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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[1]

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ABSTRACT

Northern blotting has shown that mouse small intestine contains relatively large amounts of the nuclear factor-E2 p45-related factor (Nrf) 2 transcription factor but relatively little Nrf1. Regulation of intestinal antioxidant and detoxication enzymes by Nrf2 has been assessed using a mouse line bearing a targeted disruption of the gene encoding this factor. Both *Nrf2*(−/−) and *Nrf2*(+/+) mice were fed a control diet or one supplemented with either synthetic cancer chemopreventive agents [butylated hydroxyanisole (BHA), ethoxyquin (EQ), or oltipraz] or phytochemicals [indole-3-carbinol, cafestol and kahweol palmitate, sulforaphane, coumarin (CMRN), or α-angelicalactone]. The constitutive level of NAD(P)H:quinone oxidoreductase (NQO) and glutathione *S*-transferase (GST) enzyme activities in cytosols from small intestine was typically found to be between 30% and 70% lower in samples prepared from *Nrf2* mutant mice fed a control diet than in equivalent samples from *Nrf2*(+/+) mice. Most of the chemopreventive agents included in this study induced NQO and GST enzyme activities in the small intestine of *Nrf2*(+/+) mice. Increases of between 2.7- and 6.2-fold were observed in wild-type animals fed diets supplemented with BHA or EQ; increases of about 2-fold were observed with a mixture of cafestol and kahweol palmitate, CMRN, or α-angelicalactone; and increases of 1.5-fold were measured with sulforaphane. Immunoblotting confirmed that in the small intestine, the constitutive level of NQO1 is lower in the *Nrf2*(−/−) mouse, and it also showed that induction of the oxidoreductase was substantially diminished in the mutant mouse. Immunoblotting class-α and class-μ GST showed that constitutive expression of most transferase subunits is also reduced in the small intestine of *Nrf2* mutant mice. Significantly, induction of class-α and class-μ GST by EQ, BHA, or CMRN is apparent in the gene knockout animal. No consistent change in the constitutive levels of the catalytic heavy subunit of γ-glutamylcysteinyl synthetase (GCS_h) was observed in the small intestine of *Nrf2*(−/−) mice. However, although the expression of GCS_h was found to be increased dramatically in the small intestine of *Nrf2*(+/+) mice by dietary BHA or EQ, this induction was essentially abolished in the knockout mice. It is apparent that Nrf2 influences both constitutive and inducible expression of intestinal antioxidant and detoxication proteins in a gene-specific fashion. Immunohistochemistry revealed that induction of NQO1, class-α GST, and GCS_h occurs primarily in epithelial cells of the small intestine. This suggests that the variation in inducibility of NQO1, Gsta1/2, and GCS_h in the mutant mouse is not attributable to the expression of the enzymes in distinct cell types but rather to differences in the dependency of these genes on Nrf2 for induction.

INTRODUCTION

Epidemiological studies have shown that regular dietary consumption of fruit and vegetables is associated with a reduced incidence of many forms of neoplastic disease, particularly those of the GI[3] tract (1). In humans, short-term dietary intervention with cruciferous vegetables influences drug metabolism and decreases oxidative damage to DNA (2, 3). Administration of vegetable extracts to rodents also alters drug metabolism and protects against DNA damage (4). Such extracts have been found to inhibit chemical and spontaneous carcinogenesis in laboratory animals (4, 5). The ability of edible plants to confer resistance against cancer is therefore attributed, at least in part, to the stimulation of cytoprotective mechanisms in the host.

Phytochemicals that may be responsible for the ability of plants to inhibit cancer include CMRNs, indoles, isothiocyanates, lactones, and terpenes (6). Many of these compounds not only enhance NQO and GST detoxification activities but also increase GSH levels in the liver and GI tract of experimental animals (7). The ability of phytochemicals to induce GST is not unique and is shared by synthetic chemopreventive agents such as the antioxidants BHA and EQ and the antischistosomal drug OPZ (8).

Cancer can be prevented at different stages during its development. Chemicals that inhibit initiation of cancer are designated blocking agents, whereas those compounds that inhibit the later promotion and progression stages of the disease are called suppressing agents (7). Evidence suggests that many blocking agents prevent mutagenesis, and that this is achieved by affecting transcriptional activation of antioxidant and detoxication genes through the *cis*-acting ARE found in their promoters (9, 10). The ARE enhancer was first identified in the 5′-flanking region of the rat *GSTA2* gene (11). It is also present in the flanking region of the mouse *Gsta1* gene, although in this instance, the enhancer was at first called an electrophile responsive element (12). Subsequently, AREs have also been described in promoters of genes encoding rat and human NQO1 (13, 14), human GCS_h (15), and mouse heme oxygenase 1 (16).

The consensus sequence of the ARE in the rat *GSTA2* promoter was initially defined by mutational analysis as 5′-TGACNNNGC-3′ (11). Recent work has defined the consensus of the ARE, or electrophile responsive element, from mouse *Gsta1* as 5′-$^{A}/_{G}$TGA$^{C}/_{T}$NNNGC$^{A}/_{G}$-3′ (17). Most significantly, this element shares similarity with the NF-

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[3] The abbreviations used are: GI, gastrointestinal; NQO, NAD(P)H:quinone oxidoreductase (also referred to as DT-diaphorase or NAD(P)H:menadione oxidoreductase in the literature); GST, glutathione *S*-transferase; BHA, butylated hydroxyanisole; EQ, ethoxyquin; OPZ, oltipraz; ARE, antioxidant responsive element; GCS_h, the heavy catalytic subunit of γ-glutamylcysteine synthetase; bZIP, basic leucine zipper; CNC, cap 'n' collar; KO, knockout; GCS_l, the light regulatory subunit of γ-glutamylcysteine synthetase; GS, glutathione synthase; I3C, indole-3-carbinol; C+K, cafestol and kahweol palmitate; SUL, sulforaphane; CMRN, coumarin; 3-OH CMRN; 3-hydroxy coumarin; 7-OH CMRN, 7-hydroxy coumarin; LMTN, limettin; AGLN, α-angelicalactone; DCPIP, dichlorophenol indophenol; CDNB, 1-chloro-2,4-dinitrobenzene; CYP, cytochrome P450; t-BHQ, tert-butylhydroquinone; BHT, butylated hydroxytoluene; NF, nuclear factor; Nrf, nuclear factor E2 p45-related factor; GSH, reduced glutathione.

erythroid 2 binding site 5′-$^{A}/_{G}$TGA$^{C}/_{G}$TCAGC$^{A}/_{G}$-3′ (shown in reverse orientation of the original sequence in Ref. 18) and the v-Maf (viral oncoprotein associated with musculoaponeurotic fibrosarcoma) recognition sequence 5′-TGCTGACTCAGCA-3′, otherwise called the 12-*O*-tetradecanoylphorbol-13-acetate-responsive element-Maf recognition element (T-MARE) (19).

Recognition that the ARE resembles an extended activator protein 1 binding site originally led to speculation that the bZIP proteins Jun and Fos might be involved in gene induction through this enhancer. Although this does not appear to be the case, other related transcription factors have been implicated in the process. In particular, the CNC bZIP proteins Nrf1 and Nrf2 have been shown to activate, in a dose-dependent fashion, an ARE-driven gene reporter construct when transfected into human HepG2 cells (20). These transcription factors probably bind to the ARE as heterodimers with small Maf proteins (21–23).

The most persuasive evidence that Nrf1 and Nrf2 mediate regulation of ARE-driven genes comes from mouse gene KO experiments. Several laboratories have generated murine lines possessing a targeted disruption of the genes for these factors (21, 24, 25). Examination of either embryonic fibroblast cells, in the case of *Nrf1*(−/−), or macrophages from mature animals, in the case of *Nrf2*(−/−), suggests that both transcription factors are involved in regulation of basal expression of antioxidant enzymes such as GCS_l, GS, heme oxygenase 1, and peroxiredoxin MSP23 (26, 27). The original studies by Itoh *et al.* (21) of adult *Nrf2*(−/−) mice have provided evidence that this factor also mediates induction by BHA of hepatic and intestinal NQO and GST enzymes. More recent characterization of *Nrf2*(−/−) mice has provided evidence that constitutive hepatic expression of certain α-class and μ-class GST subunits is diminished in these animals (28).

Much remains to be learned about the mechanisms that regulate detoxification and antioxidant proteins. The relative importance of Nrf1 and Nrf2 in control of basal and inducible gene expression is unclear. It is not known whether functional redundancy exists within the CNC bZIP family. Furthermore, it is unclear whether all members of the ARE gene battery are equally dependent on Nrf2 for either basal or inducible expression.

In the present study, the tissue-specific expression of Nrf1 and Nrf2 in the mouse has been examined. The data obtained suggest that the small intestine and stomach are organs where regulation of ARE-driven genes may be particularly dependent on Nrf2. In the small intestine, regulation of constitutive and inducible expression of detoxication and antioxidant enzymes by phytochemicals has been investigated using *Nrf2*(−/−) and *Nrf2*(+/+) mouse lines. NQO and GST enzyme activities have been measured in these animals to determine what impact loss of the bZIP factor has on intestinal detoxification capacity. The levels of NQO, GST, GCS_h, GCS_l, and GS proteins have also been determined in wild-type and KO mice fed either a normal diet or one supplemented with chemopreventive agent to establish whether all of the proteins are regulated in concert. Induction of these enzymes by a range of chemicals that protect against carcinogenesis has been examined to establish whether phytochemicals and synthetic antioxidants are equally effective at inducing the ARE gene battery.

MATERIALS AND METHODS

Reagents. The chemicals used were all of the highest quality grade available and, unless otherwise stated, were obtained from sources reported previously (8). The coffee-specific diterpenes C+K (a 1:1 mixture) were provided as their palmitate esters by Nestlé Research Center (Lausanne, Switzerland). Sulforaphane was purchased from LKT Laboratories (St. Paul, MN), 3-OH CMRN was from Apin Fine Chemicals (Abingdon, United Kingdom), 7-OH CMRN was obtained from Sigma Chemical Co. (Poole, United Kingdom), and both LMTN and AGLN were from Aldrich (Gillingham, United Kingdom).

Complete EDTA-free tablets of protease inhibitors were obtained from Roche Diagnostics Ltd. (Lewes, United Kingdom).

Northern Blotting. These experiments used a mouse multiple tissue Northern blot produced by OriGene Technologies Inc. (Rockville, MD) that was supplied by Cambridge Bioscience (Cambridge, United Kingdom). The tissues studied were from Swiss Webster mice between 9 and 10 months of age, and each lane of the pre-size-fractionated blot contained 2 μg of polyadenylated RNA. It was probed with random primed ^{32}P-labeled cDNAs for mouse Nrf1 (29) and Nrf2 (30) comprising the entire protein-coding regions. Washing the blot was accomplished in two stages: five 10-min washes at 20°C in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) containing 0.05% SDS were followed by two 20-min washes at 50°C in 0.1× SSC containing 0.1% SDS. Hybridizing bands were visualized by autoradiography.

Animals. Throughout this study, mice were treated as advised by regulations contained in the Animals and Scientific Procedure Act (1986) of the United Kingdom. Homozygous *Nrf2* KO and *Nrf2*(+/+) mice that were investigated in the present study have been described previously (21). Briefly, mice with disrupted *Nrf2* were generated by replacing exon 5 of the gene, which encodes the 280-amino acid COOH-terminal portion of the transcription factor, with two foreign genes: (*a*) one was a recombinant gene comprising the SV40 nuclear localization signal fused to β-galactosidase; and (*b*) the other was a neomycin resistance gene (21). The animals were genotyped by Southern blotting using full-length cDNA for murine Nrf2 (30) to probe genomic DNA, obtained from tail tips, which had been digested with *Acc*I and *Hpa*I restriction enzymes.

Male mice of approximately 10 weeks of age were used throughout this study. Both *Nrf2*(−/−) and *Nrf2*(+/+) mice were housed at a temperature range of 19°C –23°C under 12-h light/dark cycles. They were given free access to food and water. The xenobiotics were provided as dietary supplements for 14 days. Each treatment was administered to a group of three animals. Control diet comprised RM1 laboratory animal feed (SDS Ltd., Witham, United Kingdom). Treated mice were provided with RM1 diet that was supplemented as follows: BHA, 0.5% (w/w); EQ, 0.5% or 0.25% (w/w); OPZ, 0.075% (w/w); I3C, 0.5% (w/w); C+K, 0.025% (w/w); SUL, 3 μmol/gram; CMRN, 0.5% or 0.25% (w/w); 3-OH CMRN, 0.25% (w/w); 7-OH CMRN, 0.25% (w/w); LMTN, 0.25% (w/w); and AGLN, 0.25% (w/w). The relatively small number of mice available at any given time meant that the feeding studies were divided into four experimental groups. Animals in these experimental groups were given the following diets: (*a*) experiment 1, control and BHA; (*b*) experiment 2, control, EQ, OPZ, and I3C; (*c*) experiment 3, control, C+K, and SUL; and (*d*) experiment 4, control, CMRN, 3-OH CMRN, 7-OH CMRN, LMTN, and AGLN.

The health of the animals was monitored daily by measuring body weight. In compliance with United Kingdom regulations, dietary treatments that resulted in more than 20% loss of animal body weight were stopped. In such circumstances [0.5% (w/w) EQ and 0.5% (w/w) CMRN], the treatment was repeated on an additional group of mice using diet supplemented with half the dose of xenobiotic that had been used previously. Thus, in experiments 2 and 4, EQ and CMRN were both provided at a dose of 0.25% (w/w).

Once the 14-day feeding period was complete, animals were sacrificed by exposure to a rising concentration of CO_2 followed by cervical dislocation. The small intestine was removed and washed thoroughly with ice-cold PBS to remove fecal material before it was snap-frozen in liquid N_2 and stored at −70°C. All samples were analyzed within 4 weeks of storage.

Preparation of Intestinal Cytosol and Biochemical Analyses. Immediately on removal from the −70°C freezer, the small intestine from each mouse was pulverized separately using a pestle and mortar under liquid N_2. The resulting finely ground material from each sample was resuspended individually in an ice-cold 4-ml aliquot of 50 mM HEPES (pH 7.4) containing 150 mM NaCl, 1 mM DTT, and 0.05% (v/v) Tween. In all cases, this buffer was fortified with Complete EDTA-free protease inhibitor at the dose recommended by the manufacturer (1 tablet/50 ml buffer). The pulverized intestinal material was placed on ice and homogenized mechanically by three separate 30-s pulses using an Omni homogenizer. The resulting extracts were finally centrifuged (100,000 × *g*, 60 min, 4°C). The 100,000 × *g* supernatants (cytosols) were collected and retained for analyses.

Protein concentration was determined by the Bradford assay method and calibrated using BSA, as described previously (8).

NQO enzyme activity was estimated by calculating the dicoumarol-inhibitable fraction of DCPIP reductase activity (31). This was measured at 25°C in 25 mM Tris-HCl buffer (pH 7.4) containing 0.2 gram/liter BSA with 10 μM DCPIP as substrate and 100 μM NADH and 50 μM FAD as cofactors. The reaction was monitored at 600 nm and performed in both the presence and absence of 10 μM dicoumarol. The portion of total DCPIP reductase activity inhibited by dicoumarol represents that fraction contributed by NQO.

GST activity was measured using 1 mM CDNB and 1 mM GSH at 37°C in 200 mM sodium phosphate buffer (pH 6.5) (8). In all CDNB-GSH conjugating reactions, the spontaneous nonenzymatic rate was subtracted from the total observed rate.

Western Blotting. This was performed on cytosolic fractions prepared from small intestine to estimate the levels of NQO; α-class, μ-class, and π-class GST; GCS_h; GCS_l; and GS protein in samples from the mouse lines. Electrophoresis was carried out in a Bio-Rad Mini-Protean II Cell apparatus (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom) using a discontinuous buffer system. Electroblotting was performed in a Bio-Rad Mini *Trans*-Blot Cell. Aliquots (about 3 μg of protein) of intestinal samples were routinely examined by SDS-PAGE and stained with Coomassie R250 for protein immediately before immunoblotting to ensure equal loading of samples and absence of proteolysis. Details of the method of transfer of protein onto Immobilon P membrane, blocking the free protein-binding sites on the membrane, antibody incubations, and membrane washing steps have been described previously (8). The sources of the various primary antibodies used have been described elsewhere (8, 28, 32). Antibody against rat lactate dehydrogenase was used as a loading control. Cross-reacting bands were visualized by enhanced chemiluminescence and autoradiography. Blots were scanned using the UMAX Astra 1220S scanner, and the resulting images were analyzed densitometrically using Molecular Analyst software.

Immunohistochemistry. This was carried out using formalin-fixed paraffin-embedded tissue. The primary antibodies used to probe intestinal tissue sections were those used for Western blot analysis. For immunohistochemistry, the antibodies were used at a dilution of 1:50. Immunochemically cross-reacting protein was visualized using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) that employs a biotinylated second antibody complexed with horseradish peroxidase and a diaminobenzidine-based stain.

Statistical Analyses. This was determined using a two-tailed Student *t* test.

RESULTS

Tissue-specific Expression of Nrf1 and Nrf2. Northern blotting experiments for Nrf1 and Nrf2 were conducted to assess whether the two factors are coexpressed. In particular, we wished to establish whether in *Nrf1*(−/−) and *Nrf2*(−/−) mice there are particular tissues in which it is improbable that the remaining Nrf1 or Nrf2 protein can compensate for lack of the other.

Probing a mouse multiple tissue Northern blot with cDNA for Nrf1 yielded a major band of approximately 5.8 kb (Fig. 1). Additional minor hybridizing bands of about 3.5, 2.5, and 1.5 kb were also detected, but previous work indicates that the principal 5.8-kb band represents the authentic full-length Nrf1 transcript (24). The greatest levels of the 5.8-kb band were present in brain, heart, kidney, liver, skin, and testis. Substantially smaller amounts of Nrf1 mRNA were observed in small intestine, stomach, and thymus, and relatively low levels of the message were detected in lung, skeletal muscle, and spleen. The same Northern blot was probed with an Nrf2 cDNA, and in all of the mouse tissues examined, a single band of approximately 2.8 kb was observed (Fig. 1). This was considered to be authentic full-length Nrf2 mRNA because it is closely similar in size to that estimated previously (25). The greatest levels of this band were observed in kidney, liver, small intestine, stomach, and testis, with moderate amounts in heart, skin, spleen, and thymus and relatively low amounts in brain, lung, and skeletal muscle.

It is evident from blotting that Nrf1 and Nrf2 are not coordinately

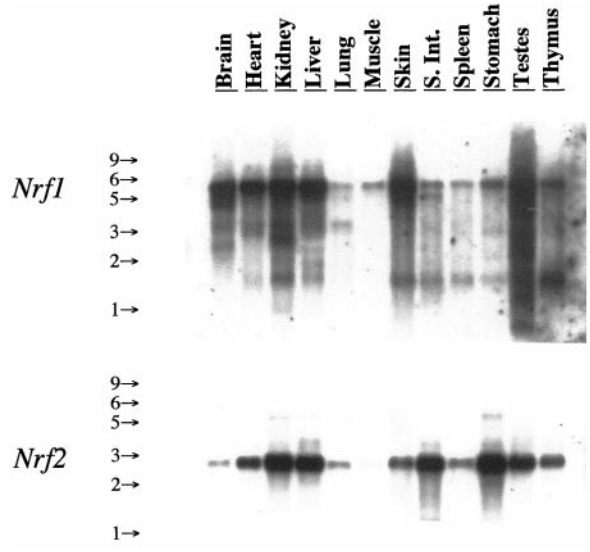

Fig. 1. Expression of Nrf1 and Nrf2 in mouse tissue. The membrane containing immobilized, prefractionated, murine multitissue RNA was probed with Nrf1 and Nrf2 cDNAs as described in the text. The positions of size markers are shown on the immediate *left* of the figure, and the identity of the cDNA used to probe the membrane for each of the Northern blots is indicated on the *far left*. The tissues from which polyadenylated RNA samples were obtained are shown at the *top*.

regulated. Together, these two bZIP factors have the potential to modify antioxidant and/or detoxification capacity in many mouse organs. Certain tissues appear to express predominantly either Nrf1 or Nrf2 (brain, skin, small intestine, and stomach), whereas other tissues express substantial amounts of both Nrf1 and Nrf2 (heart, kidney, liver, and testis). Only modest amounts of mRNA for both bZIP proteins were detected in thymus. Lastly, lung and spleen appeared to express little Nrf1 and Nrf2.

Brain and skin possess relatively high levels of Nrf1 but low levels of Nrf2. Conversely, small intestine and stomach possess relatively low levels of Nrf1 but high levels of Nrf2. These data suggest that Nrf1 may mediate adaptation to chemical and oxidative stress in brain and skin, whereas Nrf2 may mediate this response in small intestine and stomach. In tissues such as heart, kidney, liver, skin, and testis, it is possible that functional redundancy exists between these two transcription factors.

In the context of chemoprevention, the high level of Nrf2 mRNA in the small intestine and stomach suggests that this transcription factor is of particular importance in affecting protection against carcinogenesis in the GI tract of the mouse.

Impaired Tolerance of Synthetic Antioxidants and CMRN by the Nrf2(−/−) Mouse. To test whether phytochemicals modulate gene expression in a Nrf2-dependent fashion, mutant and wild-type mice were placed on diets fortified with various naturally occurring xenobiotics. The supplements included I3C and SUL, which are formed as breakdown products of glucosinolates from cruciferous vegetables, C+K, which are present in coffee beans, CMRN found in leguminous plants, and AGLN found in angelica plants. As positive controls, the synthetic antioxidants BHA and EQ, as well as the schistosomicidal drug OPZ, were incorporated into the study. To identify the metabolite(s) of CMRN responsible for increasing the expression of detoxication enzymes, the benzopyrone compounds 3-OH CMRN, 7-OH CMRN, and LMTN were also included in this study.

In the initial investigations, the inducing agents of interest were added to the RM1 diet at doses that had been used previously in mouse feeding experiments. As far as could be assessed, the

Nrf2(−/−) mice did not refuse to eat the food provided. However, they failed to thrive on certain of these diets despite consuming approximately the same amount of food as the wild-type mice. For example, after 6 days of feeding on a diet containing 0.5% (w/w) EQ, the *Nrf2* gene KO mice were found to have lost >20% body weight, whereas over an identical period, the wild-type mice lost on average only 9% body weight. Two of the four *Nrf2* KO mice on the 0.5% (w/w) EQ diet had to be sacrificed prematurely due to excessive weight loss. Although autopsy suggested excessive biliary secretion in the GI tract of these animals or impairment of readsorption of biliary secretion into the GI tract, the cause of death was not established. After 6 days, blood samples were collected from all of the mice given 0.5% (w/w) EQ, and serum alanine aminotransferase, lactate dehydrogenase, urea, and creatinine were measured. Although one *Nrf2* KO mouse had high serum levels of all these analytes, no significant difference was noted between the other *Nrf2*(−/−) and *Nrf2*(+/+) mice in their serum chemistry measurements. Thus, the reason for the toxic effects of EQ was not established. After administration of 0.5% (w/w) EQ, a separate feeding study with mice on 0.25% (w/w) EQ was undertaken. This reduced dose of EQ resulted in a loss of 13% body weight in *Nrf2* KO mice over 13 days compared with a gain of 1.1% body weight in wild-type mice over the same period.

In common with EQ, the phenolic antioxidant BHA was also not well tolerated by the homozygous *Nrf2* KO mouse. Dietary administration of 0.5% (w/w) BHA for 13 days resulted in >20% loss of body weight in *Nrf2*(−/−) mice compared with 1.5% gain of body weight in *Nrf2*(+/+) mice. By contrast, *Nrf2*(−/−) mice on 0.075% (w/w) OPZ for 13 days lost 4.3% body weight, and wild-type mice placed on the same diet for the same time gained 3.1% body weight.

Among the phytochemicals studied as inducing agents, the only problems encountered were with CMRN. After 11 days of dietary administration of this phytochemical at a dose of 0.5% (w/w), the *Nrf2* KO mice lost 21% body weight, whereas wild-type mice lost 6% body weight. The feeding schedule was stopped, and the experiment recommenced with 0.25% (w/w) CMRN. On this occasion, over a 13-day period, the *Nrf2*(−/−) mice lost 6.1% body weight, whereas the wild-type mice gained 7.2% body weight. The animals placed on diets supplemented with I3C, C+K, SUL, 3-OH CMRN, 7-OH CMRN, LMTN, and AGLN (at the doses described above) all gained weight over the 13-day feeding period.

Nrf2 Attenuates Both Constitutive and Inducible NQO and GST Enzyme Activities. NQO and GST enzyme activities were measured in the mouse intestinal cytosolic extracts from the *Nrf2*(+/+) and *Nrf2*(−/−) mice fed the various diets (Table 1). To generate sufficient numbers of mice for study and to allow the doses of EQ and CMRN to be changed, four independent feeding experiments were carried out. The enzyme activities measured in each of the four separate control groups gave reasonably consistent results, despite the fact that the experiments were conducted over a period of 6 months. Table 1 shows that both the NQO and the GST activities in mouse small intestine were significantly higher ($P < 0.01$) in the *Nrf2*(+/+) mice fed a control diet than in the *Nrf2* mutant mice provided with the same food. The constitutive NQO activity toward DCPIP in *Nrf2*(−/−) mice fed a control diet was found to be only about 45% of that in *Nrf2*(+/+) mice. Constitutive GST activity toward CDNB was also significantly higher in *Nrf2*(+/+) mice than in the KO mice, with a $P < 0.01$. However, in this latter case, the reduction in enzyme levels was less marked, with the transferase activity in *Nrf2*(−/−) mice being approximately 80% of that in *Nrf2*(+/+) mice.

As indicated above, a range of xenobiotics were tested as inducers of intestinal NQO and GST activities. Among the compounds included in this study, the quinoline EQ effected the greatest increase in activity in *Nrf2*(+/+) mice, with increases of 6.2- and 4.6-fold observed in NQO and GST activities, respectively. In agreement with work published previously, dietary administration of the phenolic antioxidant BHA increased NQO and GST activity 4.8- and 2.7-fold, respectively, in murine small intestine. In addition to the effects of synthetic antioxidants, feeding *Nrf2*(+/+) mice diets containing CMRN, AGLN, C+K, I3C, or SUL also caused a significant elevation in the oxidoreductase and transferase activities in this tissue. Among the phytochemicals studied, AGLN and CMRN proved to be the most effective inducing agents, effecting 3.1- and 2.5-fold increases in NQO activity and 2.2- and 1.8-fold increases in GST activity. It was noted that most of these xenobiotics produced a greater elevation of NQO activity than GST activity. This difference in inducibility is attributed to the fact that the coumarol-inhibited DCPIP reductase activity is specific for NQO1, whereas CDNB-GSH conjugating activity monitors a number of GST isoenzymes, some of which are not regulated by phytochemicals.

Table 1 *NQO and GST activities in the cytosolic fraction of mouse small intestine*

Diet	NQO activity[a]		GST activity[a]	
	Nrf2(+/+)	*Nrf2(−/−)*	*Nrf2(+/+)*	*Nrf2(−/−)*
Control 1	156 ± 6 (100)	71 ± 7 (46)	43 ± 0.2 (100)	31 ± 0.2 (72)
BHA	742 ± 40 (475)[b]	93 ± 6 (59)[b]	116 ± 6.0 (270)[b]	43 ± 0.5 (100)[b]
Control 2	165 ± 4 (100)	74 ± 5 (45)	53 ± 0.8 (100)	36 ± 0.4 (68)
EQ	1026 ± 76 (622)[b]	86 ± 4 (52)[c]	245 ± 6.0 (462)[b]	50 ± 1.0 (94)[b]
OPZ	212 ± 9 (128)[b]	77 ± 6 (47)	39 ± 1.0 (74)	26 ± 0.5 (49)
I3C	238 ± 12 (170)[b]	89 ± 4 (54)[c]	71 ± 0.1 (134)[b]	31 ± 0.3 (58)
Control 3	164 ± 3 (100)	75 ± 1 (45)	32 ± 0.2 (100)	28 ± 0.1 (88)
C+K	293 ± 6 (179)[b]	66 ± 3 (40)	78 ± 0.6 (244)[b]	23 ± 0.1 (72)
SUL	237 ± 15 (145)[b]	57 ± 4 (35)	49 ± 0.4 (153)[b]	20 ± 0.3 (63)
Control 4	250 ± 20 (100)	141 ± 40 (56)	35 ± 0.3 (100)	29 ± 0.4 (85)
CMRN	617 ± 50 (247)[b]	132 ± 40 (53)	65 ± 0.5 (181)[b]	39 ± 0.2 (111)[b]
3-OH CMRN	328 ± 50 (131)	92 ± 30 (37)	41 ± 0.2 (117)[b]	29 ± 0.2 (85)
7-OH CMRN	310 ± 40 (124)	122 ± 30 (49)	37 ± 0.2 (106)	30 ± 0.7 (86)
LMTN	332 ± 30 (133)[c]	95 ± 40 (38)	52 ± 0.6 (153)[b]	28 ± 0.2 (80)
AGLN	770 ± 70 (308)[b]	109 ± 40 (44)	77 ± 0.4 (220)[b]	35 ± 0.4 (100)[b]

[a] Activity is expressed as nmol/min/mg for both NQO and GST. The values presented represent mean catalytic activities (±SE) from triplicate measurements of pooled intestinal cytosol from three separate animals. The numbers in parentheses represent the mean activity value as a percentage of the value obtained with *Nrf2(+/+)* on a control diet within the same experimental group.

[b] Significantly greater from control of same genotype, $P < 0.01$.

[c] Significantly greater from control of same genotype, $P < 0.05$.

The level of induction of NQO and GST was found to be diminished in *Nrf2*(−/−) mice when compared to that observed in *Nrf2*(+/+) mice. Nevertheless, induction was not abolished in the KO animal. If allowance is made for the lower constitutive intestinal NQO and GST activity in the KO mouse, it is apparent that several of the more potent inducing agents are effective in the *Nrf2* mutant mouse. For example, Table 1 shows that administration of BHA, EQ, I3C, CMRN, or AGLN to *Nrf2*(−/−) mice causes a significant increase in either NQO or GST activity or both.

Expression of NQO and GST Subunits Is Influenced by *Nrf2* Disruption in a Gene-specific Fashion. Immunoblotting was undertaken to establish whether the alterations in detoxication enzyme activities were accompanied by changes in the amount of protein. During this study, four separate groups of mice were placed on the RM1 control diet. It was found that in all of these, NQO1 protein was present in lower amounts in intestinal cytosol of *Nrf2* null mice than in cytosol from wild-type mice (Fig. 2). Densitometric analysis of the Western blots indicated that the constitutive amount of NQO1 in the *Nrf2* KO mice is typically about 30% of that in the *Nrf2*(+/+) mice (Table 2). These analyses also revealed that most of the dietary supplements cause an elevation in NQO1 protein in the small intestine of *Nrf2*(+/+) mice, with BHA and EQ eliciting the largest increases. Administration of the same diets to *Nrf2* null mice results in a relatively minor increase in NQO1 protein. When the amount of

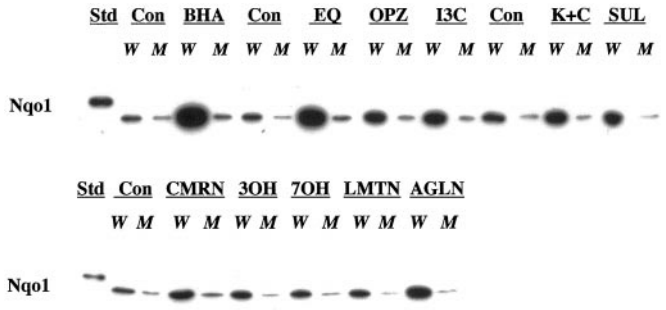

Fig. 2. Modulation of constitutive and inducible NQO1 protein levels in small intestine of wild-type and Nrf2 mutant mice fed on diets containing different xenobiotics. Both *Nrf2*(+/+) and *Nrf2*(−/−) mice were fed diets supplemented with synthetic chemopreventive agents or phytochemicals as described in "Materials and Methods." Portions (3.2 μg of protein) of cytosol from small intestine of wild-type and KO mice were subjected to Western blotting as described previously (8). Enhanced chemiluminescence was used to locate immunochemically cross-reacting polypeptides, and the results were visualized by autoradiography. Analysis of experiments 1–3 is shown in the *top panel*, whereas analysis of experiment 4 is shown in the *bottom panel*. The dietary supplements are indicated at the *top* of each membrane. In each case, recombinant rat NQO1 (with an NH_2-terminal polyhistidine tag) was included as a standard (*Std*) in *Lane 1* of the gel. The genotype of each group of mice [wild-type (*W*) and homozygous *Nrf2* mutant (*M*)] is shown *above* the appropriate lane. The results presented are typical of those obtained; they were repeated on at least three separate occasions. Closely similar patterns of cross-reacting proteins were produced on three separate occasions.

Table 2 *Quantitative analysis of NQO1 protein in intestinal cytosol from Nrf2(+/+) and Nrf2(−/−) mice given dietary supplements*

	Densitometric estimation of protein levels[a]	
Diet	*Nrf2(+/+)*	*Nrf2(−/−)*
Control 1	100	32.3 (100)
BHA	724	69.7 (216)
Control 2	100	19.6 (100)
EQ	434	54.2 (277)
OPZ	198	37.4 (191)
I3C	244	33.3 (170)
Control 3	100	27.4 (100)
C+K	152	41.3 (150)
SUL	115	10.0 (37)
Control 4	100	30.8 (100)
CMRN	208	63.2 (205)
3-OH CMRN	146	20.5 (67)
7-OH CMRN	127	21.9 (71)
LMTN	127	15.3 (50)
AGLN	255	18.8 (61)

[a] The level of NQO1 was calculated from densitometric scans of the Western blot autoradiography film. The results are typical of those from at least three separate analyses of intestinal cytosol combined from three mice. The data presented are calculated as a percentage of the value obtained from the appropriate group of *Nrf2(+/+)* mice on the RM1 control diet. Those results shown in parentheses are calculated as a percentage of the value obtained from *Nrf2(−/−)* mice fed on the control diet.

NQO1 in the small intestine of *Nrf2*(−/−) mice given BHA or EQ is compared with that in *Nrf2*(−/−) mice fed a control diet, it is apparent that the synthetic antioxidants induce the oxidoreductase about 2-fold in the KO animals. However, it is important to note that the intestinal level of NQO1 in BHA- or EQ-treated *Nrf2*(−/−) mice is lower than the normal constitutive intestinal level of NQO1 in Nrf2(+/+) mice.

Examination of class-α GST by immunoblotting revealed that the normal constitutive levels of Gsta1/2, Gsta3, and Gsta4 subunits in mouse small intestine are significantly lower in the KO mice than in *Nrf2*(+/+) mice (Fig. 3). Expression of the class-α polypeptides was increased in *Nrf2*(+/+) mice by all of the xenobiotics studied. BHA, EQ, I3C, C+K, SUL, and AGLN increased the various intestinal class-α transferases to roughly similar extents. By contrast, Gsta1/2 appeared to be induced preferentially by the benzopyrone-containing compounds. Specifically, it was observed that CMRN and its metabolites effected a greater increase in the level of Gsta1/2 than they caused in either Gsta3 or Gsta4. When allowance is made for decreased expression of class-α GST in *Nrf2*(−/−) mice on a control diet, it is apparent that the intestinal level of the Gsta1/2 subunit(s) is elevated by essentially all of the xenobiotics studied. In the *Nrf2*(−/−) mice, the Gsta3 subunit was induced in the small intestine by BHA, OPZ, and CMRN, and the Gsta4 subunit was induced by BHA, EQ, OPZ, I3C, and C+K.

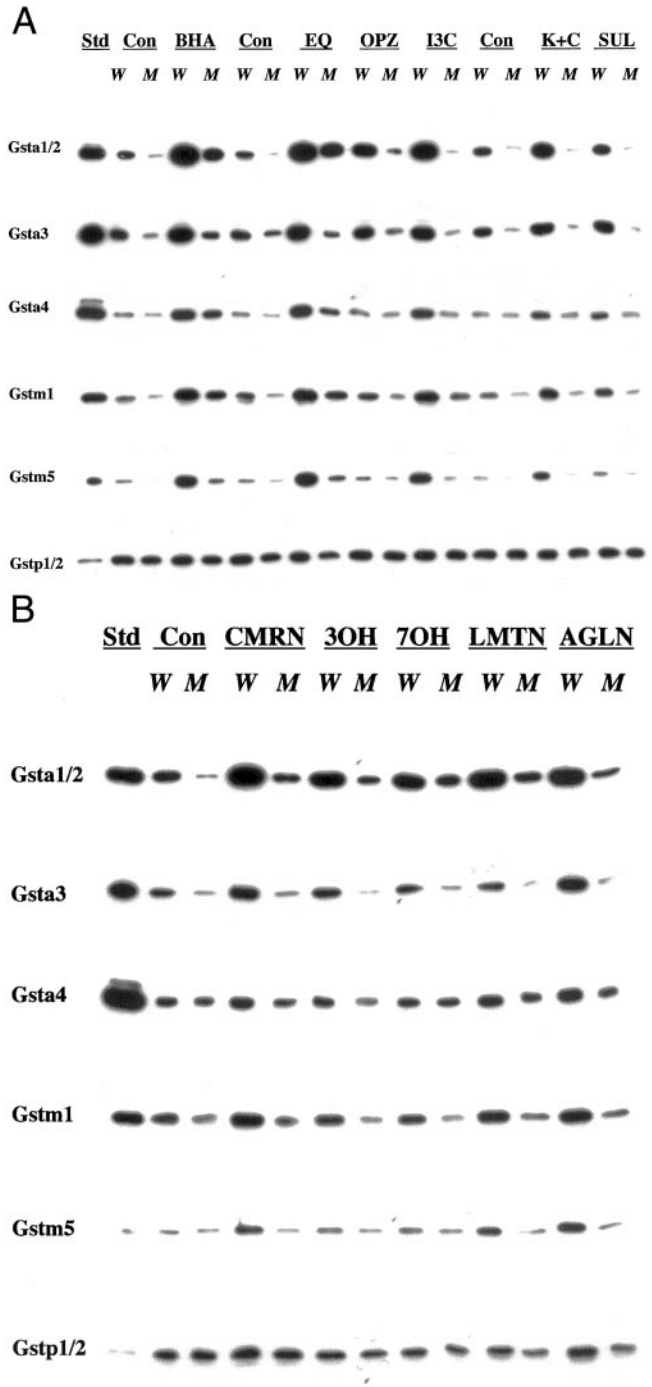

Fig. 3. Reduction of constitutive GST expression but not necessarily inducible GST expression occurs in the small intestine of Nrf2 KO mice. Wild-type (*W*) and *Nrf2* mutant (*M*) mice were fed various diets, as indicated in the text. Immunoblotting was performed using the polyclonal antibodies described elsewhere (28). The identity of the immunogens used to raise the antibodies is indicated to the *left*. Results from experiments 1–3 are presented in *A*, and results from experiment 4 are presented in *B*. An affinity-purified pool of GST was run in *Lane 1* of each analysis as a positive control (*Std*). The dietary supplements and the genotype of the mice are indicated at the *top* of each panel. The immunoblotting data are typical of those obtained and were repeated on at least three separate occasions.

Table 3 *Quantitative analysis of GST polypeptides in intestinal cytosol from Nrf2(+/+) and Nrf2(−/−) mice given dietary supplements*

	Densitometric estimation of protein levels[a]											
	Gsta1/2		Gsta3		Gsta4		Gstm1		Gstm5		Gstp1/2	
Diet	*Nrf2(+/+)*	*Nrf2(−/−)*	*Nrf2(+/+)*	*Nrf2(−/−)*	*Nrf2(+/+)*	*Nrf2(−/−)*	*Nrf2(+/+)*	*Nrf2(−/−)*	*Nrf2(+/+)*	*Nrf2(−/−)*	*Nrf2(+/+)*	*Nrf2(−/−)*
Control 1	100	24.7 (100)	100	9.3 (100)	100	50.5 (100)	100	30.8 (100)	100	13.0 (100)	100	82.0 (100)
BHA	475	201.0 (815)	320	42.7 (486)	402	234.0 (464)	317	177.0 (576)	648	169.0 (1300)	110	86.1 (105)
Control 2	100	24.9 (100)	100	28.2 (100)	100	43.8 (100)	100	25.9 (100)	100	29.7 (100)	100	73.4 (100)
EQ	294	152.0 (610)	293	15.7 (55)	446	201.0 (460)	212	166.0 (643)	645	25.2 (85)	90	61.9 (85)
OPZ	162	74.5 (300)	106	43.0 (153)	136	97.2 (223)	96	40.1 (155)	109	56.7 (191)	104	96.7 (132)
I3C	230	79.2 (320)	220	17.3 (61)	376	108.2 (247)	189	82.0 (317)	514	71.0 (239)	103	89.8 (122)
Control 3	100	47.9 (100)	100	24.0 (100)	100	77.8 (100)	100	43.1 (100)	100	21.4 (100)	100	90.0 (100)
C+K	220	56.5 (120)	266	5.5 (23)	221	181.2 (233)	204	45.5 (106)	374	13.1 (61)	92	100.0 (111)
SUL	139	22.5 (47)	177	13.0 (54)	191	84.2 (108)	140	31.5 (73)	91	17.2 (80)	91	72.3 (80)
Control 4	100	21.2 (100)	100	37.1 (100)	100	78.5 (100)	100	51.2 (100)	100	84.0 (100)	100	116.0 (100)
CMRN	269	75.8 (360)	184	56.4 (152)	139	82.4 (105)	222	55.3 (108)	364	88.2 (105)	161	144.0 (124)
3-OH CMRN	186	56.4 (266)	134	22.5 (61)	100	58.8 (76)	105	19.7 (38)	174	94.3 (112)	113	82.1 (71)
7-OH CMRN	157	88.8 (420)	95	32.2 (87)	96	89.5 (114)	86	16.3 (32)	187	148.5 (177)	92	76.3 (66)
LMTN	215	90.3 (426)	98	17.8 (48)	141	87.9 (112)	163	38.0 (74)	297	105.3 (125)	103	64.5 (56)
AGLN	208	54.3 (256)	198	26.7 (72)	161	78.5 (100)	205	39.3 (77)	410	106.7 (127)	155	78.5 (67)

[a] The levels of the class-α, class-μ and class-π transferase subunits were calculated from densitometric scans of the Western blot autoradiography film. Results are expressed as a percentage of the value obtained for the *Nrf2(+/+)* mice within each experimental group that were fed a control diet. Results in parentheses are expressed as a percentage of *Nrf2(−/−)* mice on a control diet.

Blotting of class-μ transferases demonstrated that the constitutive amount of the Gstm1 subunit in the small intestine of the *Nrf2* mutant was only 30–40% of that observed in the *Nrf2*(+/+) mouse (Fig. 3, Table 3). This transferase was induced by the majority of the xenobiotics studied, although it is noteworthy that neither OPZ nor the two CMRN metabolites (3-OH CMRN and 7-OH CMRN) increased intestinal Gstm1 levels. Administration of BHA, EQ, and I3C caused a large induction of Gstm1 in the KO mouse. In contrast, administration of CMRN, which increased intestinal Gstm1 in *Nrf2*(+/+) mice, did not serve as an inducer in *Nrf2*(−/−) mice. Western blotting for Gstm5 revealed significant variability in the levels of this polypeptide in mice fed a control diet. The reason for this is uncertain, but because Gstm5 is regarded as being a "testis-specific" transferase (33), it probably represents a relatively minor form in the small intestine. Nonetheless, it is apparent that the intestinal levels of this transferase can be increased by many of the xenobiotics studied.

The constitutive levels of class-π Gstp1/2 subunits were not altered substantially in the *Nrf2* mutant mouse. Furthermore, this class of transferase was not inducible in the small intestine. Dietary administration of CMRN and AGLN effected approximately 1.5-fold induction, but the other compounds studied did not influence the expression of class-π GST, at least not in male mice.

Regulation of GSH Biosynthetic Proteins in Nrf2(−/−) Mice. Immunoblotting for GCS_h, GCS_l, and GS was undertaken because these proteins are responsible for *in vivo* production of the endogenous antioxidant GSH. Under the conditions used, the regulatory GCS_l subunit was not detected in murine small intestine. However, both the catalytic GCS_h subunit and GS were readily detected. The constitutive levels of neither of these latter proteins appeared to differ when the two mouse lines were compared, although significant inter-individual variation was noted. Large levels of induction of GCS_h were observed in *Nrf2*(+/+) mice fed diets containing either BHA or EQ (Fig. 4). By contrast, I3C, C+K, CMRN, 7-OH CMRN, LMTN, and AGLN elicited modest induction of GCS_h in these mice. In *Nrf2* mutant mice, GCS_h proved to be essentially uninducible by these compounds. Modest induction of GS was observed in *Nrf2*(+/+) mice by BHA, EQ, C+K, and SUL, and this was not entirely abolished in the KO mice.

Cell-specific Expression of Antioxidant and Detoxication Enzymes in Mouse Small Intestine. Immunohistochemistry was performed to determine whether the variable effects of Nrf2 on the inducible levels of NQO, GST, and GCS might be due to the enzymes being expressed in different cell types of the GI tract. To answer this question, sections of formalin-fixed small intestine from *Nrf2*(+/+) and *Nrf2*(−/−) mice fed either a control diet or one containing BHA were probed with various antibodies. Immunostaining sections from mutant or wild-type mice given either of the two types of diet yielded similar results when probed with antisera against NQO1, Gsta1/2, or GCS_h. In all cases, immunoreactivity was largely restricted to the epithelial cell layer lining both the villi and crypts. The immunostain for NQO1 and Gsta1/2 appeared to be stronger in epithelial cells of the intestinal villi than in the crypts, but this difference was less obvious for GCS_h (Fig. 5). However, in all three cases, immunostaining of tissue from either mutant or wild-type mice was most intense on the apical membranes of epithelial cells (Fig. 5). Significantly, none of these enzymes appeared to be expressed in cells associated with the lamina propria of the intestinal villi. The pattern of staining was identical in animals fed a control diet and those administered a BHA-containing diet. These data indicate that the variable levels of induction of NQO1, Gsta1/2, and GCS_h in the *Nrf2*(−/−) mouse are not attributable to individual enzymes being expressed in different cell types. Furthermore, there is no evidence that the residual induction of Gsta1/2 in *Nrf2*(−/−) mice is due to the transferase being expressed in a different population of cells than in wild-type mice.

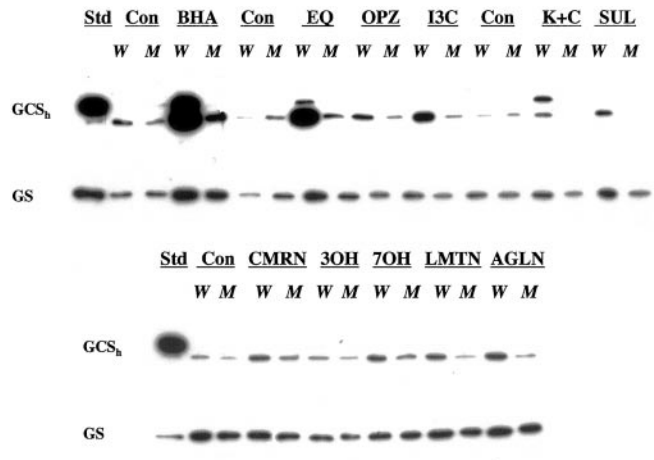

Fig. 4. Attenuation of inducible expression of GSH-biosynthetic enzymes in Nrf2 KO mice. Cytosolic extracts from the small intestine of *Nrf2*(+/+) and *Nrf2*(−/−) mice treated with dietary supplements were subjected to Western blotting as described in the legends to Figs. 2 and 3. The antibodies used against GCS_h and GS are described in Ref. 32.

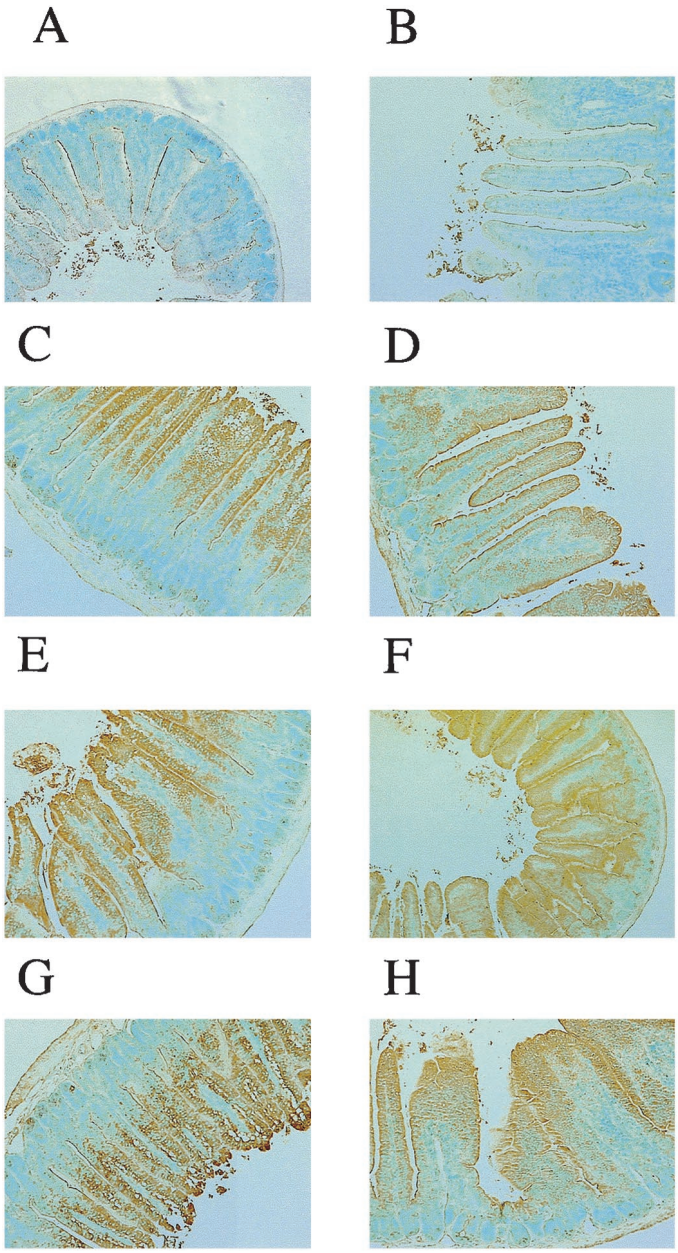

Fig. 5. Expression of antioxidant and detoxication enzymes in mouse small intestine is restricted to epithelial cells Formalin-fixed sections of small intestine from *Nrf2*(+/+) mice fed a diet containing BHA were probed with (*A*) preimmune serum, (*C*) anti-NQO1 serum, (*E*) anti-GCS_h serum, and (*G*) anti-Gsta1/2 serum. Similar intestinal sections from *Nrf2*(−/−) mice were also probed with (*B*) preimmune serum, (*D*) anti-NQO1 serum, (*F*) anti-GCS_h serum, and (*H*) anti-Gsta1/2 serum. Cross-reacting protein was visualized using secondary antibodies coupled to horseradish peroxidase with diaminobenzidine as substrate. The sections were counterstained with methyl green.

DISCUSSION

Phytochemicals as Chemopreventive Agents. Dietary choice can exert both a beneficial as well as a deleterious effect on the incidence of malignant disease (34). A diet containing too much fat, along with a lack of fiber, is associated with an increased likelihood of tumorigenesis (35). Consumption of food contaminated with mycotoxins (36) or cooked foods that contain heterocyclic amines and polycyclic aromatic hydrocarbons (37, 38) also contributes to the development of cancer. Set against these risk factors, diets enriched with particular types of fruit and vegetables protect against certain types of malignant disease (1, 34). This study is concerned with the mechanisms by which dietary cancer chemopreventive compounds induce cytoprotective enzymes in the GI tract.

Contribution of CNC bZip Transcription Factors to Regulation of Cytoprotective Enzymes. Many cancer chemopreventive blocking agents are either thiol-active chemicals or are metabolized to such compounds (9, 10, 39). Induction of antioxidant and detoxication enzymes by anticarcinogens appears to be a form of adaptation to metabolic stress (10). Evidence gathered by various laboratories suggests that Nrf1 and Nrf2 both mediate cellular adaptation to oxidative stress through regulation of ARE-driven gene expression (20, 21, 26, 27). Our Northern blotting experiments indicate that Nrf1 and Nrf2 are widely distributed in the mouse, but they are not coexpressed. Thus, the contribution of these two bZIP factors to adaptive responses to stress may vary in a tissue-specific fashion. Fig. 1 indicates that Nrf2 is possibly of particular importance in the GI tract because relatively little Nrf1 mRNA was detected in small intestine or stomach.

Homozygous *Nrf2* KO Mice Are Sensitive to Certain Xenobiotics. Evaluation of the role of Nrf2 in regulating enzyme induction affected by chemopreventive agents has been greatly facilitated through the availability of a gene KO mouse. In this investigation, mice were given free access to food and water, and their ability to tolerate the various diets was monitored by measurement of body weight. Dietary administration of BHA, EQ, and CMRN at a dose of 0.5% (w/w) to *Nrf2* KO mice caused substantially greater weight loss than was observed in wild-type animals. Previous work has shown that high doses of BHA or EQ can cause pathological changes in the forestomach and kidney of rodents (40, 41), and CMRN causes liver damage (42). Presumably the increased loss of weight in *Nrf2*(−/−) mice fed diets containing BHA, EQ, or CMRN is due to their reduced capacity to detoxify these xenobiotics. All three compounds are extensively metabolized *in vivo* by CYP isoenzymes. In rodents, BHA is *O*-demethylated to form t-BHQ (28), EQ is *O*-deethylated to 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline (43), and CMRN is hydroxylated at the 3, 5, 7, and 8 positions (42). Once formed, these metabolites are conjugated with glucuronic acid, GSH, or sulfate before elimination from the body. The metabolites of BHA, EQ, and CMRN responsible for their toxicity in the KO mouse have not been identified. However, it is likely that reduced expression or failure to induce NQO and the enzymes that catalyze drug conjugation reactions results in the mutant mouse being more sensitive to potentially harmful chemicals. This hypothesis is supported by the observation that overexpression of GST and GCS in tumor cell lines is associated with resistance to cancer chemotherapeutic drugs (44).

In view of the fact that BHA, EQ, and CMRN are CYP substrates, it is also possible that their biotransformation produces reactive oxygen species as by-products of the reaction (45). Generation of such species may be responsible for the sensitivity of *Nrf2* KO mice to these chemopreventive agents. A decrease in the ability of *Nrf2*(−/−) mice to synthesize GSH would increase their sensitivity to reactive oxygen species arising from CYP-catalyzed reactions. The *Nrf2*(−/−) mice are sensitive to pulmonary toxicity caused by BHT (46). The data on BHT, together with our results using BHA and EQ, suggest that *Nrf2* mutant mice readily suffer tissue damage when given synthetic aromatic antioxidants.

Nrf2 Regulates Constitutive Expression of NQO and Certain GST Subunits in Small Intestine. Significant differences exist between the NQO activity in the small intestine of the two mouse lines fed a control diet. Under constitutive conditions, the amount of NQO1 protein in intestinal cytosol of homozygous gene KO mice is only about 30% of that in the wild-type (Table 2). Furthermore, GST activity in the same samples was also lower in the small intestine of *Nrf2* KO mice than in the wild-type mice. Immunoblotting with

antibodies against various GST subunits demonstrated that the reduced expression of the transferases in the *Nrf2* null mice was not uniform across the superfamily (Table 3). Like NQO1, the expression of Gsta1/2 and Gsta3 polypeptides was reduced in *Nrf2* mutant mice to about 30% of the level found in wild-type mice. The amount of Gstm1 protein was also lower in *Nrf2* KO mice than in wild-type mice, but in this case, the reduction to about 50% of the amount observed in the wild-type is not as pronounced as was observed for NQO1. Western blots showed that expression of both Gsta4 and Gstm5 was lower in *Nrf2*(−/−) mice, but interindividual differences were observed, making interpretation of the data difficult. Lastly, the level of class-π GST was not altered in the small intestine of mutant male mice.

Nrf2 Contributes to Induction of NQO1 and GCS_h, but not GST Subunits, in Small Intestine. Induction of NQO1 by synthetic antioxidants and phytochemicals was substantially impaired in *Nrf2* KO mice. When allowance is made for the lower constitutive level of NQO1 in *Nrf2*(−/−) mice, it is apparent that a small increase in the amount of the oxidoreductase can occur in the mutant. However, the maximum amount of NQO1 observed in the KO mouse treated with chemopreventive agents was not sufficient to cause the level of the oxidoreductase to exceed that in wild-type mice on a control diet. It is concluded that Nrf2, either directly or indirectly, normally mediates the major portion of inducible expression of NQO1 in the small intestine.

The contribution of Nrf2 to inducible gene expression by synthetic antioxidants is not limited to NQO1 but was also found with the GCS_h subunit. In the small intestine of wild-type mice, GCS_h proved to be markedly increased by BHA and EQ, whereas in the KO animal, the responsiveness of the subunit was largely abolished.

By contrast with NQO1 and GCS_h, induction of Gsta1/2 by xenobiotics was not diminished in *Nrf2*(−/−) mice. Diets containing BHA, EQ, or CMRN achieved the greatest increase in intestinal Gsta1/2 in wild-type mice. In *Nrf2* mutant mice, administration of these xenobiotics caused an equally substantial increase in Gsta1/2. The levels of Gsta4 and Gstm1 were also increased markedly in the KO mice by most of the xenobiotics that served as inducers in the wild-type mice. Thus, Nrf2 does not contribute substantially to the increased expression of Gsta1/2, Gsta4, or Gstm1 in the small intestine by BHA, EQ, OPZ, CMRN, or AGLN.

Molecular Basis for Influence of Nrf2 on Gene Expression. The ARE was originally identified as a unique enhancer because of its responsiveness to the BHA metabolite t-BHQ (47), a feature that distinguishes it from other enhancers such as the xenobiotic responsive element and 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (48). Mutation analysis of the ARE has shown that *in vitro* it controls both basal and inducible gene expression (11). Our demonstration that the levels of NQO1 and Gsta1/2 are reduced in the mutant mouse fed a control diet is consistent with Nrf2 mediating regulation of their basal expression in an ARE-dependent fashion. In the KO mice, the induction of NQO1 by BHA was largely abolished, a finding that is again consistent with Nrf2 playing either a direct or indirect role in drug-induced transcriptional activation of ARE-driven genes. However, induction of Gsta1/2 by BHA was not abolished in *Nrf2*(−/−) mice (Table 3). It is possible that the *Gsta1/2* genes contain enhancers other than the ARE that respond to BHA, but there is no evidence in the literature that this is in fact the case. Indeed, the 5′-flanking region of *Gsta1* contains a functional ARE (12, 17). Should the transcriptional activation of *Gsta1* by BHA occur through this enhancer, it is presumably mediated by a bZIP transcription factor other than Nrf2. Members of the CNC bZIP family, NF-erythroid 2-p45, Nrf1, Nrf3, Bach1, and Bach2 (28) are obvious candidates.

The fact that the metabolite of BHA, t-BHQ, can generate free radicals by redox cycling might implicate NF-κB in the induction of Gsta1/2 in *Nrf2*(−/−) mice. The two other xenobiotics that served as good inducers of Gsta1/2 are EQ and CMRN, and although their metabolites cannot redox-cycle, they can cause oxidative stress by depleting GSH. A case can therefore also be made that these inducers will activate NF-κB. The possibility that *Gsta1* responds to this additional transcription factor warrants further investigation.

It is perplexing that whereas induction of NQO1 by BHA is significantly impaired in *Nrf2*(−/−) mice, Gsta1/2 remains inducible by BHA in the mutant animals. This finding could be used to support the hypothesis that induction of GST in KO mice does not involve the ARE, but this conclusion is not necessarily correct. The difference in Nrf2 dependence for induction of these proteins by BHA might reflect differences in the sequence context of the AREs in the *NQO1* and *Gsta1* promoters. It must be pointed out that in the case of the mouse *NQO1* gene, the regulatory region has not been thoroughly characterized (49). However, the 5′-flanking regions of both the rat and human *NQO1* genes contain AREs (13, 14). In both instances, the enhancers have been found to lie downstream from an inverted repeat (5′-AGTCAC-3′) that allows the formation of a 13-bp palindrome (5′-AGTCACAGTGACT-3′), of which the 6 bp in the 3′ half encompass the ARE (13, 14). This type of motif represents a high-affinity binding site for Nrf2 (22). It is therefore reasonable to assume that the function of the palindromic-type ARE is likely to be highly dependent on Nrf2. Although it has yet to be established, it is likely that the mouse *NQO1* promoter contains a similar palindromic-type ARE, as do the promoters of the orthologous rat and human genes.

The promoter of *Gsta1* contains a perfect ARE (5′-GTGACAAAGCA-3′) located 4 bp downstream from a closely related imperfect ARE sequence [5′-ATGACATTGCT-3′ (12, 17)]. The region encompassing both sequences is unable to form a palindromic structure. The type of ARE found upstream of *Gsta1* is part of a head-to-tail tandem direct repeat, and it is not bound by Nrf2 as tightly as the bZIP protein binds the palindromic ARE (22). It is possible that this type of tandem repeat ARE can recruit other CNC transcription factors besides Nrf2 (see above). If this proposal is indeed correct, the reason why the additional factor can compensate for Nrf2 in terms of mediating inducible gene expression but cannot compensate for Nrf2 in normal constitutive circumstances requires explanation. It could be argued that the putative additional factor is itself inducible. In any event, it will be important to establish whether there are functionally distinct types of ARE. If so, the possibility that they display selectivity in the bZIP proteins they recruit and the protein kinase signaling pathways they respond to (for examples, see Ref. 50) merits investigation.

Concluding Comments. The present study has demonstrated that the constitutive expression of NQO and GSH-associated enzymes is reduced substantially in the small intestine of the homozygous *Nrf2* KO mouse. Furthermore, induction of NQO and GCS_h is substantially impaired in the KO mouse. These results suggest that the *Nrf2*(−/−) mouse will provide a useful model to study the contribution of enzyme induction to cancer chemoprevention. Important questions remain to be answered regarding the number of genes that can be included in the ARE gene battery and possible heterogeneity in their response to xenobiotics.

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