*	M32599	Forward: 5′-CTTCATTGACCTCAACTACATGGT-3′	680
Human *GAPDH*	M33197	Reverse: 5′-TTCACCACCTTCTTGATGTCATC-3′	

such as *Gsta1* [14], *NQO1* [NAD(P)H:quinone oxidoreductase] [15] and GCLC (GCL catalytic subunit) [16]. Recently, it has been proposed that the sequence of the minimal ARE enhancer is (a/g)TGA(C/T/G)nnnGC(a/g) [17]. The bZIP (basic-region leucine-zipper) factor Nrf2 (nuclear factor-erythroid 2 p45-related factor 2), in combination with a small Maf protein, mediates transcriptional activation of genes via the ARE [18,19]. Accumulated data from studies of *Nrf2*-knockout mice and overexpression of *Nrf2* in human hepatoma cells have established that Nrf2 is an essential ARE-binding factor that is involved in both constitutive and inducible gene expression [20–23].

Using the herbicide 2,4,5-T (trichlorophenoxyacetic acid), we have previously demonstrated a model of the co-induction of *Mrp2* and *GCLC* genes *in vivo* in the mouse liver that is associated with increased GSH synthesis and biliary GSH output [24]. Our observations are consistent with the hypothesis that the Mrp2 transporter plays a crucial role in the secretion of biliary GSH, and may be involved in co-ordinated changes in the expression of protective genes in the liver in response to exposure to xenobiotics. The role of Nrf2 in the regulation of *Mrp2* gene expression has not been reported previously. Thus we tested the hypothesis that the xenobiotic-mediated induction of *Mrp2* is also dependent on the ARE–Nrf2 cellular detoxification pathway.

MATERIALS AND METHODS

Animal models

Female CF1 mice between 8 and 10 weeks of age (25–28 g) were used in the present study. All mice were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (publication no. 86-23). For 4 days, control mice were fed a commercial rodent diet (Prolab RMH 3000; PMI Nutritional International), whereas the diet of the mice in the experimental groups was supplemented with 0.75 % BHA (butylated hydroxyanisole) that had been dissolved previously in ethanol. Bile flow, biliary bile acids and the concentrations of GSH, GSSG and total glutathione (GSH plus GSSG) were analysed in control and BHA-treated mice as described previously [24–27].

Cell cultures and xenobiotics

Mouse Hepa 1-6 and human HepG2 hepatoma cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l D-glucose (Invitrogen), 10 % (v/v) foetal bovine serum (Invitrogen), 100 units/ml penicillin G, 100 units/ml streptomycin sulphate and 0.25 μg/ml amphotericin B (Invitrogen). BHA, 2,4,5-T, 2AAF (2-acetylaminofluorene) and β-NF (β-naphthoflavone) were obtained from Sigma Chemicals. All compounds were dissolved in DMSO, and the final DMSO concentration in all treatment conditions was 0.1 % in complete medium.

Northern blot analysis and run-on assay

Total RNA was isolated from mouse liver or cells using the guanidinium/phenol method [28], resolved (10 μg/lane) on a 1.5 % agarose/2.2 M formaldehyde gel and transferred on to nylon filters (NEN Research Products). The membranes were hybridized with mouse or human ^{32}P-labelled DNA probes. The DNA probes were synthesized by RT (reverse transcription)–PCR with specific primers using total RNA from mouse liver or HepG2 cells. The *Gsta1*/*Gsta2* probe was synthesized with primers that recognize both *Gsta1* and *Gsta2* mouse cDNAs (Table 1). The annealing temperature in all PCRs was 58 °C. The DNA probes obtained by RT–PCR were radiolabelled using a random primer labelling system (Promega). A *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) DNA probe was used to normalize the RNA loading.

A run-on assay using *Mrp2*, *GCLC*, *Gsta1*/*Gsta2* and *GAPDH* mouse DNA probes was performed as described previously [24,26]. The ø-X174 plasmid DNA was used as a control for non-specific binding in the assay.

Western blot analysis

Proteins were separated by SDS/PAGE (10 % gels). Liver plasma membrane fractions enriched in the bile canalicular domain were prepared from control and BHA-treated animals [26]. The Mrp2 protein content was determined by immunoblotting using the anti-Mrp2/cMOAT monoclonal antibody M2 III-5 as described previously [24]. Detection of P-glycoprotein, for controlling membrane protein loading, was performed using the monoclonal antibody C219. GCLC and GST alpha-class protein contents were determined in the cytosolic fraction of liver homogenates [26] using a polyclonal antibody against human GCLC and human polyclonal anti-GST A1-1 (Calbiochem) respectively. Protein loading levels were normalized to the immunodetection of albumin.

Isolation, cloning, sequencing and characterization of the 5′-flanking region of the mouse *Mrp2* gene

The 5′-flanking region of the mouse *Mrp2* gene was isolated using nested PCR using *Mrp2*-specific primers and adaptor primers from mouse libraries of uncloned adaptor-ligated genomic DNA fragments (BD Genome Walker Kit; BD Biosciences), according to the manufacturer's instructions. The following *Mrp2*-specific

primers near the translation start site were designed: 5′-GTAGAGTTGCAGAATTCGTCCATCG-3′ (GSP1N: +23 to −2) and 5′-CGTGATTTCCAGGGCACTTCTGTTA-3′ (GSP2N: −1 to −24), which included the XhoI adapter sequence at the 5′ end. A 4-kb fragment of the 5′-flanking region of *Mrp2* was amplified and then digested with MluI and XhoI enzymes to obtain a fragment of approx. 2 kb. This isolated fragment corresponds to the upstream sequence, and extends downstream to nucleotide −1 relative to the translation start site for the *Mrp2* gene. The 2-kb fragment was ligated directionally into two reporter plasmids: *LUC* (luciferase) reporter gene vector pGL3 enhancer and *CAT* (chloramphenicol acetyltransferase) reporter gene vector (Promega), which had been predigested with the same enzymes.

To functionally characterize the isolated 5′-flanking region, a series of 5′-end deletions of the insert in p-2kb-LUC were constructed using the Erase-a-Base System (Promega) according to the manufacturer's instructions. All of the deleted inserts were sequenced using specific pGL3 primers (RVprimer3 and GLprimer2), and the complete nucleotide sequence of the 2-kb insert obtained corresponded exactly to 1995 bp of the 5′-flanking region of *Mrp2*. The 285-bp fragment upstream of the translation start site was obtained by digestion of p-2kb-CAT with XbaI and XhoI, and was cloned into the NheI/XhoI site in pCAT-basic, yielding the construct p-285-CAT.

The transcription start site of the *Mrp2* gene was determined using the Primer Extension System-AMV Reverse Transcriptase System (Promega). Briefly, 10 μg of total RNA isolated from mouse liver and gene-specific primers GSP1N and GSP2N (end-labelled with [α-^{32}P]ATP using T4 polynucleotide kinase) were used according to the manufacturer's instructions. The primer-extended cDNA products were analysed on a denaturing 6 % polyacrylamide gel alongside the sequencing reaction of p-2kb-LUC with the same primers. The location of the mRNA 5′-end was determined by co-migration with the p-2kb-LUC nucleotide sequence. The transcription initiation site was located 100 bases upstream of the translation start site.

Nrf2 expression plasmids

The full-length cDNA for mouse Nrf2 (GenBank® accession number U20532), a dominant-negative mutant (Nrf2-DN, amino acids 296–581; corresponding to the DNA-binding and leucine zipper dimerization domain [29]) and the N-terminal transactivation domain (Nrf2-TA, amino acids 1–296) [30] were generated by PCR with specific primers from mouse liver cDNA. The amplified products were cloned into pcDNA4/His Max TOPO (Invitrogen). The full-length Nrf2 cDNA was performed with the following primers: forward, 5′-CAGCAGGACATGGATTTGATTGAC-3′ (289–312 bp), and reverse, 5′-AGAAAAGGCTCCATCCTCCCGAAC-3′ (2044–2067 bp). Nrf2-DN was amplified using the following primers: 5′-TGTGAC**A**TGTCACTGTGTAAAGC-3′ (1180–1202 bp; where the underlining indicates a change from C to A to create a translation start site) and the Nrf2 reverse primer. To obtain Nrf2-TA, the following primers were used: the Nrf2 forward primer and 5′-GGGTTGAAAGCTT**A**ACACAGTGACAG-3′ (1186–1212 bp; where the underlining indicates a change from T to A to create a stop codon sequence).

Transfections and reporter assays

Hepa 1-6 cells were grown in complete medium, and, 24 h before transfection, the cells were seeded on 12-well plates at a density of 1×10^5 cells per well. Cells were transfected with 2.6 μg of DNA, 3.5 μl of Lipofectamine™ and 6 μl of Lipofectamine™ Plus reagent (Invitrogen). The total DNA included 0.5 μg of reporter plasmid (p-insert-LUC or p-insert-CAT) and 0.1 μg of transfection control plasmid [pCMV-βgal (Clontech) or pRL-TK *Renilla* (Promega)] with or without 2 μg of the expression plasmid (p-Nrf2, p-Nrf2-DN or p-Nrf2-TA). The total amount of DNA in each transfection assay was kept constant by using pBR322 plasmid. After 4 h of incubation, the transfection medium was replaced with complete medium or supplemented with each of the tested compounds at various concentrations. Cells were harvested after 24 h, and cell extracts were assayed for enzyme-specific activity of the reporter gene used. LUC activities were normalized to the luminescence of the *Renilla* internal control, and the activities were measured by the Dual-Luciferase Reporter Assay system (Promega). The CAT activity was determined by the CAT enzyme assay system with reporter lysis buffer (Promega), and was normalized to β-galactosidase activity as determined by the β-galactosidase enzyme assay system with reporter lysis buffer (Promega). All experiments were repeated at least three times.

Isolation of nuclear extracts and EMSAs (electrophoretic mobility-shift assays)

Nuclear protein extracts were isolated from HepG2 and Hepa 1-6 (DMSO control and BHA- or β-NF-treated) cells and from Hepa 1-6 cells overexpressing the DNA-binding and leucine zipper dimerization domain (Nrf2-DN). Briefly, cells were collected by centrifugation at 800 ***g*** for 5 min at 4 °C and resuspended in 400 μl of ice-cold buffer [10 mM Hepes (pH 7.8), 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM PMSF and 0.5 mM DTT (dithiothreitol)]. After 15 min of incubation on ice, Nonidet P40 was added to a final concentration of 0.6 %, and the cells were vortex-mixed and centrifuged for 1 min at 16 000 ***g***. The nuclear pellet was extracted with 50 μl of ice-cold buffer [10 mM Hepes (pH 7.8), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT and 25 % glycerol] for 30 min at 4 °C on a rocking platform, and debris was removed by centrifugation at 16 000 ***g*** for 20 min at 4 °C. The protein concentration was determined using the Bradford assay (Bio-Rad).

A double-stranded DNA probe containing the *ARE-1* and *ARE-2* sequences or the corresponding *ARE-1* mutant oligonucleotide (ARE-1M) was end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and used for gel shift analysis. The DNA probes were 5′-ACTGGGA**TGACATAGC**ATTCATC-3′ (ARE-1) and 5′-TGCTTGC**TGACATGGC**AAACATC-3′ (ARE-2), where the boldface indicates the ARE core sequence; or 5′-ACTGGGAGTCAGACGCATTCATC-3′ (ARE-1M; underlining indicates the ARE core mutation). The reaction mixture containing 2 μl of 5 × gel-shift binding buffer (Promega), 5 μg of nuclear protein extract and sterile water to a total volume of 9 μl was incubated at room temperature (22 °C) for 10 min. The probe (1 μl; 2×10^5 c.p.m.) was then added, and DNA-binding reactions were performed overnight at 4 °C. For competition experiments, unlabelled *Mrp2* ARE probe was added at a 10-, 25- or 50-fold molar excess before the labelled probe. For the supershift assay, an anti-Nrf2 antibody (sc-722X; Santa Cruz Biotechnology) was added after 4 h of the binding reaction at 4 °C. DNA–protein interactions were detected by electrophoresis on a non-denaturing 5 % polyacrylamide gel in 0.5× TBE buffer. Gels were dried, and DNA–protein complexes were visualized by autoradiography.

RESULTS

BHA induces gene expression of *Mrp2*, *GCLC* and *Gsta1*/*Gsta2* in the mouse liver

BHA, a classical activator of Nrf2, has been used to study the activation of GCLC [31] and Gsta1/Gsta2 [21] by ARE-dependent

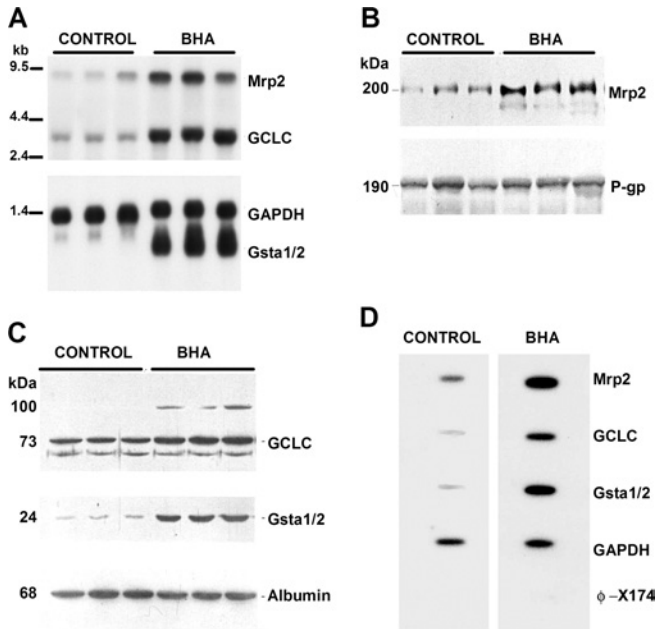

Figure 1 Co-induction of *Mrp2*, *GCLC* and *Gsta1/Gsta2* genes by BHA in the mouse liver

Total RNA and proteins were isolated from the livers of control and BHA-treated animals. The levels of *Mrp2*, *GCLC* and *Gsta1/Gsta2* mRNAs and proteins were determined by Northern (**A**) and Western (**B** and **C**) blotting respectively, as described in the Materials and methods section. (**D**) Nuclear run-on assay. Isolated nuclei from hepatocytes of control and BHA-treated mice were incubated in the presence of [α-^{32}P]UTP. The nuclear-labelled RNA was used as a hybridization probe against the specific DNA probes that were immobilized on a nylon membrane (ϕ-X174 plasmid DNA was included to assess non-specific hybridization). Sizes of bands are given in either kb or kDa, as indicated. P-gp, P-glycoprotein.

mechanisms. The effect of BHA on *Mrp2* gene expression was assessed here *in vivo*. As shown in Figure 1(A), the mRNA levels for the *Mrp2* and *GCLC* genes were higher in the livers of BHA-treated mice than in control mice. *Gsta1/Gsta2* mRNA was not detected in the livers of control mice and, as expected, was strongly induced in the livers of BHA-treated mice. The *Gsta3* mRNA level did not change in treated mice, as assessed by RT–PCR (results not shown).

Western blotting was used to determine whether increased levels of hepatic *Mrp2*, *GCLC* and *Gsta1/Gsta2* mRNAs were associated with increased expression of the encoded products. The Mrp2 protein content in the canalicular domain of hepatocytes was 3-fold higher in BHA-treated mice than in control animals, as shown in Figure 1(B). The contents of GCLC and Gsta1/2 proteins were also increased (1.8- and 11-fold respectively) in the cytosolic fraction of proteins in the livers of BHA-treated mice (Figure 1C).

The relative transcription rates of *Mrp2* were also examined using a nuclear run-on assay with nuclei isolated from control and BHA-treated mice livers. As seen in Figure 1(D), the transcription rates of *Mrp2*, *GCLC* and *Gsta1/Gsta2* were higher in BHA-treated mice than in control mice. No changes in the level of GAPDH-labelling RNA transcript, a gene of constitutive expression, were detected. This result indicates that BHA also induces the transcription of the *Mrp2* gene.

The hepatocellular concentration of total glutathione was 2-fold higher in BHA-treated mice than in control animals, suggesting an increase of GSH synthesis: total glutathione was 15.49 ± 0.16 compared with 7.99 ± 1.82 μmol/g of wet liver, and GSSG was 0.30 ± 0.49 compared with 0.48 ± 0.38 μmol/g of wet liver

Table 2 Effects of BHA on bile composition

Data are means $\pm$ S.D. ($n = 6$). *$P < 0.05$; **$P < 0.005$ relative to control (Student's t test).

	Bile flow (μl/min per g of liver)	Bile salts (nmol/min per g of liver)	Total glutathione (nmol/min per g of liver)	GSSG (nmol/min per g of liver)	GSH (nmol/min per g of liver)
Control	1.86 ± 0.25	82.9 ± 23.5	7.53 ± 2.22	2.38 ± 0.70	5.15 ± 1.87
BHA	2.54 ± 0.41**	89.6 ± 36.3	12.62 ± 1.87*	3.74 ± 0.51*	8.89 ± 1.73*

($P < 0.05$, $n = 3$). As evident from Table 2, the bile flow was significantly higher in BHA-treated mice, without changes in the biliary output of bile salts. Remarkably, a significantly higher biliary GSH output was observed in BHA-treated mice, indicating that the newly synthesized Mrp2 transporter was functionally incorporated into the canalicular domain of hepatocytes.

Taken together, these results suggest that BHA induces not only Phase II detoxifying enzymes (Gsta1/Gsta2) and the rate-limiting enzyme of GSH synthesis (GCLC) in the mouse liver as described previously [21,31], but also the Mrp2 transporter via transcriptional activation of the specific genes.

Co-induction of *Mrp2* and *GCLC* genes by xenobiotics in hepatoma cell lines

The above *in vivo* observations of the co-induction of *Mrp2* and *GCLC* genes in the mouse liver were extended to a cell culture model using the human (HepG2) and mouse (Hepa 1-6) hepatoma cell lines. Cells were cultured with classical activators of the ARE–Nrf2 pathway, such as the synthetic flavonoid β-NF and the antioxidant BHA [16,31], as well as chemical inducers of the *Mrp2* gene: 2,4,5-T [24] and 2AAF [32]. The mechanisms of induction of these two compounds remain undefined. Exposure of Hepa 1-6 and HepG2 cells for 24 h to different concentrations of all of the compounds revealed dose-dependent increases in the levels of *Mrp2* and *GCLC* mRNAs (Figures 2A and 2B). The induction of *Mrp2* as well as *GCLC* was time-dependent in the Hepa 1-6 cell line (Figures 2C and 2D). The compounds 2,4,5-T, 2AAF and BHA induced both genes with a similar pattern: induction was first detected at 12 h after treatment, with the maximal induction (2–2.5-fold) occurring after 24 h. The β-NF-mediated induction of both genes showed a similar pattern in this cell line, but the induction occurred as early as 6 h after treatment, and peaked at 12 h.

The similar patterns of co-induction of *Mrp2* and *GCLC* genes in human and mouse hepatoma cell lines by classical activators of Nrf2 (BHA and β-NF) and the chemical inducers (2,4,5-T and 2AAF) suggests conserved molecular mechanisms of transcriptional activation, which may be mediated by the ARE–Nrf2 pathway.

The 5′-flanking region of the mouse *Mrp2* gene contains two ARE sequences

To determine the presence of ARE sequences in the 5′-flanking region of the mouse *Mrp2* gene, a 1995-bp fragment was cloned and sequenced as described in the Materials and methods section. The BLAST program revealed that the isolated fragment exhibited 99.8 % nucleotide identity with a clone of genomic DNA (GenBank® accession number AC132251) located in mouse chromosome 19. As expected, several consensus motifs for the potential transcription-factor-binding sites were identified using the program TRANSFAC [33]. The isolated region contains liver-abundant transcription-factor-binding sites for C/EBP (CCAAT/

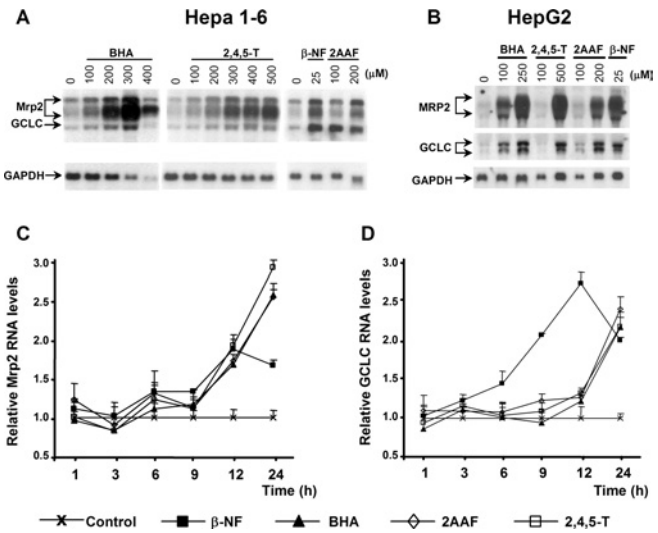

Figure 2 Dose-dependence and time course of the induction of *Mrp2* and *GCLC* mRNAs by xenobiotics in hepatoma cell lines

Mouse Hepa 1-6 cells (**A**) and human HepG2 cells (**B**) were exposed to different concentrations of xenobiotics (BHA, 2,4,5-T, 2AAF or β-NF) for 24 h. Total RNA (10 μg) from control (DMSO) and treated cells was subjected to Northern blot analysis using specific probes for mouse or human genes. In order to determinate the time course of the induction of *Mrp2* (**C**) and *GCLC* (**D**) mRNAs, Hepa 1-6 cells were incubated in medium containing 0.1 % DMSO (control), 25 μM β-NF, 250 μM BHA, 500 μM 2,4,5-T or 200 μM 2AAF. At various intervals up to 24 h, RNA was harvested, and the specific mRNA levels were determined by Northern blotting. The relative contents of *Mrp2* and *GCLC* were determined using the PhosphorImager system. The mRNA levels were normalized to values observed in control cells, and are plotted as the relative amounts of mRNA. Results are means $\pm$ S.D. for four independent experiments.

enhancer-binding protein), HNF-3β (hepatocyte nuclear factor 3β), HNF-4 and PXR (pregnane X receptor)/CAR (constitutively active receptor)/FXR (farnesoid X receptor) (ER-8: everted repeat 8) [34], and ubiquitous factors expressed in multiple tissues including AP-1 (activator protein 1), NF-Y (nuclear factor Y), NF-κB (nuclear factor κB) and SP-1 (specificity protein 1). Interestingly, two ARE-like sequences were found in the isolated region: one at positions -1391 to -1381 (*ARE-2*; cTGACatgGCa, with lower-case letters indicating non-conserved bases) and the other at positions -95 to -85 (*ARE-1*; aTGACataGCa) from the transcription start site. The *ARE-1* sequence is identical with the previously reported minimal ARE enhancer sequence (a/g)TGA(C/T/G)nnnGC(a/g) [17]. In contrast, the *ARE-2* sequence differs from the consensus sequence in the first base. TATA box sequences were not found in the 5′-flanking region of the mouse *Mrp2* gene. The complete sequence of the isolated fragment and the putative binding sites for the transcription factors are shown in Figure 3 (GenBank® accession number AY905402).

Deletion analysis of the 5′-flanking region of the *Mrp2* gene

In order to identify the sequences in the 5′-flanking region of the *Mrp2* gene that mediate constitutive gene expression, *LUC* or *CAT* reporter gene assays with constructs containing the complete isolated flanking region or 5′-unidirectionally deleted inserts were performed in Hepa 1-6-transfected cells. As shown in Figure 4(A), the deleted constructs p $-$ 1598/+ 99-LUC, p $-$ 995/+ 99-LUC, p $-$ 445/+ 99-LUC and p $-$ 255/+ 99-LUC exhibited LUC activity similar to that of the full-length construct (p $-$ 1895/+ 99-LUC). However, the construct p $-$ 94/+ 99-LUC resulted in a 70 % decrease in the LUC activity ($P < 0.05$, $n = 3$). Interestingly, this construct contains the *ARE-1* sequence truncated in the first base pair. These results suggest that the *ARE-2* sequence is not relevant to the constitutive *Mrp2* gene expression. On the other hand, the activity of a minor construct (p $-$ 185/+ 99-CAT) was similar to the activity of the p $-$ 255/+ 99-LUC (78–80 %) (Figure 4B), indicating that positions -185 to $+99$ of the 5′-flanking region contain the elements that are responsible for the basal transcriptional activity of the *Mrp2* gene. This region contains the putative binding sites for the transcription factors C/EBP, SP-1, NF-κB and ARE-1, as shown in the representative map of the *Mrp2* 5′-flanking region illustrated in Figure 4(C).

Activity of the *Mrp2* reporter gene constructs is induced by xenobiotics

To determine whether the isolated 5′-flanking region of the *Mrp2* gene contains the sequences that mediate the chemical induction of the *Mrp2* gene, Hepa 1-6 cells were transfected with either the construct containing the full region and both ARE-like elements (p $-$ 1895/+ 99-CAT) or the construct responsible for the basal transcriptional activity of the *Mrp2* gene (p $-$ 185/+ 99-CAT). The transfected cells were exposed to the following xenobiotics: 250 μM BHA, 500 μM 2,4,5-T or 25 μM β-NF, or to 0.1 % DMSO as a control. After 24 h, the CAT reporter activity of both constructs was measured in cell extracts (Figure 5A). We observed that all of the compounds studied induced CAT activity (at different levels) in both constructs compared with control (DMSO). Interestingly, the relative amount of induced CAT activity (p $-$ 1895/+ 99-CAT or p $-$ 185/+ 99-CAT) was similar in both constructs for each compound tested, suggesting that the proximal region, containing the *ARE-1* sequence, is responsible for the xenobiotic-mediated induction of the *Mrp2* gene (Figure 5B). These experiments also suggest that the *ARE-2* sequence is not involved in the xenobiotic-mediated induction of this gene.

Overexpression of *Nrf2* enhances the activity of the *Mrp2* 5′-flanking region

Transient co-transfection assays were used to determine whether the ARE-like sequences present in the *Mrp2* gene are activated directly by Nrf2. The two constructs of the *Mrp2* 5′-flanking region, p $-$ 1895/+ 99-CAT or p $-$ 185/+ 99-CAT, were co-transfected into Hepa 1-6 cells with either an *Nrf2* expression plasmid or one of two *Nrf2*-mutant-expressing plasmids (p-Nrf2-TA or p-Nrf2-DN). Overexpression of the Nrf2 protein significantly enhanced the CAT activity of both constructs compared with controls (Figure 6): 2.2-fold for p $-$ 1895/+ 99-CAT and 3.5-fold for p $-$ 185/+ 99-CAT compared with control ($P < 0.05$, $n = 3$). On the other hand, overexpression of both Nrf2-TA and Nrf2-DN mutant proteins significantly decreased CAT activity to below basal values (to less than 50 % in both constructs). Taken together, these results indicate that the *ARE-1* sequence (located at positions -95 to -85 relative to the transcription start site of *Mrp2*) is the only ARE sequence activated by the Nrf2 transcription factor.

ARE sequences of the 5′-flanking region of the *Mrp2* gene bind Nrf2 complexes

EMSAs were performed to characterize the protein binding of *ARE-1* and *ARE-2* sequences present in the 5′-flanking region of the *Mrp2* gene. Nuclear extract proteins obtained from Hepa 1-6 cells (control) or transfected cells overexpressing Nrf2-DN, and oligonucleotides (23 nucleotides long) containing *ARE-1* or *ARE-2* sequences, were used in the assay. As shown in Figure 7(A), nuclear extract proteins from control Hepa 1-6 cells bind, with different affinities, to the *ARE-1* and *ARE-2* sequences, determining a main DNA–protein complex (band a in Figure 7A).

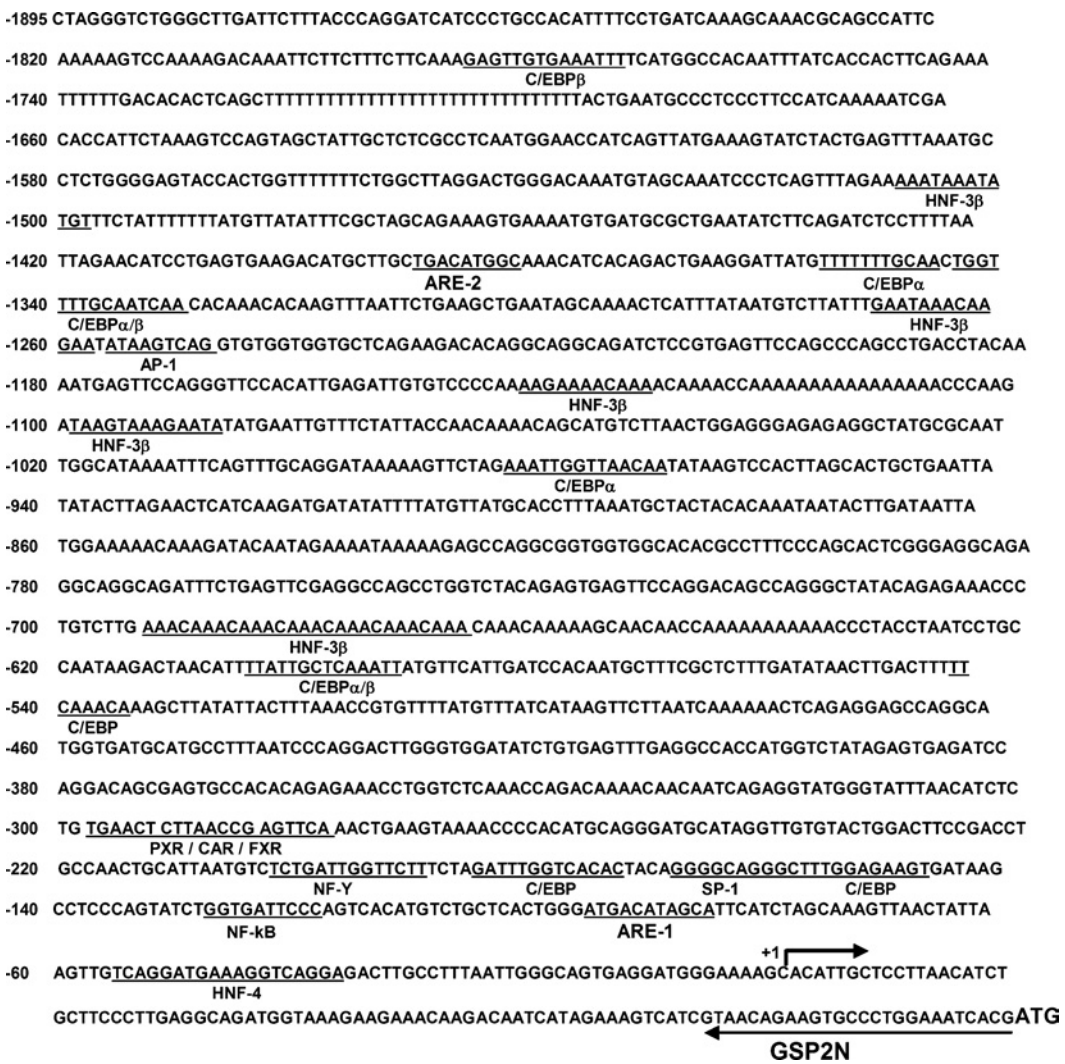

Figure 3 Nucleotide sequence of the 5′-flanking region of the *Mrp2* gene

The nucleotide sequence of the 5′-flanking region of the mouse *Mrp2* gene from nucleotides −1895 to +103 is shown with numbering relative to the transcription start site (+1, arrow). The consensus binding sites for putative regulatory elements are underlined, and the respective transcription factors are given above the sequences. The positions of the putative *ARE-1* (−95 to −85) and *ARE-2* (−1391 to −1381) sequences are indicated. The cloning primer (GSP2N) is also indicated by an arrow, and the ATG start codon is shown in boldface. The full nucleotide sequence is available at GenBank® under accession number AY905402. The 5′-flanking region of the mouse *Mrp2* gene was isolated, cloned and sequenced as described in the Materials and methods section. AP-1, activator protein 1.

Only *ARE-1* showed an additional upper DNA–protein complex (Figure 7A, band b). These bands are specific, because they are effectively competed for by an excess amount of corresponding unlabelled ARE and by Nrf2-DN protein in the nuclear extract (Figure 7A, band c). This result indicates that both ARE sequences are recognized by the DNA-binding and leucine zipper dimerization domain (Nrf2-DN) of Nrf2 (Figure 7A, band c), and also suggests that *ARE-1* has a higher affinity for forming different Nrf2–protein complexes (Figure 7A, bands a and b) than does *ARE-2*. In addition, to demonstrate that the *ARE-1* sequence binds specifically to Nrf2 complexes, the *ARE-1* core was replaced by the mutant core of the human *NQO1* ARE sequence, which has been used previously to assess the specificity of binding of Nrf2 to the ARE sequences [35]. As shown in Figure 7(B), when the EMSA was performed using the mutated ARE-1M oligonucleotide, only a weak main DNA–protein complex (band a) was detected using both control and Nrf2-DN nuclear extracts. The absence of additional bands (Figure 7B, bands b and c) in this assay suggests that the *ARE-1* sequence specifically binds Nrf2–protein complexes. We propose that the main complex (Figure 7B, band a) is formed by the *ARE-1* sequence binding the heterodimer of Nrf2 with Maf proteins, and the upper complex (Figure 7B, band b) corresponds to a second complex in which additional unknown proteins have been recruited. The three DNA–protein complexes (Figure 7B, bands a, b and c) were effectively competed with the unlabelled ARE-1 oligonucleotide in both nuclear extracts.

To assess whether Nrf2 activated by β-NF and BHA is able to bind the *Mrp2* ARE sequences, an EMSA was performed using nuclear extracts isolated from HepG2 cells treated with 25 μM β-NF or 250 μM BHA for 3 h. Remarkably, as shown in Figure 8(A), the presence of β-NF and BHA increased the binding of Nrf2–protein complexes to the *ARE-1* sequence, indicating that *ARE-2* has a lower affinity for forming Nrf2–protein complexes than does *ARE-1*. The presence of Nrf2 in *ARE-1*–protein complexes was determined by a supershift assay using anti-Nrf2 antibody

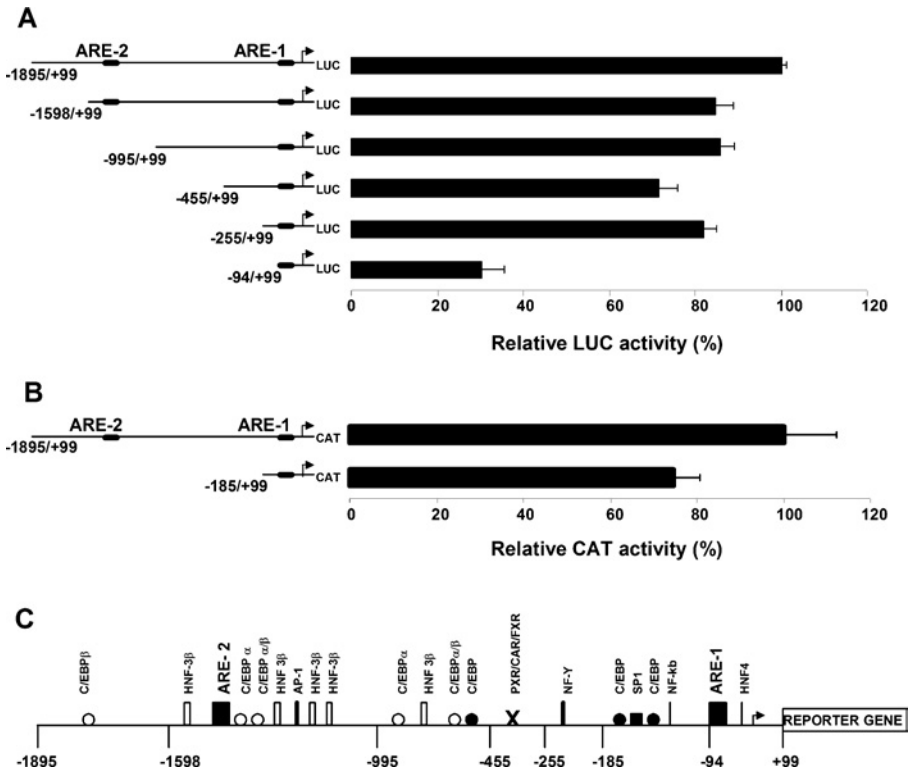

Figure 4 Deletion analysis of the 5′-flanking region of the *Mrp2* gene

The 5′-flanking region constructs were transiently transfected into Hepa 1-6 cells, and after 24 h the LUC (**A**) and CAT (**B**) activities were determined. The results are expressed as the percentage activity of the entire isolated 5′-flanking region (with p − 1895/+ 99-LUC or p − 1895/+ 99-CAT normalized to 100 %), after subtraction of the activity of the empty plasmid reporter. Transfections were performed in triplicate, and results are means ± S.D. (**C**) Schematic representation of the 5′-flanking region of the *Mrp2* gene. The reporter gene constructs and the putative binding sites for transcription factors, including *ARE-1* and *ARE-2* sequences, are shown. AP-1, activator protein 1.

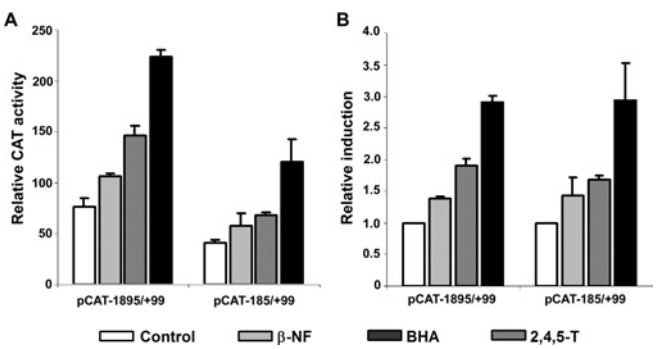

Figure 5 Activity of the *Mrp2* reporter gene constructs induced by xenobiotics

(**A**) Hepa 1-6 cells were transfected with constructs containing either both ARE sequences (p − 1895/+ 99-CAT) or only the *ARE-1*-like sequence (p − 185/+ 99-CAT). CAT activity was measured after treatment with 250 μM BHA, 500 μM 2,4,5-T or 25 μM β-NF for 24 h. (**B**) Results expressed as the induction relative to the activity of the construct treated with vehicle (0.1 % DMSO). Results are means ± S.D. for three independent experiments.

(SC-722x) and nuclear extracts isolated from HepG2 cells treated with 250 μM BHA for 3 h. As shown in Figure 8(B), *ARE-1* bound more protein complex from BHA-treated cells than control nuclear extracts. The addition of anti-Nrf2 antibody resulted in weak supershift bands in both extracts, indicating that Nrf2 is present in the *ARE-1*–protein complex. The supershift assay was also performed with nuclear extracts isolated from Hepa 1-6 cells.

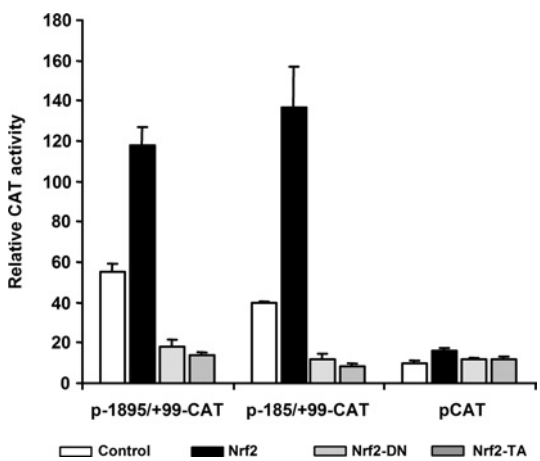

Figure 6 Overexpression of *Nrf2* enhances the activity of the *Mrp2* 5′-flanking region

The construct p − 1895/+ 99-CAT or p − 185/+ 99-CAT was co-transfected into Hepa 1-6 cells with either an *Nrf2* expression plasmid or one of two Nrf2-mutant expression plasmids: p-Nrf2-TA or p-Nrf2-DN. The activity is shown relative to that obtained with the plasmid pBR322 used as a control. Results are means ± S.D. for six independent experiments.

However, we were unable to obtain supershift bands in Hepa 1-6 cells (results not shown) because this antibody did not bind reliably to mouse Nrf2; this behaviour has also been reported by other authors [36].

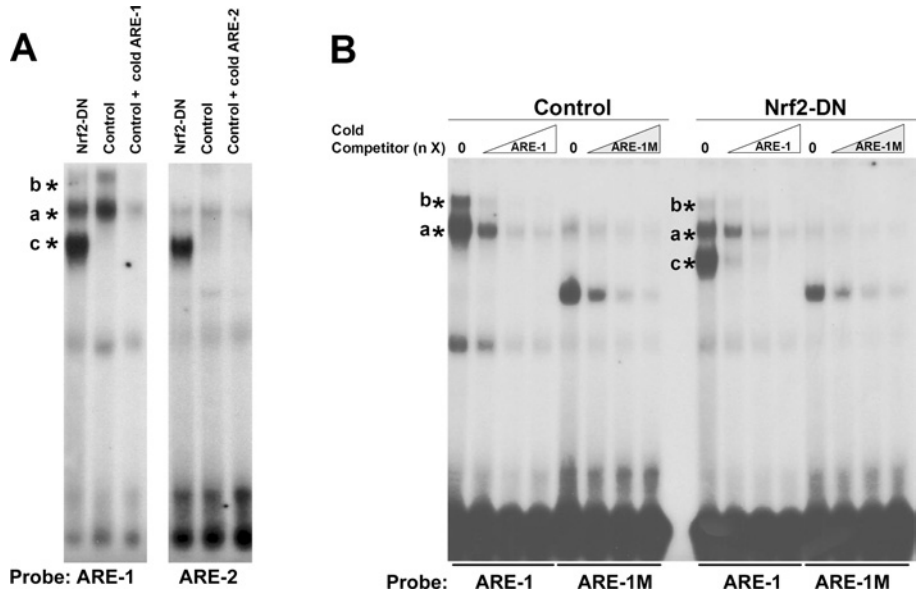

Figure 7 ARE sequences of the 5′-flanking region of the *Mrp2* gene bind to the Nrf2–protein complexes

(**A**) EMSA was performed using nuclear extracts (5 μg) obtained from control Hepa 1-6 cells or cells overexpressing Nrf2-DN, and the radiolabelled probe containing the *ARE-1* or *ARE-2* sequence. For competition experiments, the unlabelled (cold) probe was included at a 50-fold molar excess. (**B**) EMSA was performed using nuclear extracts (5 μg) from Hepa 1-6 cells (control) or cells overexpressing Nrf2-DN and the radiolabelled probe containing the *ARE-1* or mutant *ARE-1* sequence (ARE-1M). For competition experiments, the unlabelled (cold) probe was included at a 10-, 25- or 50-fold molar excess. The positions of the DNA–protein complexes are indicated by asterisks (bands a, b and c).

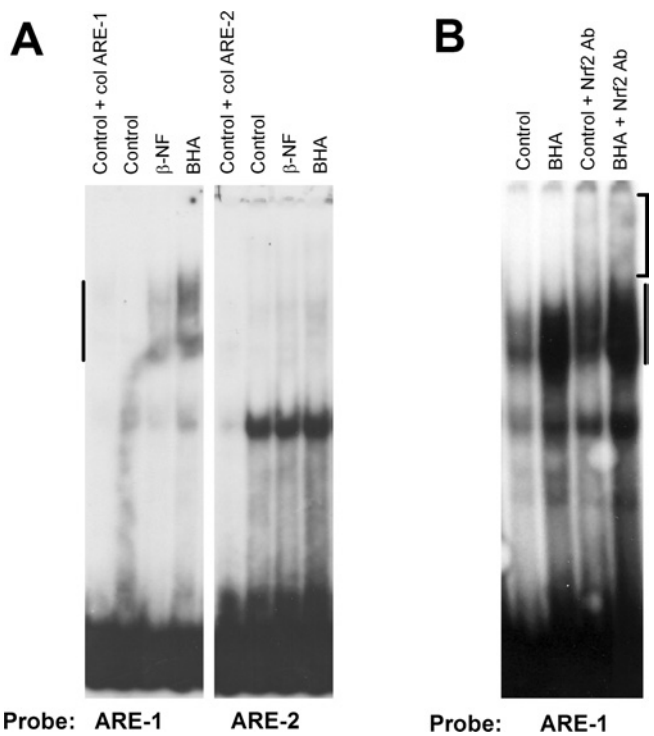

Figure 8 Xenobiotics increased the binding of Nrf2–protein complexes to the *ARE-1* sequence

(**A**) EMSA was performed using nuclear extracts (5 μg) obtained from control HepG2 cells or those treated with 25 μM β-NF or 250 μM BHA for 3 h and the radiolabelled probe containing the *ARE-1* or *ARE-2* sequence. For competition experiments, the unlabelled (cold) probe was included at a 50-fold molar excess. (**B**) A supershift assay was performed using nuclear extracts (15 μg) from HepG2 cells (control) or those treated with BHA and the radiolabelled probe containing the *ARE-1* sequence in the presence or absence of anti-Nrf2 antibody (Ab). The shifted bands are denoted by lines, and supershifted bands are denoted by the bracket.

DISCUSSION

The following observations made in the present study demonstrate that several xenobiotics induce *Mrp2* gene expression via the ARE–Nrf2 pathway: (i) two ARE sequences were identified in the 5′-flanking region of the mouse *Mrp2* gene, (ii) the mouse *Mrp2* promoter activity was significantly enhanced either by overexpression of Nrf2 protein or by the Nrf2 activators BHA or β-NF, and (iii) the *ARE-1* sequence appears to be the only ARE sequence that is activated by Nrf2. In addition, the co-induction of *Mrp2* and *GCLC* gene expression by several compounds was similar in human and mouse hepatoma cell lines. Interestingly, the Nrf2 activator BHA appears to induce not only Phase II detoxifying enzymes and the rate-limiting enzyme of GSH synthesis in the mouse liver, but also the Mrp2 transporter via transcriptional activation of the specific genes, resulting in increased GSH synthesis and biliary GSH output.

Our observations are consistent with previous reports that Nrf2 activators, such as sulphoraphane and the metabolite of BHA, t-BHQ (t-butylhydroquinone), increase the levels of *Mrp2*, *GSTA1/GSTA2* and *NQO1* mRNAs in a dose- and time-dependent manner in rat and human hepatocytes. In those studies, the *Mrp2* induction was associated with increased cellular production of ROS [37,38]. It has also been proposed that the intracellular levels of ROS regulate *MRP1* and *GCLC* gene expression, based on experiments using cultured hepatoma cells treated with pro-oxidants such as t-BHQ and menadione [39]. The term ‘oxidative stress’ encompasses a broad spectrum of conditions that change the cellular redox status, such as an increased production of free radical species within the cell or by pro-oxidant compounds that are thiol-reactive and mimic an oxidative insult [13]. In the present study, we tested not only activators of Nrf2, such as BHA and β-NF, but also the chemicals 2,4,5-T and 2AAF, which are well-known *Mrp2* inducers [24,32]. Interestingly, the observed pattern of co-induction of *Mrp2* and *GCLC* genes was similar for all of the xenobiotics tested, both in human and mouse cultured cell lines,

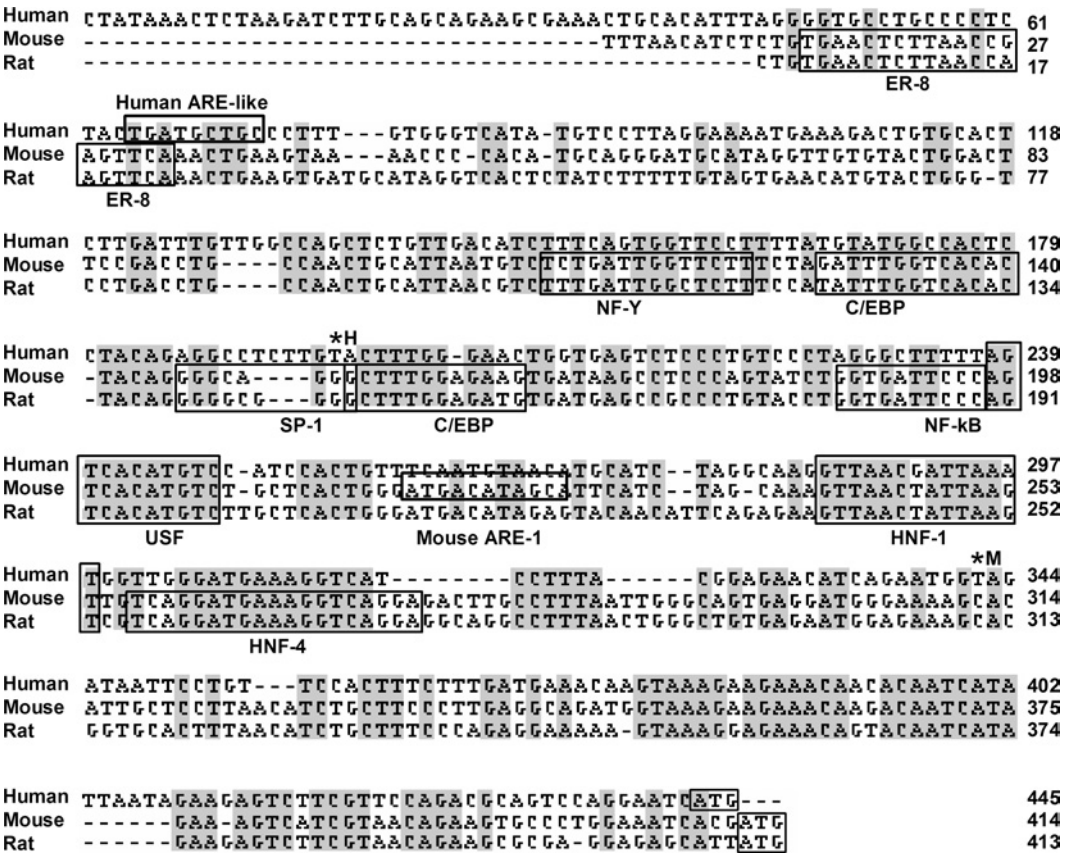

Figure 9 Sequence alignment of the human, mouse and rat *MRP2*/*Mrp2* 5′-flanking regions

ClustalW alignment of the proximal 5′-flanking region of each gene is shown. Shaded boxes indicate nucleotides that are identical across all three species. The conserved regions containing transcription-factor-binding sites and mouse *ARE-1* and human ARE-like core sequence are boxed. The transcription start sites for mouse and human are indicated by *M and *H respectively. USF, upstream stimulatory factor.

suggesting that the xenobiotic-mediated inductions of these genes share a common molecular mechanism of activation in response to oxidative/electrophilic stress.

The transcription factor Nrf2 binds the ARE sequences, regulating a battery of detoxifying and antioxidant genes. Genes now recognized as being induced via the ARE–Nrf2 pathway include those that encode a panel of enzymes that are associated with GSH synthesis, redox proteins with active thiol moieties and drug-metabolizing enzymes [23]. A recent study using microarray analysis has revealed that Nrf2 ablation down-regulates many detoxification genes in the liver of *Nrf2*-knockout mice [40], including the *Mrp3* and *Mrp6* genes, suggesting that the ARE–Nrf2 pathway is involved in the regulation of these genes. Interestingly, it was also found recently that the constitutive and inducible expression levels of *Mrp1* mRNA and protein were significantly lower in *Nrf2*-knockout mouse embryo fibroblasts than in wild-type cells [41], suggesting that Nrf2 is required for the constitutive and inducible expression of MRP family proteins.

The role of the ARE–Nrf2 pathway may also extend to the regulation of the human *MRP1* gene, which is the homologue of *MRP2* in the basolateral domain of polarized cells. In fact, two ARE sequences (located at positions −1843 to −1833 and −499 to −490) have been identified in the 5′-flanking region of the *MRP1* gene [41], which may be involved in the t-BHQ- and quercetin-mediated induction of the gene [37]. It has also been demonstrated that the region at positions −678 to −118 from the translation start site of the human *MRP2* gene contains the elements that are responsible for the basal expression of the gene. Putative binding sites for C/EBPβ, HNF-1 and HNF-3β, as well as an ARE site (−336 to −328), have been identified in this region [42–44]. Our analysis of the sequences of the 5′-flanking region of the human *MRP2* gene (GenBank® accession number AF144630) revealed an additional ARE site not reported previously (at positions −2865 to −2856), indicating that the distribution of ARE sequences in the 5′-flanking region is similar in human *MRP2* and mouse *Mrp2* genes. On the other hand, we only found two ARE-like sequences: at positions −1211 to −1201 and −1754 to −1744 from the translation start site of the rat *Mrp2* gene (GenBank® accession number AF261713). Thus only human *MRP2* and mouse *Mrp2* genes contain at least one ARE sequence in the 5′-proximal region near to the transcription start site.

Based on the premise that functionally important regulatory sites may be conserved between species, we compared the sequences of the promoters of mouse, rat and human *MRP2*/*Mrp2* genes around the mouse *Are-1* sequence. The promoter regions that are homologous with *Mrp2* in more distantly related species have not been reported. As evident in Figure 9, we found 19% sequence identity between the *MRP2*/*Mrp2* promoter regions of these three species, which share only the binding site sequences for the transcription factors HNF-1 and USF (upstream stimulatory factor). In contrast, the mouse and rat *Mrp2* promoter regions retained more than 60% sequence identity, sharing putative binding sites for HNF-4, NF-κB, C/EBP, SP-1, NF-Y and PXR/

CAR/FXR (ER-8). The human ARE-like sequence is located in a different region of the mouse *ARE-1* sequence, as illustrated in Figure 9, and its functional role remains to be defined.

Our observations, together with those of a recent study [45], strongly suggest that MRP family members in mouse, such as Mrp2–Mrp6, share conserved mechanisms of gene activation via the ARE–Nrf2 cellular detoxification pathway in response to exposure to xenobiotics. The role of the ARE–Nrf2 pathway in the mechanism(s) of gene activation in human members of the MRP family also remains to be defined.

We have observed that BHA induces significant functional changes in biliary GSH secretion and bile flow *in vivo*. The increased bile flow observed in BHA-treated mice was probably due to an increased synthesis and output of GSH. Two lines of evidence support this interpretation: (i) it is well known that GSH is the major driving force of bile-acid-independent flow [6–8] and (ii) no changes in bile salt output were observed in treated mice (Table 2). Our observations of *Mrp2* gene induction in mouse liver and increased GSH synthesis and biliary GSH output are similar to our previous findings using 2,4,5-T in mice [24]. These results agree with studies performed *in vivo* and *in vitro* that demonstrate the role of Mrp2 in the excretion of GSH [3–5]. In the liver, the ARE–Nrf2 detoxification pathway may be an interesting therapeutic target for many clinical conditions, such as the chemoprevention of liver carcinogenesis due to fungal aflatoxins, the prevention of liver-transplantation-associated reperfusion injury and the treatment of cholestatic diseases with impaired bile flow, including biliary stones.

In summary, we have characterized the 5′-flanking region of the mouse *Mrp2* gene for the first time, and have demonstrated that xenobiotic induction of the *Mrp2* gene is regulated via the ARE–Nrf2 cellular detoxification pathway.

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