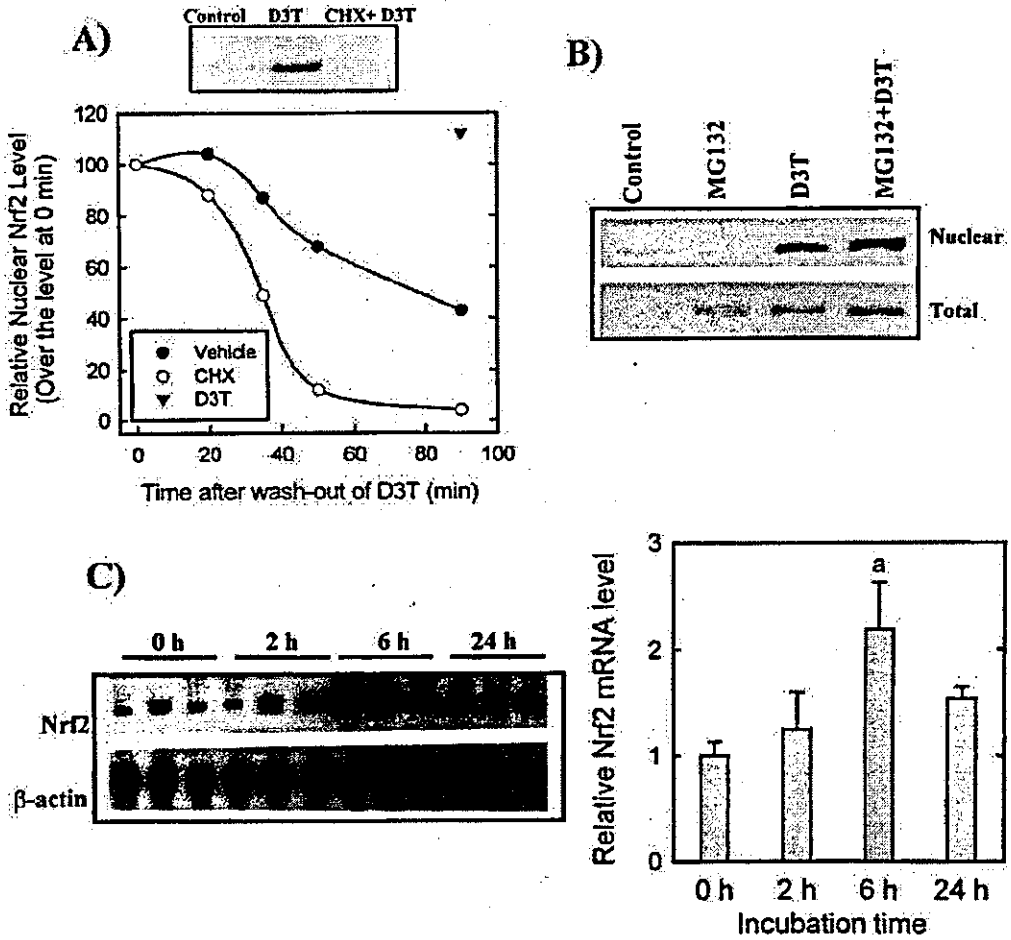

FIG. 2. Effects of protein synthesis inhibitor, CHX, and proteasome inhibitor, MG132, on the accumulation of Nrf2 and effect of D3T on levels of Nrf2 mRNA. (A) In the upper panel, cells were treated with vehicle, D3T (10 μM), or D3T plus CHX (1 μg/ml) for 3 h. Immunoblotting with Nrf2 antibody was carried out with nuclear extracts. In the lower panel, cells were washed after treatment with D3T for 3 h, and either vehicle or CHX was added to the culture medium for the indicated times. ▼, Level of nuclear Nrf2 after incubation with D3T for 4.5 h without washout at 3 h. Nuclear Nrf2 levels were measured by immunoblot analysis of three pooled samples at each time point. (B) Nrf2 levels after treatment for 6 h with vehicle, MG132 (15 μM), D3T (10 μM), or MG132 plus D3T were measured in nuclear and total cellular fractions. Each lane contains three pooled samples, and three separate immunoblots were carried out. (C) Nrf2 mRNA levels measured in PE cells by Northern blot hybridization. The histogram on the right represents the mean ± the standard error (SE) from three different experiments.

transfected into PE cells, and the luciferase activity was measured after treatment with D3T. Luciferase activity of blank plasmid pGLbasic was not influenced by D3T treatment in these cells. However, luciferase activity derived from the *nrf2* promoter was consistently doubled after treatment with D3T for 5 h (Fig. 3B). The *nrf2* promoter-derived luciferase activity was not elevated by longer incubations (i.e., 24 h) with D3T (data not shown). Several nested deletion fragments differing only in their 5′ ends were constructed and transfected into PE cells. These modified promoters contained AREL2-deleted (−599 to −35) and AREL1- and AREL2-deleted (−429 to −35) promoter fragments (Fig. 3A). Induction of luciferase activity by D3T was lost in both of these constructs (Fig. 3B). This result suggested that the region between −599 and −1065, which includes the AREL2 sequence, could mediate activation of the *nrf2* promoter by D3T.

ARE-like sequences regulate the *nrf2* promoter and bind Nrf2. Two sequences containing AREL2 (−848 to −684) and AREL1 (−574 to −403) were amplified from the *nrf2* promoter and ligated to pTATALuc+ for enhancer analysis. The AREL2 containing sequence (pTATA AREL2) could be activated modestly (50%) but significantly ($P < 0.05$) by D3T compared to dimethyl sulfoxide-treated cells (Fig. 4A). However, pTATA AREL1 was not activated by D3T. To verify this result, a full-length promoter containing mutated AREL2 (TG ACTGTGGC → GTCCTGTGGC; MutAREL2) was constructed and transfected into PE cells. Luciferase activity of mutated AREL2 promoter was not increased by treatment with D3T (Fig. 4B). Mutation of AREL1 (TGACTCCGC → GTCCTCCGC; MutAREL1) also abolished inducibility of the wild-type promoter by D3T. Thus, AREL2 mediates a weak induction, but AREL2 alone is not sufficient to activate fully this promoter by D3T treatment.

EMSA analysis was carried out to establish the protein-

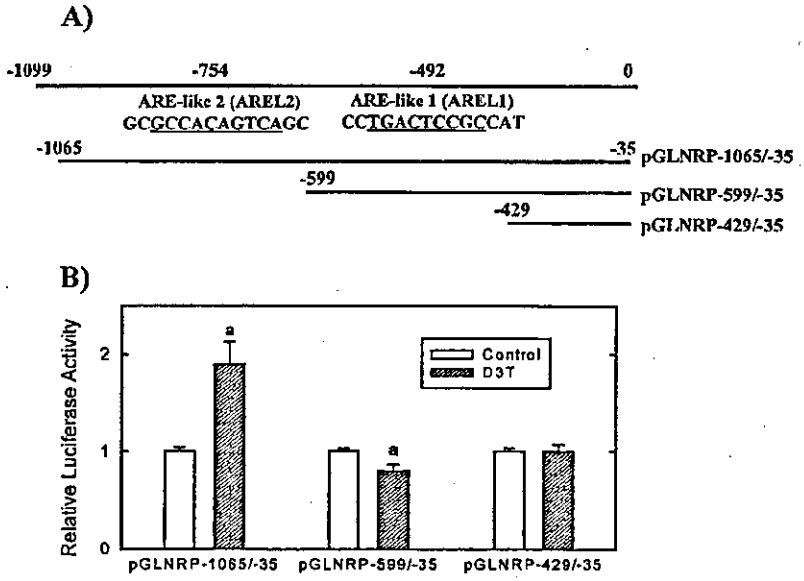

FIG. 3. Effect of D3T on *nrf2* promoter activity in PE cells. (A) A 1-kb portion of the promoter of *nrf2* was isolated from murine liver and ligated into a luciferase reporter vector (pGLNRP−1065/−35) to monitor the activity of this promoter. ARE-like sequences (AREL1; −492, AREL2; −754) are found in the promoter of Nrf2. Different 5′-deletion constructs (pGLNRP−599/−35 and pGLNRP−429/−35) were also prepared by PCR. (B) Luciferase reporter plasmids containing the *nrf2* promoter (pGLNRP−1065/−35) or truncated promoter constructs (pGLNRP−599/−35 and pGLNRP−429/−35) were transiently transfected into PE cells, and the luciferase activities were measured after treatment with D3T (10 μM) for 5 h. Luciferase activities were normalized by cotransfecting *Renilla* luciferase control vectors. Values are means ± the SE from five to seven different experiments. a, $P < 0.05$ compared to vehicle-treated control.

binding patterns of AREL1 and AREL2. AREL1 and AREL2 sequences from the *nrf2* promoter were end labeled with [^{32}P]ATP and incubated with nuclear extract isolated from PE cells. As shown in Fig. 5A, excess amounts (200-fold) of cold AREL1 (lane 1) and AREL2 (lane 6) inhibited the binding of nuclear proteins to these sequences. Competition with cold human *NQO1* ARE (lanes 3 and 8) and the NF-E2 (lanes 5 and 10) consensus sequence also inhibited binding, whereas the AP-1 (lanes 4 and 9) consensus sequence did not. Nuclear extracts isolated from PE cells treated with either vehicle or D3T were then used for gel shift analyses with AREL1 and AREL2. Total binding of nuclear extract protein to AREL2 (Fig. 5B, lane 4) was substantially increased with nuclear extract isolated from D3T-treated mice compared to vehicle-treated mice (lane 3). No differential effect of D3T treatment on nuclear protein binding to the AREL1 was observed (lanes 1 and 2). Immunodepletion with Nrf2 antibodies of nuclear extracts from D3T-treated cells greatly diminished protein binding to AREL2 (Fig. 5B, lanes 5 and 6) and *NQO1* ARE (not shown) but not AREL1 (not shown). Collectively, these results indicated that common factors, including Nrf2, may bind to the AREs of phase 2 genes and the ARE-like sequences of the *nrf2* promoter. The results of a ChIP assay are shown in Fig. 5C. Nrf2 antibody precipitated portions of the promoter of *nrf2* containing the AREL2 sequence in D3T-treated PE cells. This antibody also precipitated the ARE sequence of *GST Ya*, a well-characterized binding motif for Nrf2, but did not precipitate the promoter for unrelated genes such as β-*actin* and the transcription factor *GATA-1*. In contrast, GATA-1 antibody precipitated the *GATA-1* gene hematopoietic enhancer of the *GATA-1* promoter but not the ARE or AREL2 of the murine *GST* and *nrf2* promoters, respectively, in D3T-treated PE cells. Thus, Nrf2 can bind specifically to a region of its own promoter containing the AREL2 after D3T treatment of cells.

Overexpression of Nrf2 activates *nrf2* promoter activity through AREL2. To probe the effects of Nrf2 on its own regulation through ARE-like sequences of its promoter, wild-type or mutant Nrf2 and MafK were overexpressed in PE cells, and the activity of a *nrf2* promoter-luciferase reporter was measured. The activity of full-length *nrf2* promoter (pGLNRP−1065/−35) doubled compared to blank plasmid-transfected cells when wild-type Nrf2 was overexpressed. This effect of overexpression of Nrf2 was identical in magnitude to the effect of D3T treatment on full-length promoter activity. However, overexpression of mutant Nrf2 decreased the activity of this promoter to <50% of its basal activity (Fig. 6A). Coexpression of MafK with wild-type Nrf2 increased promoter activity by sixfold compared to blank plasmids, but coexpression of mutant Nrf2 (in which the transactivation domain was deleted), together with wild-type MafK, produced only a 2.5-fold increase in promoter activity. Similar results were also seen with an AREL2-containing reporter construct (pTATAAREL2) (Fig. 6B). However, the full-length promoter containing a mutated AREL2 (MutAREL2) sequence was not activated by overexpression of Nrf2, whereas mutation of the AREL1 sequence (MutAREL1) had no effect on activation by Nrf2 overexpression (Fig. 6B). Collectively, these results suggest that Nrf2 can activate its own promoter, albeit weakly, through interaction with an AREL2 within its promoter.

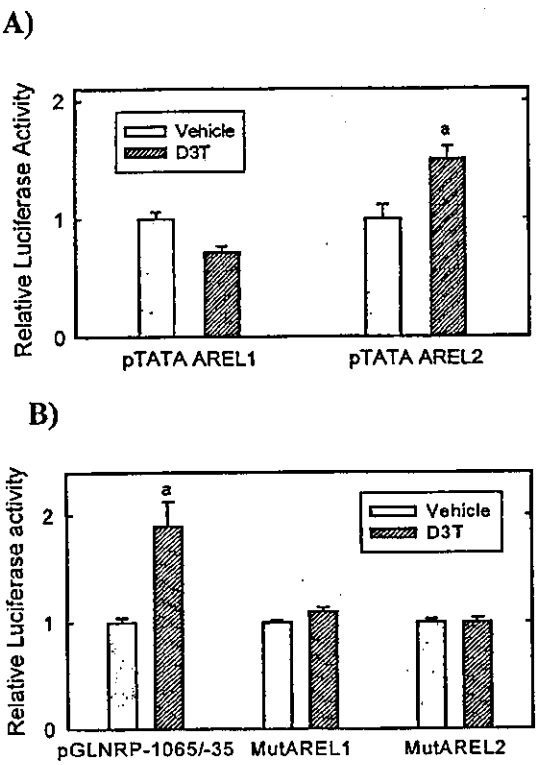

FIG. 4. Effects of D3T on ARE-like-mediated luciferase activity and site-directed mutagenesis studies of the *nrf2* promoter. (A) Luciferase plasmids containing ARE-like sequences from *nrf2* promoter (pTATA AREL1 and pTATA AREL2) were transfected, and the luciferase activities were measured after D3T treatment for 5 h. Luciferase activities were normalized by measuring the *Renilla* luciferase activity from a cotransfected reporter vector. Values are means ± the SE from four different experiments. a, $P < 0.05$ compared to vehicle-treated control. (B) Mutated AREL1- or AREL2-containing promoters were transfected and luciferase activities measured following treatment with D3T for 5 h. Values are means ± the SE from five different experiments. a, $P < 0.05$ compared to vehicle-treated control.

DISCUSSION

Induction of phase 2 enzymes, which neutralize reactive electrophiles and act as indirect antioxidants, occurs in response to a wide array of cancer chemopreventive agents. Although the biological half-lives of these inducers may be measured in seconds to hours, the pharmacodynamic half-life of enzyme induction is typically several days. Thus, transient exposure to an inducer activates a protracted counterattack protective system that guards against subsequent assaults by electrophiles and free radicals. Understanding the key determinants in the regulation of this protective system should provide insights into optimizing the selection and utilization of pharmacological or dietary inducers for the prevention of cancer and other pathological states driven by elevated or sustained exposures to reactive intermediates formed from exogenous and endogenous compounds.

Nrf2 is a critical transcription factor in the regulation of both basal and inducible expression of many phase 2 and antioxidative genes (27, 34, 39). Nrf2 is sequestered in the cytoplasm by the actin-binding protein Keap1 (20). Upon stimulation of cells with inducers, Nrf2 dissociates from Keap1 and translocates to the nucleus, where it interacts with AREs found in the promoter region of many phase 2 genes. This translocation is driven by a nuclear localization signal in Nrf2 but also appears

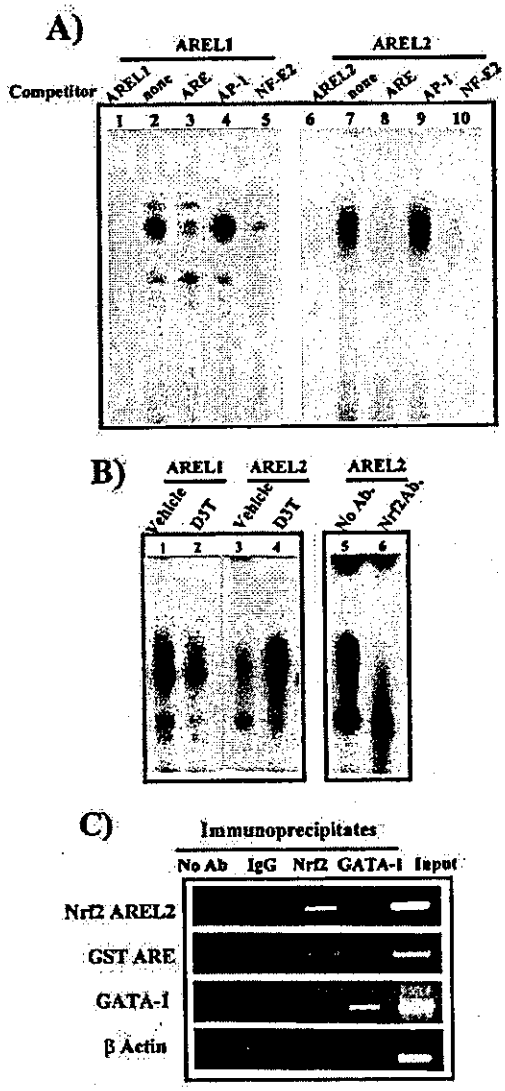

FIG. 5. EMSA analyses with AREL1 and AREL2 sequences and ChIP assay. (A) Competitive binding of AREL1 (lanes 1 to 5) and AREL2 (lanes 6 to 10) with consensus sequences of human *NQO1* ARE, AP-1, or NF-E2 with nuclear extracts from D3T-treated cells. (B) Total binding of nuclear extracts from vehicle- or D3T-treated cells to AREL1 (lanes 1 and 2) and AREL2 (lanes 3 and 4). The effects of immunodepletion of nuclear extracts from D3T-treated cells with Nrf2 antibody are shown in lanes 5 and 6. Each lane represents three pooled samples. (C) ChIP assay performed in D3T-treated PE cells with Nrf2 antibody. Intact protein-DNA complexes were cross-linked by adding formaldehyde into the culture medium. Immunoprecipitates from control (incubated without antibody) and nonspecific immunoglobulin (IgG)-, Nrf2 antibody-, or GATA-1 antibody-incubated cells were isolated and analyzed by PCR with primers specific for the *GST*, *nrf2*, *GATA-1*, and β-*actin* promoters. The supernatant from the control nuclear extract was prepared as the total input of chromatin, and 0.1% of total input was used as the input DNA. Each sample was prepared from three pooled plates.

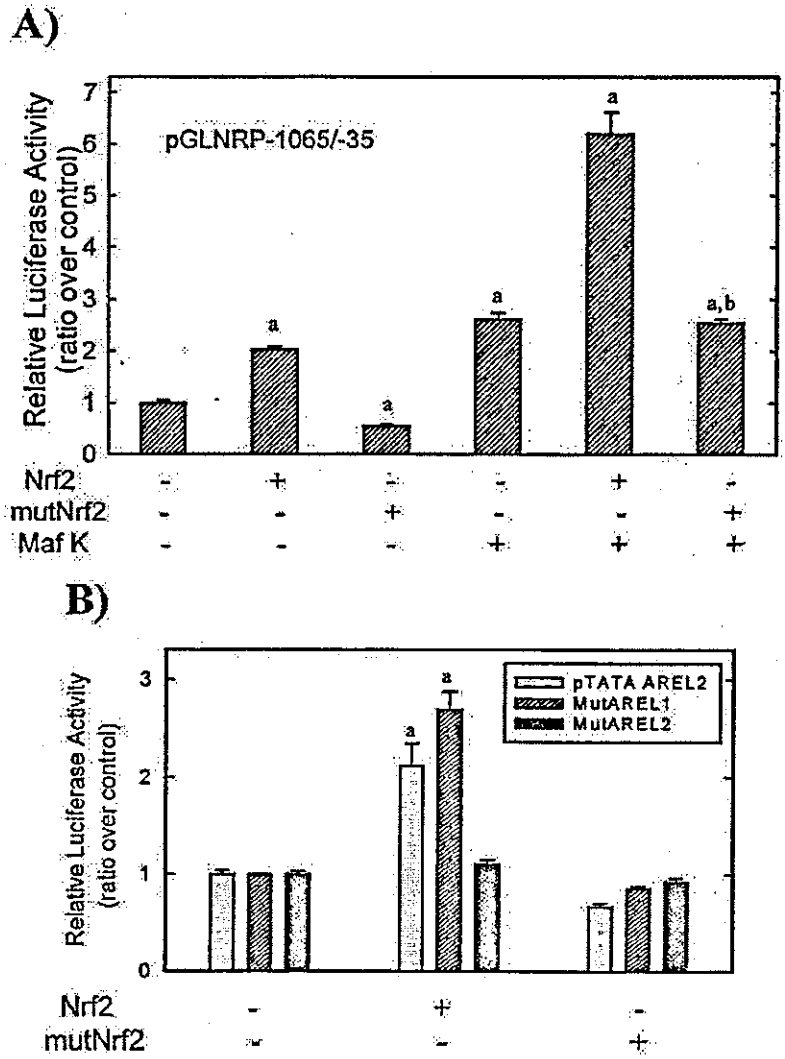

FIG. 6. Effect of overexpression of Nrf2 on the activity of the *nrf2* promoter. (A) *Nrf2* promoter luciferase activity was measured after transfection of wild-type or mutant Nrf2 with or without MafK. Values are means ± the SE from four different experiments. a, $P < 0.05$ compared to blank plasmid-transfected control. b, $P < 0.05$ compared to the wild-type Nrf2 and MafK-transfected group. (B) Luciferase activities of pTATA AREL2, mutated AREL2 promoter (MutAREL2), or mutated AREL1 promoter (MutAREL1) after overexpression of wild-type and mutant Nrf2. Values are means ± the SE from 4 different experiments. a, $P < 0.05$ compared to blank plasmid-transfected control.

to be facilitated through phosphorylation by several kinases (16, 45). Our results indicate that the nuclear accumulation of Nrf2 is rapid and persistent and can be mediated by inducers of distinct chemical classes. Enhanced nuclear accumulation of Nrf2 in response to inducers does not appear simply to be due to translocation of preexistent Nrf2 from the cytoplasm. First, quiescent PE cells have very low levels of Nrf2 that are barely detectable by Western blotting and that cannot account fully for the elevated amount of Nrf2 seen in nuclei after treatment with an inducer. Second, concomitant increases in total cellular amounts of Nrf2 are seen with the nuclear accumulation, suggesting that amplified de novo synthesis of the transcription factor is occurring. Treatment of cells with CHX blocks the accumulation of Nrf2 (nuclear and total) by D3T. Third, D3T treatment in combination with a proteasome inhibitor enhanced the nuclear accumulation of Nrf2 compared to treatment with a proteasome inhibitor alone. Fourth, Nrf2 mRNA and protein levels were elevated 6 h after treatment in these cells. These results suggest that dissociation of Nrf2 from Keap1 leads to an initial elevation of Nrf2 in the nucleus within 20 to 60 min. However, given the short half-life of Nrf2 (~20 min [Fig. 2A]), the predominant factor driving the sustained accumulation and transactivation of phase 2 and/or antioxidative genes results from enhanced de novo synthesis.

The sequence of murine *nrf2*, including 1 kb of the 5′-flanking region, has been reported and contains multiple SP-1 and AP-2 sites, as well as two ARE-like sequences located at −492 and −754 from the start codon. One motif has a perfect ARE (AREL1; TGACTCCGC) consensus sequence, while the second one has one more base before the GC box (AREL2;

TGACTGTGGC). The activity of a luciferase reporter construct containing the 1-kb promoter (pGNLRP−1065/−35) transfected into PE cells could be doubled by treatment with D3T. Studies with several nested deletion fragments differing only in their 5′ ends indicated that deletion of AREL2 (−599/−35) or AREL2 and AREL1 (−429/−35) eliminated dithiolethione inducibility. Mutation of the core sequence of either AREL2 or AREL1 in the full-length promoter obviated activation by D3T. A reporter construct containing an AREL2 (−848 to −684) ligated to pTATAluc+ was partially activated by D3T, while a comparable construct with AREL1 was not. Collectively, these studies document that both AREL1 and AREL2 are necessary to fully activate Nrf2 expression by D3T.

Nrf2, a cap'n'collar bZIP transcription factor, forms heterodimers with other proteins, especially of the small Maf class and other bZIP transcription factors (15, 19, 39). Diminished binding of nuclear proteins to AREL1 or AREL2 after the addition of excess *NQO1* ARE or NF-E2 suggests common factors may bind to these sequences. EMSA conducted on nuclear proteins isolated from D3T-treated cells demonstrated a marked increase in binding to AREL2-containing oligonucleotides compared to vehicle control, while no such differential was observed with AREL1-containing oligonucleotides. Treatment of EMSA incubations with Nrf2 antibody diminished the total protein binding to AREL2 through immunodepletion. Binding of Nrf2 to ARE-like sequences of its promoter was directly confirmed by a ChIP assay with Nrf2 antibody. This experiment demonstrated that Nrf2 associated with a region of the *nrf2* promoter that included or was adjacent to AREL2. The likely involvement of Nrf2 in its own regulation is also supported by experiments in which either wild-type or mutant Nrf2 was overexpressed in PE cells. Overexpression of wild-type Nrf2 activated promoter activity twofold, while mutant Nrf2, which has no transactivation domain, did not increase promoter activity. Coexpression of MafK with Nrf2 in PE cells substantially enhanced the activation of the Nrf2 promoter. Mutation of three bases in the AREL2 of the full-length promoter-reporter construct eliminated responsiveness to overexpression of Nrf2, whereas mutation in AREL1 did not.

In PE cells, Nrf2 can bind to and enhance the activity of its own promoter. However, the extent of activation of the *nrf2* promoter by enzyme inducers (or forced Nrf2 expression) is less than seen with typical ARE-containing promoters found in murine *GST Ya* or rat *NQO1*. The murine *GST Ya* and rat *NQO1* genes have repeated AREs in their promoters. Mutation studies have shown that multiple AREs are necessary for maximal activation of these enhancers (12, 42). AREL2 in the *nrf2* promoter is a single, imperfect ARE since it has one more base before the GC box. This degeneration from the consensus ARE sequence may induce different binding affinities to transcription factors and account for the weak responsiveness of the *nrf2* promoter to inducers. Several reports have suggested that different combinations of the bZIP transcription factors have different binding affinities to DNA. Ryseck and Bravo (36) showed that Jun family proteins have different binding affinities to TRE (TPA response element) and CRE (cyclic AMP response element) motifs depending upon their partners. TRE (TGACTCA) and CRE (TGACGTCA) have very similar sequences; however, the Jun-Fos dimer has a higher affinity to TRE than CRE, while the Jun-ATF (activation transcription factor) dimer binds more efficiently to CRE than TRE. Kataoka et al. (23) also suggested that TRE-type MARE (Maf response element) and the CRE-type MARE are recognized with different affinities by different combinations of bZIP proteins, including Maf. Similar conclusions hold for the regulation of detoxifying genes. Small differences in the AREs found in detoxifying genes seem to be related to differential responsiveness to bZIP transcription factors. The promoter of the γ-glutamylcysteine ligase heavy chain has several AREs, but a single ARE acts as a *cis*-acting element (32) and is activated by Nrf2 overexpression while inhibited by overexpression of MafK or MafG in human hepatoma cells (43). Jeyapaul and Jaiswal (21) have shown that Nrf2 and c-Jun are important in regulating the basal and inducible levels of γ-glutamylcysteine ligase heavy chain by β-naphthoflavone. Activation of the ARE of human *NQO1* was repressed by expression of small Maf proteins such as MafK and MafG in human hepatoma cells, whereas the expression of c-Jun did not increase activity of an *NQO1* ARE-derived luciferase reporter (8). Activity of a *GST Ya* ARE luciferase reporter was also repressed by expression of MafK in PE cells (M.-K. Kwak and T. W. Kensler, unpublished data). In contrast, expression of reporter genes linked to the *thioredoxin* ARE (TGAGTCGT) and *p53* ARE (TG ACTCTGC) was increased by MafK expression (13, 25). These results suggest that the composition of the transcription complex can be varied depending upon the individual genes and the means of stimulation. In the case of *nrf2*, Nrf2 associates with the AREL2 in its own promoter and MafK facilitates activation of this promoter.

This mechanism of autoregulation of gene expression can be seen for several other transcription factors. For example, *GATA-1*, which is essential for hematopoietic cell differentiation, also has GATA-binding sequences in its promoter region that have been shown to be critical for regulation of this gene (33). NF-κB also positively regulates its transcription by binding to an NF-κB regulating element in its promoter (29). NF-κB levels are controlled through binding with its inhibitor I-κB in the cytoplasm. Stimuli such as oxidative stress can trigger degradation of I-κB by phosphorylation, allowing NF-κB to be translocated into the nucleus (22). While control of trafficking is the main pathway for the regulation of NF-κB, transcriptional activation of NF-κB is also observed. The bZIP proteins c-Jun and c-Fos can also autoregulate their expression (2, 40). It is also probable that bZIP transcription factors, including Nrf2, can cross talk with each other. Venugopal and Jaiswal (38) have reported that human c-Jun has an ARE (TGACTTCGGC) and suggested the involvement of an ARE-mediated induction of this protein. D3T induces c-Jun expression. Thus, increased nuclear Nrf2 accumulation in response to D3T may also induce other transcription factors such as Jun, which in turn contribute to binding and activation of AREL1 and AREL2 with Nrf2.

In summary, Nrf2 appears to autoregulate its expression through weak ARE-like *cis*-elements in its promoter, thereby greatly extending the duration if not the magnitude of its transactivating action. Under quiescent or nonstressed situations, PE cells maintain low levels of this rapidly turned

over transcription factor. Upon exposure to stressor molecules, such as electrophiles or free radicals, release of this constitutive Nrf2 from Keap1 initiates signaling for the induction of protective genes. Amplification of this counterattack response occurs through transactivation of the *nrf2* gene, leading to increased synthesis of Nrf2. Saturation of the cytoplasmic tether of Nrf2, Keap1, allows for enhanced nuclear accumulation of Nrf2 and protracted activation of phase 2 genes. Signaling for increased synthesis of Nrf2 is ultimately attenuated, even in the face of continued challenge with inducers. Although the mechanism underlying this dampening response is unclear, posttranslational modification of Nrf2 through phosphorylation or other means may mark the transcription factor for altered disposition. This multifaceted pathway for the regulation of Nrf2 levels in cells provides a tightly controlled mechanism to modulate the expression of genes that protect against an array of endogenous and exogenous assault molecules.

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Identification of Nrf2-regulated Genes Induced by the Chemopreventive Agent Sulforaphane by Oligonucleotide Microarray[1]

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ABSTRACT

Electrophiles formed during metabolic activation of chemical carcinogens and reactive oxygen species generated from endogenous and exogenous sources play a significant role in carcinogenesis. Cancer chemoprevention by induction of phase 2 proteins to counteract the insults of these reactive intermediates has gained considerable attention. Nuclear factor E2 p45-related factor 2 (Nrf2), a bZIP transcription factor, plays a central role in the regulation (basal and or inducible expression) of phase 2 genes by binding to the "antioxidant response element" in their promoters. Identification of novel Nrf2-regulated genes is likely to provide insight into cellular defense systems against the toxicities of electrophiles and oxidants and may define effective targets for achieving cancer chemoprevention. Sulforaphane is a promising chemopreventive agent that exerts its effect by strong induction of phase 2 enzymes via activation of Nrf2. In the present study, a transcriptional profile of small intestine of wild-type (*nrf2* +/+) and knock out (*nrf2* −/−) mice treated with vehicle or sulforaphane (9 μmol/day for 1 week, p.o.) was generated using the Murine Genome U74Av2 oligonucleotide array (representing ~6000 well-characterized genes and nearly 6000 expressed sequence tags). Comparative analysis of gene expression changes between different treatment groups of wild-type and *nrf2*-deficient mice facilitated identification of numerous genes regulated by Nrf2 including previously reported Nrf2-regulated genes such as *NAD(P)H:quinone reductase* (*NQO1*), *glutathione S-transferase* (*GST*), *γ-glutamylcysteine synthetase* (*GCS*), *UDP-glucuronosyltransferases* (*UGT*), *epoxide hydrolase*, as well as a number of new genes. Also identified were genes encoding for cellular NADPH regenerating enzymes (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme), various xenobiotic metabolizing enzymes, antioxidants (glutathione peroxidase, glutathione reductase, ferritin, and haptaglobin), and biosynthetic enzymes of the glutathione and glucuronidation conjugation pathways. The data were validated by Northern blot analysis and enzyme assays of selected genes. This investigation expands the horizon of Nrf2-regulated genes, highlights the cross-talk between various metabolic pathways, and divulges the pivotal role played by Nrf2 in regulating cellular defenses against carcinogens and other toxins.

INTRODUCTION

There is considerable epidemiological evidence suggesting an association of consumption of fruits and vegetables with reduced incidence of cancer, especially in the gastrointestinal tract (1). Induction of phase 2 enzymes by phytochemicals represents an important mechanism for achieving protection against cancer (2). Although phase 2 enzymes have been traditionally recognized as those catalyzing the conjugation of endogenous ligands, glutathione and glucuronic acid, to endo- and xenobiotic substrates, this classification is expanding to include proteins that catalyze a wide variety of reactions that confer cytoprotection against the toxicity of electrophiles and reactive oxygen species (3). The growing list of phase 2 proteins includes NAD(P)H:quinone reductase (NQO1), epoxide hydrolase, dihydrodiol dehydrogenase, γ-GCS,[3] heme oxygenase-1, leukotriene B_4 dehydrogenase, aflatoxin B_1 dehydrogenase, and ferritin (3). These proteins, which enhance resistance to different toxicants, are regulated by a common element (5′-$A^{/G}$ TGA $C^{/T}$ NNN GC $A^{/G}$-3′) in their promoter region that is termed the ARE (4). The mechanisms that regulate the expression of phase 2 genes through the ARE are the subject of intensive investigation (5).

It has been demonstrated clearly that Nrf2, a bZIP transcription factor, translocates into the nucleus and binds to the ARE in conjugation with small Maf proteins after activation by chemopreventive agents and that Nrf2 plays a central role in the constitutive and inducible expression of several phase 2 proteins (6–11). Higher sensitivity of *nrf2*-deficient mice to liver damage by acetaminophen (12), pulmonary injury by butylated hydroxytoluene (13), increased DNA adducts in lungs after exposure to diesel exhaust (14), and gastric tumors by benzo(*a*)pyrene (7) have been attributed to decreased basal expression of phase 2 enzymes in various organs (8, 10, 15). The induction of several phase 2 genes (*e.g.*, *GST* and *NQO1*) by butylated hydroxyanisole, oltipraz, and sulforaphane is also dependent on Nrf2 (7, 10). Collectively, these studies clearly indicate the pivotal role of this transcription factor in the regulation of phase 2 proteins and, thus, chemoprevention against xenobiotic toxicities.

Molecular epidemiological studies have indicated that consumption of dietary isothiocyanates are effective in decreasing the relative risk of colorectal cancer (16) and lung cancer (17, 18). Among the isothiocyanates, sulforaphane (*R*-1-isothiocyanato-4-methylsulfinylbutane) is the most potent inducer of phase 2 proteins (19–21) and functions to activate Nrf2 (10, 11). Sulforaphane effectively reduces colonic aberrant crypt foci formation in carcinogen-treated rats (22). Identification of cytoprotective genes, which are directly or indirectly dependent on Nrf2 for transcriptional activation in response to promising chemopreventive agents such as sulforaphane, will facilitate the understanding of molecular downstream effectors of chemoprevention. By feeding sulforaphane p.o. to wild type and *nrf2*-deficient mice, we have identified several genes in a target organ, the small intestine, using oligonucleotide arrays that are dependent on Nrf2 for their basal and/or inducible expression. This study expands the horizon of Nrf2-regulated protective proteins and identifies novel downstream mediators for chemoprevention by sulforaphane and, presumably, other classes of enzyme inducers.

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[3] The abbreviations used are: γ-GCS, γ-glutamylcysteine synthetase; γ-GCS(h), heavy catalytic subunit of γ-GCS; EST, expressed sequence tag; ALDH, aldehyde dehydrogenase; ARE, antioxidant response element; HSP, heat shock protein; G6PDH, glucose-6-phosphate dehydrogenase; GPX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase; NQO1, NAD(P)H:quinone reductase; Nrf2, nuclear factor E2 p45-related factor 2; 6PGDH, 6-phsophogluconate dehydrogenase; UGT, UDP-glucuronosyltransferase.

MATERIALS AND METHODS

Reagents. L-Sulforaphane was purchased from LKT Laboratories (St. Paul, MN). A nucleotide removal kit was procured from Qiagen (Valencia, CA). NADPH, malate, glucose-6-phosphate, 1-chloro-2,4-dinitrobenzene, *p*-nitrophenylacetate, NAD^+, and menadione were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals. Experiments were conducted in accordance with the standards established by the United States Animal Welfare Acts, set forth in NIH guidelines and the Policy and Procedures Manual of the Johns Hopkins University Animal Care and Use Committee. *Nrf2*-deficient ICR mice were generated as described (8). Genotypes of homozygous wild-type and *nrf2*-deficient mice (10 weeks of age) were confirmed by PCR amplification of genomic DNA extracted from blood or liver. PCR amplification was carried out by using three different primers, 5′-TGGACGGGACTATTGAAGGCTG-3′ (sense for both genotypes), 5′-CGCCTTTTCAGTAGATGGAGG-3′ (antisense for wild type), and 5′-GCGGATTGACCGTAATGGGATAGG-3′ (antisense for LacZ), as described previously (7).

Female mice [wild-type *nrf2* (+/+) and *nrf2* (−/−) deficient], 10 weeks of age, were maintained on an AIN 76A diet and water *ad libitum* and housed at a temperature range of 20–23°C under 12-h light/dark cycles. The mice were grouped into four groups ($n = 3$): I, control (*nrf2* +/+) wild type; II, treatment (*nrf2* +/+) wild type; III, control knock out (*nrf2* −/−); and IV, treatment knock out (*nrf2* −/−). The control and treatment groups were administered either vehicle (corn oil) alone or sulforaphane (9 μmol/mouse/day) by gavage (0.2 ml), respectively, for 7 consecutive days. Body weights were recorded to monitor the health of animals. Animals were sacrificed by cervical dislocation 24 h after the last dose. The small intestine was removed and washed thoroughly with ice-cold PBS to remove the fecal material and frozen in liquid nitrogen before storage at −80°C until further use.

Northern Blotting. Total RNA from the intestine was extracted with TRIzol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. Total RNA (10 μg) was separated on 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Nytran Super Charge; Schleicher and Schuell, Dassel, Germany), and UV-cross-linked. Probes for NAD(P)H:quinone oxidoreductase (NQO1), GST Ya, γ-GCS (regulatory subunit), UDP-glucuronosyltransferases (UGT1A6), malic enzyme, glutathione reductase, and β-actin were generated by PCR from the cDNA of murine liver. These PCR products were radiolabeled with [α-^{32}P]dCTP using a random primers DNA labeling kit (Invitrogen, San Diego, CA). Northern hybridization was done using QuickHyb (Stratagene, Carlsbad, CA) as per the manufacturer's protocol. After hybridization, the membranes were washed twice in 0.2% SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% (w/v) SDS at room temperature for 15 min and finally in 0.1% SSC/0.1% SDS at 60°C for 45 min. The membranes were exposed to a phosphorimager screen, and radioactivity was visualized and quantified with a BAS1000 Bioimaging system (Fuji Photo Film, Tokyo, Japan). Levels of RNA were quantified and normalized for RNA loading by stripping and reprobing the blots with a probe for β-actin.

Transcriptional Profiling by Oligonucleotide Microarray. Total RNA was purified with the RNeasy Mini kit (Qiagen, Valencia, CA) after isolation with TRIzol reagent as described above and was used for experiments with Murine Genome U74A version 2 GeneChip arrays (Affymetrix, Santa Clara, CA), which contain probes for detecting ~6000 well-characterized genes and 6000 expressed sequence tags (ESTs). Briefly, double-stranded cDNA was synthesized from 15 μg of total RNA with SuperScript Choice System (Invitrogen) by using oligo(dT)$_{24}$ primers with a T7 RNA polymerase promoter site added to its 3′ end (Genset Corp., La Jolla, CA). The isolated cDNA was then labeled to generate biotinylated cRNA *in vitro* and amplified using the BioArray T7 RNA polymerase labeling kit (Enzo, Farmingdale, NY). After purification of the cRNA by RNeasy Mini kit, 20 μg of cRNA were fragmented at 94°C for 35 min. Approximately 12.5 μg of fragmented cRNA was used in a 250-μl hybridization mixture containing herring sperm DNA (0.1 mg/ml; Promega Corp., Madison, WI), plus bacterial and phage cRNA controls (1.5 pmol of BioB, 5 pmol of BioC, 25 pmol of BioD, and 100 pmol of Cre) to serve as internal controls for hybridization efficiency as directed by the manufacturer (Affymetrix). Aliquots (200 μl) of the mixture were hybridized onto the array for 18 h at 45°C in a GeneChip Hybridization Oven 640 (Affymetrix). Each array was washed and stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and amplified with biotinylated anti-streptavidin antibody (Vector Laboratory, Burlingame, CA) on the GeneChip Fluidics Station 400 (Affymetrix). Each array was scanned with the GeneArray scanner (Agilent Technologies, Palo Alto, CA) to obtain image and signal intensities.

Data Analysis Using Affymetrix Software. Scanned output files were analyzed with the Affymetrix Microarray Suite 5.0 and normalized to an average intensity of 500 independently, before comparison. To identify differentially expressed transcripts, pairwise comparison analysis were carried out with Data Mining Tool 3.0 (Affymetrix). The analysis compares the differences in values of perfect match to mismatch of each probe pair in the baseline array to its matching probe pair on the experimental array. *P*s were determined by the Wilcoxon's signed rank test and denoted as increase, decrease, or no change. Analysis using Data Mining Tool also provides the signal log ratio, which estimates the magnitude and direction of change of a transcript when two arrays are compared (experimental *versus* baseline). We have converted the signal log ratio output into fold change for convenience using the formula recommended by Affymetrix:

$$\text{Fold change} = \begin{cases} 2^{\text{Signal Log Ratio}}, & \text{Signal Log Ratio} > 0 \\ (-)\,2^{-(\text{Signal Log Ratio})}, & \text{Signal Log Ratio} < 0 \end{cases}$$

In the present study, we performed nine pairwise comparisons for each group (experimental, $n = 3$ *versus* baseline, $n = 3$). Only those altered genes that appeared in at least seven of the nine comparisons were selected. This conservative analytical approach was used to limit the number of false positives. In addition, we also performed a Mann-Whitney pairwise comparison test in Data Mining Tool to rank the results by concordance as a calculation of significance (*P*) of each identified change in gene expression. The ESTs obtained in the data were searched for their recent annotation using the "Analysis Center" at the Affymetrix site (www.netaffx.com).

Enzyme Activity Assays. Total GST activity was measured in cytosolic fractions (100,000 × *g*) in the presence of 0.1% BSA with 1-chloro-2,4-dinitrobenzene as a substrate (23), whereas NQO1 activity was determined using menadione as substrate (24). Activity of G6PDH was determined from the rate of glucose 6-phosphate-dependent reduction of $NADP^+$ (25). Malic enzyme activity was measured from the rate of malate-dependent $NADP^+$ reduction (26). Carboxylesterase activity was determined by measuring the hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol at 410 nm (27). UDP-glucose dehydrogenase activity was measured by the reduction of NAD^+ in the presence of UDP-glucose at 340 nm (28). Protein concentration was determined by using the Bio-Rad DC reagent and BSA as the standard.

Statistics. The values for enzyme-specific activities are mean ± SE, and the Student *t* test was used to analyze the statistical significance.

RESULTS

Microarray Analysis. The transcriptional profile of a target organ, the small intestine of *Nrf2* wild-type and -deficient mice with or without sulforaphane treatment was generated using the Murine Genome U74Av2 chip. The obtained transcriptional profile was logically analyzed to derive the set of genes regulated by Nrf2. We performed three categories of comparisons (Fig. 1) to accomplish the objective. Because Nrf2 is well established in the positive regulation of genes (15), we have concentrated only on up-regulated genes.[4] The gene profile that emerged after comparative analysis was further filtered. The genes that appeared in at least seven of the nine comparisons that showed fold changes >1.5 and had *P*s of ≤0.05 were selected. These cutoff values gave a conservative estimate of the number of genes in each category, and only those genes complying with the criteria were considered further.

Wild-Type Control and *Nrf2*-deficient Control. In this category, the altered gene profile reflects the effect of disruption of Nrf2. Overall, there were 45 genes and 27 ESTs with higher expression in

[4] The comprehensive lists of all up-regulated and down-regulated genes in the comparisons are reported in http://commprojects.jhsph.edu/ehs/biswal_data.xls.

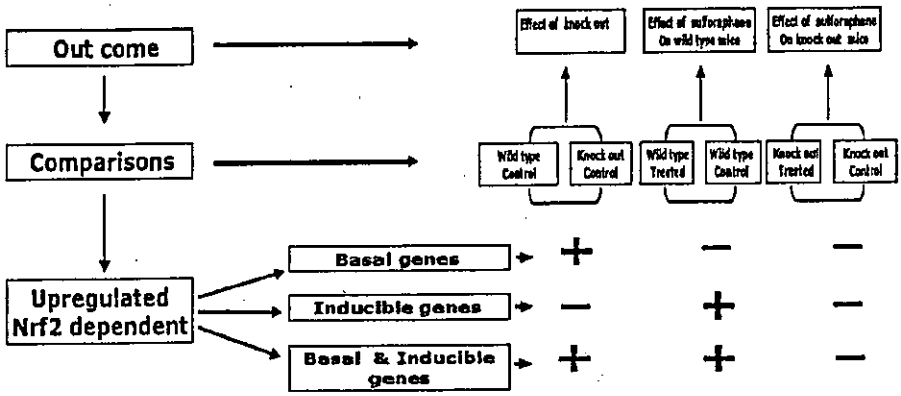

Fig. 1. Schematic depicting the strategy for comparison and analysis of transcriptional profiles obtained from microarray using the Affymetrix Murine Genome U74A version 2 arrays that contain probes for detecting ~6000 well-characterized genes and 6000 expressed sequence tags. *Wild type, nrf2* +/+ mice; *knock out, nrf2* −/− mice.

the wild-type mice. Prominent genes showing large fold changes were *carboxyl esterase* (15-fold), *epoxide hydrolase* (12-fold), various GSTs (3–6-fold), *malic enzyme* (3.5-fold), *UGT* (8-fold), *aldehyde dehydrogenase II* (4.6-fold), and *NQO1* (3-fold).

Wild-Type Treated and Wild-Type Control. The altered transcriptional profile in this comparison group is the result of treatment with sulforaphane. Thirty-three genes and 17 ESTs were induced by sulforaphane. The most responsive genes in this class of comparison were *GSTs* (2.5–6-fold), catalytic subunit of *GCS* (4-fold), *fibroblast growth factor related protein* (3-fold), *carboxyl esterase* (4-fold), and *NQO1* (2.5-fold).

***Nrf2*-deficient Treated and *Nrf2*-deficient Control.** This profile also includes genes induced by sulforaphane; however, Nrf2 does not regulate them. There were 59 genes and 62 ESTs, of which the most responsive genes were histone genes (*H2B* and *H2A*; 5-fold) and mouse immunoglobulin-active λ-1-chain V-region (*V-J*) gene (2.5-fold).

Nrf2-dependent Genes. To identify the gene targets of Nrf2 based on the transcriptional pattern of gene expression, we adopted a specific strategy of analysis that is depicted in Fig. 1. Our analysis revealed 77 up-regulated genes whose expression was influenced by Nrf2 (Table 1). They can be classified into three different categories based on their dependence on Nrf2. For basal expression, the expressions of this class of genes were elevated in wild-type control mice compared with *nrf2*-deficient mice and were not effected by sulforaphane in either genotype, suggesting that these genes require Nrf2 for their basal but not inducible expression. For inducible expression, this subset of genes was elevated in the sulforaphane-treated, wild-type group relative to vehicle-treated, wild-type and *nrf2*-deficient mice and were not induced in sulforaphane-treated, *nrf2*-deficient mice, demonstrating their dependence on Nrf2 for induction. For basal and inducible expression, this class of genes was elevated in vehicle-treated, wild-type mice when compared with the *nrf2*-deficient mice and selectively induced in response to sulforaphane in the wild-type mice only. Thus, these genes are dependent on Nrf2 for basal as well as inducible expression. All genes belonging to these three groups are presented in Table 1, together with fold change and mode of dependence on Nrf2. Genes elevated in the *nrf2*-deficient, treated and *nrf2*-deficient, control comparison are considered to be Nrf2-independent genes and are included as supplementary data at the web site.

We have further categorized the Nrf2 up-regulated genes based on their functions (Table 1). The majority of the up-regulated genes at the inducible and/or basal level are associated with various metabolic reactions (hydrolysis, reduction, oxidation, and conjugation with endogenous ligands: glutathione and glucuronide) involved in detoxication of electrophiles and free radicals. Antioxidative genes such as *glutathione peroxidase, glutathione reductase, ferritin*, and *haptaglobin* and the genes encoding NADPH-generating enzymes, *i.e., glucose 6-phosphate dehydrogenase, malic enzyme*, and *6-phosphogluconate dehydrogenase* were also dependent on Nrf2 for expression.

Validation of Microarray Data by Northern Blot. Four well-characterized Nrf2 regulated genes, *NQO1, GST Ya, γ-GCS(h)*, and *UGT 1A6* and two new genes identified by this screening, *malic enzyme* and *glutathione reductase*, were selected for verification of the transcriptional changes using Northern hybridization. The fold changes [NQO1, 2.5-fold; GST Ya, 2 fold; γ-GCS(h), 3-fold; UGT 1A6, 1.4-fold; malic enzyme, 1.8-fold; and glutathione reductase, 2-fold] in sulforaphane-treated, wild-type mice compared with vehicle-treated, wild-type mice are in close agreement with the oligonucleotide array data (Fig. 2*A*). Furthermore, lower expression of these genes in *nrf2*-deficient mice compared with wild-type mice is consistent with the transcriptional changes observed by microarray.

Enzyme Assays of Selected Genes. Six genes were selected for enzymatic assays (Figs. 2*B* and 3). NQO1 and GST enzyme activities were induced 1.6- and 1.3-fold, respectively, by treatment of wild-type mice with sulforaphane. Moreover, their basal activities were significantly lower in the untreated *nrf2*-deficient mice when compared with the untreated wild-type mice. Malic enzyme and glucose 6-phosphate dehydrogenase activities were induced to 1.8- and 10.3-fold, respectively, and there were significant differences in basal activities between the wild-type and knock-out mice. There was 1.3-fold difference in the basal activity of UDP-glucose dehydrogenase between wild-type and *nrf2*-deficient mice, and significant induction was seen with sulforaphane treatment in wild-type mice. Carboxylesterase activity showed a modest induction of 1.3-fold in response to sulforaphane only in wild-type mice (Fig. 3). In general, the fold increases in enzyme activities were lower than those observed for the increases in transcript levels.

DISCUSSION

Previous studies using biochemical and Northern blot analyses in wild-type and *nrf2*-deficient mice have demonstrated that Nrf2 controls the constitutive expression of antioxidative enzymes such as the light regulatory subunit of γ-glutamylcysteine synthetase, heme oxygenase 1, and peroxiredoxin MSP23 (29) and also mediates induction of hepatic and intestinal NQO1 and GST enzymes by butylated hydroxytoluene, oltipraz, and sulforaphane (8, 11). In the present investigation, we have compared the transcriptional profile of small intestine of *nrf2* wild-type and *nrf2*-deficient mice with and without sulforaphane treatment, a potent chemopreventive agent that activates the Nrf2 pathway (30), and identified several new target genes that are positively regulated at the basal and/or inducible level by Nrf2. Interestingly, most of the proteins that are positively regulated through

Table 1 *Nrf2 up-regulated genes obtained from the screening*

Group/Class and accession no.	Gene	Nrf2 regulated expression[a]	Fold change ± SE
Hydrolysis			
Y12887	*Carboxyl esterase*	B & I	15.2 ± 3.7 and 4 ± 0.3
U89491	*Epoxide hydrolase*[b]	B & I	11.8 ± 1.6 and 1.6 ± 0.1
M29961	*γ-Glutamyl peptidase*	I	1.7 ± 0.15
Reduction			
U31966	*Carbonyl reductase*	B	1.9 ± 0.1
AI840094	*Aflatoxin aldehyde reductase*[c]	B	1.62 ± 0.1
U12961	*NQO1*[b]	B & I	3.7 ± 0.2 and 2.4 ± 0.2
U04204	*Aldose reductase (fibroblast growth factor regulated protein)*	B & I	2.9 ± 0.2 and 3.1 ± 0.2
AB027125	*Aldo-keto reductase*	I	1.5 ± 0.1
Oxidation			
U96401	*Aldehyde dehydrogenase*[d]	B	2.4 ± 0.2
U07235	*Aldehyde dehydrogenase*	B	1.7 ± 0.1
AI848045	*Monoamine oxidase*[c]	B	1.7 ± 0.1
AI197481	*Amino oxidase*[c]	B	1.6 ± 0.1
AA596710	*NADP-dependent leukotreine B4 12-hydroxydehydrogenase*[c]	B & I	1.7 ± 0.1 and 2.5 ± 0.1
Glucuronidation pathway			
XO6358	*UDP-glucuronosyl-transferase 2 family*[b]	B	8.0 ± 0.7
AF061017	*UDP-glucose dehydrogenase*	B	1.5 ± 0.1
Glutathione transferases			
AW124337	*Rat glutathione S-transferase*[b,c]	B	1.9 ± 0.1
J04696	*GST class mu (GST5-5)*[b]	B	1.9 ± 0.1
AI326397	*GST M2* (muscle)[b,c]	B	1.8 ± 0.1
AI843448	*Microsomal GST 3*[b,c]	B	1.7 ± 0.1
J03952	*GST GT8.7*[b]	B & I	2.7 ± 0.2 and 4.6 ± 0.2
J03953	*GST GT9.3*[b]	B & I	6.1 ± 0.4 and 2.4 ± 0.1
AI841270	*GST mu 1*	B & I	4.7 ± 0.3 and 2.8 ± 0.2
X65021	*GST α3*[b]	I	4.9 ± 0.6
JO3958	*GST α2* (Yc2)[b]	I	1.6 ± 0.5
L06047	*GST α1* (Ya)[b]	I	1.8 ± 0.1
AA919832	*Microsomal GST 2*[b,c]	I	1.8 ± 0.1
Glutathione synthesis			
U95053	*GCS, regulatory*[b]	I	2.4 ± 0.2
U85414	*GCS, catalytic*[b]	I	4.1 ± 0.4
Antioxidants			
X61399	*Glutathione peroxidase*[c]	B	1.7 ± 0.1
AV097950	*Ferritin*[c,d]	B	1.6 ± 0.1
AI841295	*Haptaglobin*[c]	B	1.6 ± 0.1
AI851983	*GR*[c]	I	1.7 ± 0.1
Protective proteins			
AA833514	*Multidrug resistance protein*[c]	B	1.6 ± 0.1
AW120711	*HSP 40 (DnaJ)*[c]	I	1.8 ± 0.1
NADPH regenerating enzymes			
Z11911	*G6PDH*	B	1.7 ± 0.1
J02652	*Malic enzyme*	B	3.4 ± 0.2
AW120625	*6PGDH*[c]	I	1.6 ± 0.1
Metabolic enzymes			
AI790931	*Fructose bisphosphatase*[c]	B	1.6 ± 0.1
U67611	*Transaldolase*	I	1.5 ± 0.1
U05809	*Transketolase*	I	1.5 ± 0.1
Inflammatory suppressive gene			
AW046181	*Glucocorticoid-regulated kinase*[c]	I	1.7 ± 0.1
Miscellaneous			
AI841464	*Tryptophan hydrolase*[c]	B	1.5 ± 0.1
X64837	*Ornithine aminotransferase*	B	1.5 ± 0.1
AI849587	*Calcium channel, voltage dependent*[c]	B	1.6 ± 0.1
AI842432	*Phosphoglucomutase*[c]	B	1.7 ± 0.1
AF071068	*Aromatic amino acid decarboxylase*	I	1.9 ± 0.1
AI845584	*Dual-specificity protein tyrosine phosphatase*[c]	I	1.7 ± 0.1
AJ238636	*Nucleoside diphosphatase (ER-UDPase gene)*	I	1.9 ± 0.1
AF042491	*Membrane-associated progesterone receptor component*	I	1.6 ± 0.1
EST (unannotated)			
AW123697		B	1.6 ± 0.1
AW125453		B	1.9 ± 0.2
AI788959		B	1.6 ± 0.1

[a] B, basal gene; I, inducible gene; B & I, basal and inducible gene.
[b] Genes containing ARE, which are known to be regulated by Nrf2.
[c] Annotated ESTs.
[d] Genes with ARE.

Nrf2 are implicated directly or indirectly in counteracting the cellular stress induced by a wide spectrum of electrophiles and free radicals (Table 1).

Fig. 1 depicts the strategy and the outcomes of the comparisons of transcriptional profiles obtained from the different treatment groups. The strategy we used of comparative analysis of transcriptional profile resulted in both up-regulated and down-regulated genes in each category of comparison. Our focus in the present investigation has been only on genes that are positively regulated by Nrf2, because most of the detoxifying phase 2 proteins depend on this transcription factor for their constitutive and or inducible synthesis. The down-regulated genes in sulforaphane treated/vehicle control comparison using *nrf2*-deficient mice cannot be attributed to Nrf2; however, the role of Nrf2 in the repression of these genes that are present exclusively in "wild

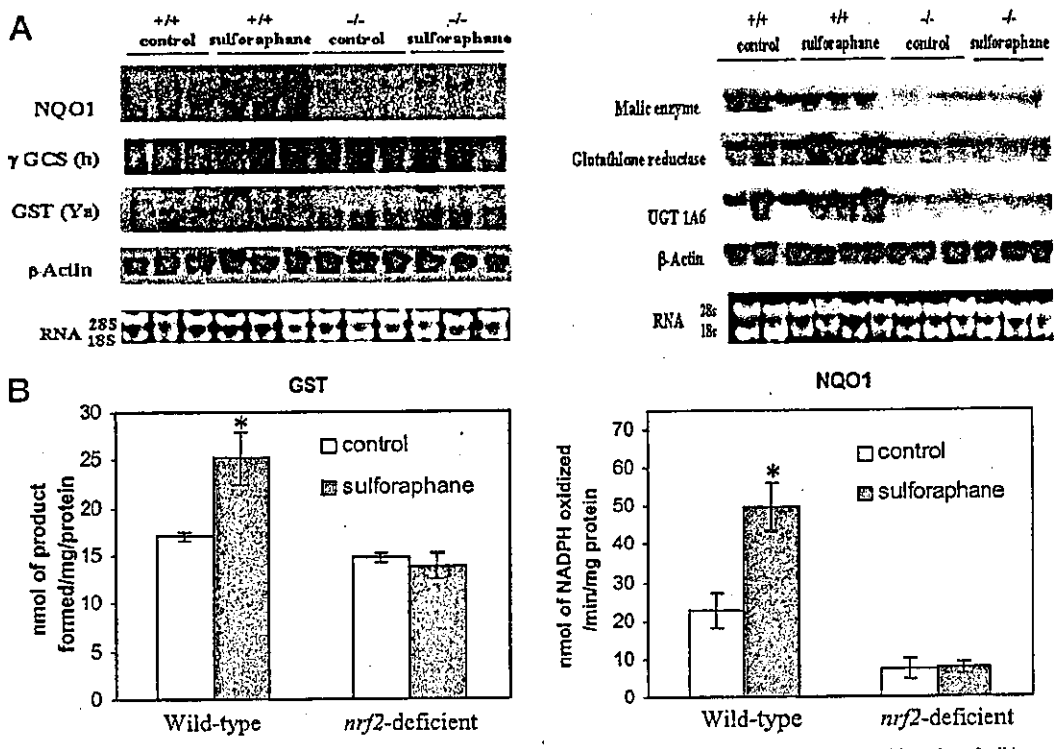

Fig. 2. *A*, Northern blot analysis of mRNA levels of NQO1, GST (Ya), GCS (h), UGT, malic enzyme, and glutathione reductase in small intestine of wild-type [*nrf2* (+/+)] and *nrf2*-deficient [*nrf2* (−/−)] mice treated with either vehicle (corn oil) or sulforaphane to show the basal and inducible expression. *B*, GST and NQO1 activities in the cytosolic fractions of small intestine of wild-type [*nrf2* (+/+)] and *nrf2*-deficient [*nrf2* (−/−)] mice treated with vehicle (corn oil) or sulforaphane. The activities of both enzymes are elevated only in sulforaphane-treated *nrf2*+/+ mice. Values represent means ($n = 3$); *bars*, SE. *, significantly greater from control of same genotypes, $P < 0.05$ (analyzed by Student's *t* test).

type treatment/wild type control" comparisons cannot go unnoticed. It is reported that the balance in expression of Nrf2 and its small maf binding proteins can affect the positive or negative regulation of some genes (31).

Two major cellular pathways of detoxication, *i.e.*, glucuronidation and glutathione conjugation, appeared to be even more dependent on Nrf2-regulated genes than thought previously. The glucuronidation pathway enhances the elimination of many lipophilic xenobiotics and

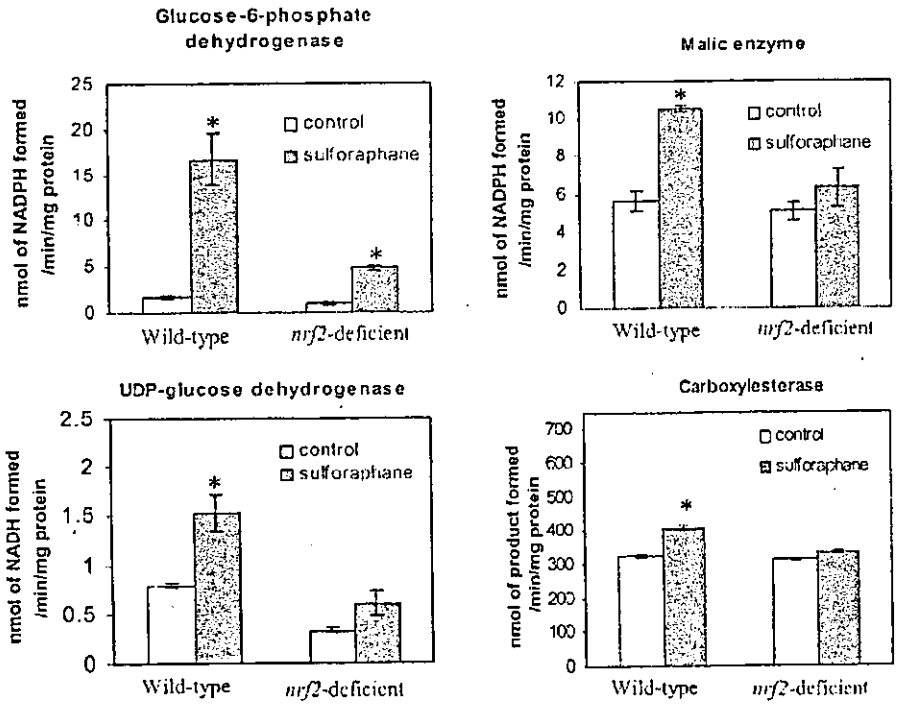

Fig. 3. Specific activities of selected enzymes in the cytosolic fractions of small intestine of wild-type [*nrf2* (+/+)] and *nrf2*-deficient [*nrf2* (−/−)] mice treated with sulforaphane. The activities of all of the enzymes are elevated only in sulforaphane-treated *nrf2* +/+ mice. Values represent means ($n = 3$); *bars*, SE. *, significantly greater from control of same genotypes, $P < 0.05$ (analyzed by Student's *t* test).

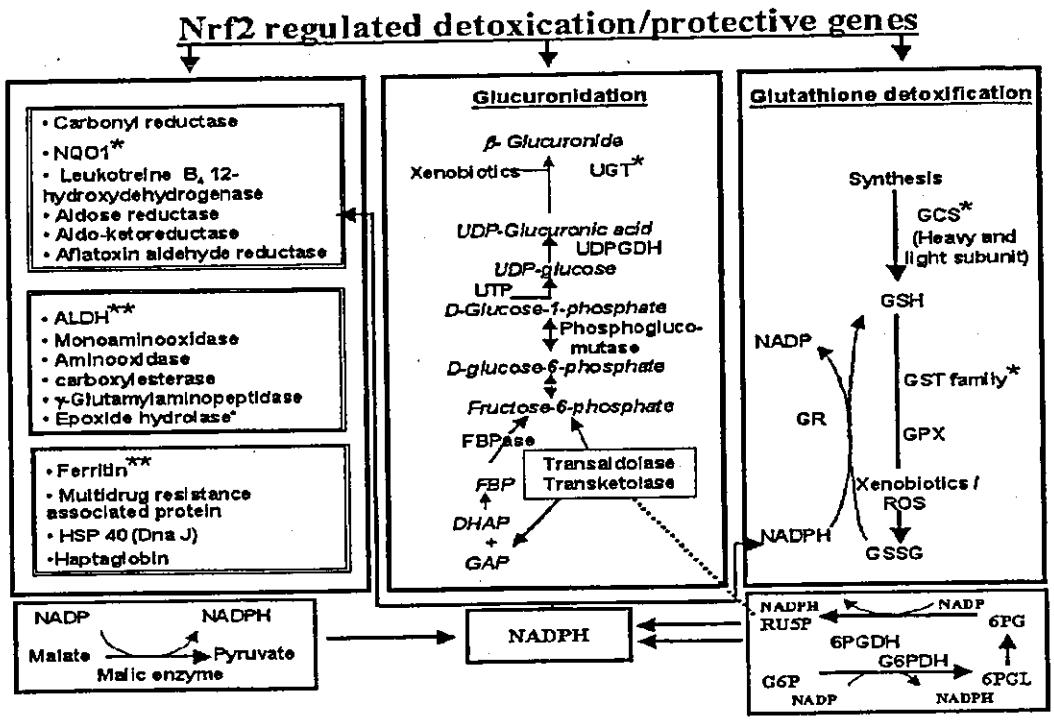

Fig. 4. Probable network of Nrf2-regulated genes involved in the detoxication process. *, genes containing ARE that are known to be regulated by Nrf2. **, genes with ARE.

endobiotics by conversion to more water-soluble compounds. Glucuronidation requires the cofactor UDP-glucuronic acid, and the reaction is catalyzed by a family of UGTs, some members of which are well-characterized Nrf2-regulated genes (32). In the present investigation, we found several new Nrf2-dependent genes (*transketolase, transaldolase, fructose bisphosphatase, phosphoglucomutase,* and *UDP-glucose dehydrogenase*), which are associated with metabolic pathways that may directly or indirectly aid the glucuronidation process (Fig. 4). Transketolase and transaldolase catalyzes the formation of fructose-6-phosphate from the products derived from ribulose-5-phosphate. Furthermore, fructose 1,6-bisphosphatase also catalyzes the conversion of fructose 1,6-bisphosphate to fructose-6-phosphate. Both these reactions may increase the influx of fructose-6-phosphate, which after conversion to glucose-6-phosphate forms glucose-1-phosphate by the action of phosphoglucomutase (Fig. 4). The UDP-glucose that provides the glucuronic acid for UGT-mediated conjugation with xenobiotics is formed from glucose-1-phosphate by UDP-glucose dehydrogenase.

GSTs constitute a family of enzymes that detoxify xenobiotics by conjugating glutathione to a range of electrophilic substrates. The cytosolic GSTs are currently divided into at least eight classes on the basis of their physical and chemical properties (33). In our study, we have found several Nrf2-dependent genes coding for isozymes of GST, including *GST mu (GST5-5), GST mu 1, GST GT8.7, GST GT9.3, GST α3, GST α2 (Yc2), GST α1 (Ya).* In addition, a few ESTs that were homologous to human GST [GST M2 (muscle), microsomal GST 3, and microsomal GST 2] and rat GST required Nrf2 for either basal or inducible expression.

Glutathione using genes such as *GR* and *GPX* [other than the well-known Nrf2 targets, *GST* (33) and *γ-GCS* (34)] were up-regulated in wild-type mice. *GR,* which is involved in the production of reduced glutathione by using NADPH, is up-regulated only in wild-type sulforaphane-treated mice, suggesting the role of Nrf2 in its induction, whereas *GPX,* which is involved in detoxifying various free radicals and peroxides by consuming glutathione (35), was found to be elevated only in the wild-type control mice, indicating the dependence of Nrf2 for basal expression. Lack of or low expression of all these glutathione-associated genes in *nrf2*-deficient mice undoubtedly makes them more susceptible to xenobiotic toxicities (12, 14).

Other than NQO1 (36) and epoxide hydrolase (3), which are known Nrf2 targets, additional targets of xenobiotic detoxication genes regulated by Nrf2 obtained from the screening can be grouped as oxido-reductase, hydrolytic, and oxidative detoxication enzymes. Carbonyl reductase, aldose reductase (fibroblast growth factor regulated protein), aldo-keto reductase, and aflatoxin aldehyde reductase belong to a class of NADPH-dependent oxido-reductases catalyzing the reduction of aldehyde and keto groups of several endogenous and exogenous compounds. The broad range of substrates includes acrolein, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, prostaglandins, steroids, pterins, and biogenic amines (37–42). Carboxylesterase and peptidases belong to the hydrolytic class of detoxication enzymes. Carboxylesterase hydrolyzes xenobiotics containing functional groups such as carboxylic esters (procaine), amides (procainamide), and thioesters (spironolactone; Ref. 43). Several oxidative enzymes, such as aldehyde dehydrogenase, monoamine oxidase, and amino oxidase, are also regulated by Nrf2. ALDH enzymes are involved in the oxidation of xenobiotic aldehydes (44) and also possess esterase activity. *ALDH2,* one of the genes identified in our investigation, is a mitochondrial enzyme that appears to be regulated by Nrf2. It is primarily responsible for oxidizing simple aldehydes such as acetaldehydes (45). Interestingly, the presence of AREs in the 5′ upstream region of ALDH was shown recently (46). This observation is consistent with our findings and suggests the positive regulation by Nrf2 on these classes of genes. Monoamine oxidase, an integral protein of the mitochondria outer membrane is a flavoenzyme that is dependent on Nrf2 for basal expression. It catalyzes the oxidation of structurally diverse amines and xenobiotics such as the neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (47). Leukotriene B_4 hydroxydehy-

drogenase is another basal and inducible oxidoreductase regulated by Nrf2 that has been found to be effective in catalyzing the hydrogenation and detoxication of wide variety of cytotoxic and mutagenic α,β-unsaturated aldehydes and ketones that are major toxic environmental pollutants and products of lipid peroxidation (48–50).

The genes coding for NADPH generating enzymes, such as *G6PDH* and *malic enzyme*, were elevated only in the wild-type control mice, suggesting the dependence of these genes on Nrf2 for basal expression. Recently, *malic enzyme* has been reported to contain an ARE in its promoter region, which corroborates well with our observation (51). However, another NADPH-generating enzyme, 6PGDH, was up-regulated in wild-type mice in response to sulforaphane, indicating that its induction is mediated by Nrf2 (52, 53). Increased formation of NADPH may prove to be beneficial because it is involved in the microsomal monooxygenation of xenobiotics, reductive biosynthesis, maintenance of redox state, and also acts as a potent antioxidant (direct and indirect; Ref. 54). The hexose shunt enzymes, G6PDH and 6PGDH, are also responsible for generating the ribose-5-phosphate necessary for nucleic acid biosynthesis and repair. The coordinated expression of all of these genes involved in detoxication, antioxidant status, and repair suggests an important role for Nrf2 in regulating the cellular defenses against carcinogenic challenges by increasing the reductive capacity of the cell. The microarray data were verified by the observed increases in enzyme activity of G6PDH and malic enzyme in the intestine of wild- type mice treated with sulforaphane (Fig. 3). Although the impact of Nrf2 genotype on the transcript levels of the genes coding G6PDH and malic enzyme appeared to be on basal expression, the activities of these enzymes were elevated in wild-type mice by sulforaphane treatment (nearly 10-fold and 2-fold higher when compared with vehicle, respectively). Also, there was significant increase in G6PDH activity in *nrf2*-deficient mice after treatment with sulforaphane, suggesting that the induction of this enzyme is partly controlled by other transcription factors.

Apart from enzymes, several cytoprotective proteins involved in lessening electrophile toxicity and oxidative stress appear to be regulated through Nrf2. Ferritin (light chain), an antioxidant, known to possess an ARE (55), requires Nrf2 for basal expression. HSP 40, a cofactor for HSP 70, the expression of which is up-regulated by a variety of cellular stresses (56), requires Nrf2 for induction. Another antioxidant protein, haptaglobin, an acute phase protein capable of binding to hemoglobin, thus preventing iron loss and renal damage, is also dependent on Nrf2 for basal expression (57). Multidrug resistance protein is elevated only in wild-type control, suggesting that Nrf2 contributes to its basal expression. Multidrug resistance protein is a ubiquitously expressed protein with several physiological functions, such as protection against heavy metal oxyanions, modulation of the activity of ion channels, and transport of leukotriene C_4 and other glutathione conjugates and glucuronides (58).

We have identified a number of Nrf2-dependent genes (for either basal or inducible expression) that are involved in different biological functions such as *ornithine aminotransferase* [protects against ammonia intoxication (59)], *tryptophan hydrolase*, *aromatic amino acid decarboxylase*, *nucleoside diphosphatase*, *putative membrane-associated progesterone receptor*, and *glucocorticoid-regulated kinase*. The screening also enabled us to identify genes that are induced in response to sulforaphane but not regulated by Nrf2 (genes up-regulated in *nrf2*-deficient treated/*nrf2*-deficient control comparison).

The time of sampling point is very critical to monitor the transcriptional activation of any gene. Probably in the present investigation at the selected sampling point (24 h after the last dose), there may be certain genes whose transcriptional activity might have returned to basal expression levels; such differential outcome between transcript and protein levels has been observed with hepatic GST Ya in oltipraz-treated rats (60). Similarly, with G6PDH and malic enzyme, enzyme activities were high at the selected time point after sulforaphane treatment, but the transcript levels were basal as determined by our microarray data analysis. In addition to the dynamic influence of time of sampling, potency and efficacy of different Nrf2 activators will vary with chemical class and target tissue. Thus, studies of the transcriptional profiles with different Nrf2 activators in various tissues at a range of sampling points may result in identification of additional sets of Nrf2-dependent genes that our study is unable to reveal.

This study expands the scope of the positive, coordinated regulation of a wide variety of cellular defense proteins by Nrf2 and underscores the potential of Nrf2 activation as a strategy for achieving cancer chemoprevention. The genes regulated by Nrf2 include detoxication enzymes as well as antioxidative and cytoprotective proteins that can collectively alleviate the toxicities mediated by a broad range of electrophiles and reactive oxygen species. Future studies aimed at searching for AREs in the promoter of these target genes of Nrf2 will help in deciphering the direct and indirect role of this transcription factor in these actions. In conclusion, this study expands the molecular basis by which the cancer preventive agents such as sulforaphane exert protective efficacy against a broad spectrum of exogenous and endogenous toxicants. Furthermore, understanding of the downstream molecular targets of these anticarcinogens will facilitate their development and use in clinical interventions (61).

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Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system

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Abstract

***Background*: The transcription factor Nrf2 and its negative regulator Keap1 play important roles in transcriptional induction of a set of detoxifying and anti-oxidant enzymes. To gain an insight into our present enigma as to how cells receive oxidative and electrophilic signals and transduce them to Nrf2, we have developed a zebrafish model system for molecular toxicological studies.**

***Results*: We systematically cloned zebrafish cytoprotective enzyme cDNAs and found their expression to be efficiently induced by electrophilic agents. We consequently identified the presence of Nrf2 and Keap1 in zebrafish. Both loss- and gain-of-function analyses demonstrated that Nrf2 is the primary regulator of a subset of cytoprotective enzyme genes, while Keap1 suppresses Nrf2 activity in zebrafish. An ETGE motif, critical for the Nrf2–Keap1 interaction, was identified in the Neh2 domain of Nrf2 by reverse two-hybrid screening and found to be indispensable for the regulation of Nrf2 activity in zebrafish.**

***Conclusion*: Taken together, these results indicate that the Nrf2-Keap1 system is highly conserved among vertebrates and that the interface between Nrf2 and Keap1 forms an important molecular basis of this regulatory system.**

Introduction

Mammalian cells have developed two primary lines of defence against electrophilic carcinogen metabolites and reactive oxygen species, the major causes of malignancy and cellular damage. These lines of defence consist of a high cellular level of anti-oxidants, such as glutathione (GSH), and a family of phase II detoxification enzymes, including glutathione *S*-transferases (GST) and NAD(P)H: quinone oxidoreductase (NQO1). Several lines of evidence indicate that an elevation in the levels of phase II enzymes and GSH protects against chemical carcinogens (Talalay *et al.* 1995). A wide variety of chemical agents can induce the transcription of phase II and anti-oxidant enzymes and genes encoding GSH biosynthetic enzymes, such as γ-glutamylcysteine synthetase (γGCS), which catalyses the rate-limiting reaction in *de novo* GSH synthesis. These inducers include oxidizable diphenols and quinones, Michael reaction acceptors, isothiocyanates, trivalent arsenicals, and hydroperoxides. Interestingly, these inducers do not share any substantial similarity in their structures, but they are all electrophiles capable of reacting with sulfhydryl groups.

Extensive biochemical analyses on the regulatory regions of genes encoding phase II enzymes revealed that the inducible expression is primarily mediated by a *cis*-acting regulatory sequence known as the anti-oxidant responsive element (ARE), or electrophile-responsive element (Primiano *et al.* 1997). Several years later, we discovered Nrf2 in our quest for transcription factors that bind to the ARE. Nrf2, a member of the Cap'n'Collar (CNC) family of transcription factors possessing a basic region-leucine zipper (bZip) structure, targets the ARE and transactivates genes encoding phase II enzymes (Itoh *et al.* 1999a; Hayes *et al.* 2000). This idea was supported by the severe impairment observed in the electrophile-induced activation of a battery of phase II enzymes and GSH biosynthetic proteins in viable Nrf2-deficient mice (Itoh *et al.* 1997; Ishii *et al.* 2000; McMahon *et al.* 2001). Moreover, it has recently been demonstrated that Nrf2-deficient mice are highly sensitive to carcinogen and/or oxidative stress. For instance, Nrf2-null mutant mice are susceptible to benzo[*a*]pyrene-induced neoplasia in

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the forestomach (Ramos-Gomez *et al.* 2001), diesel exhaust-induced hyperplasia and oxidative DNA adduct formation in the lung (Aoki *et al.* 2001), butylated hydroxytoluene-induced pulmonary injury (Chan & Kan 1999), and acetaminophen-induced hepatotoxicity (Enomoto *et al.* 2001; Chan *et al.* 2001).

Our recent studies suggest that Nrf2 activity is controlled by an interaction between Nrf2 and a cytoskeleton-associated protein called Keap1 (Kelch-like ECH Associating Protein 1)(Itoh *et al.* 1999b). Nrf2, which is normally localized in the cytoplasm, translocates to nuclei when cells are exposed to electrophiles. Even in the absence of an electrophilic exposure, forcing the expression of Nrf2 in cultured cells by-passed its cytoplasmic regulation, allowing Nrf2 to induce ARE-dependent gene expression. This induction was reduced by the simultaneous over-expression of Keap1 and de-repressed in the presence of electrophiles. These findings indicate that Nrf2 and Keap1 play critical roles in the induction of phase II enzymes and thus protection against malignancy.

Unravelling the molecular basis of the enzymatic induction defence mechanism will greatly accelerate the search for non-toxic cancer chemoprotective agents that potently induce phase II enzymes (Fahey & Talalay 1999; Kensler *et al.* 1999). The most important issue for solving this mechanism is a clarification of the regulatory mode of Nrf2 activation. For instance, what is the sensor molecule for a wide variety of electrophilic agents and how does this sensor transduce signals to Nrf2?

The zebrafish *Danio rerio* has emerged as an excellent model organism in which to study vertebrate biology. We hypothesized that zebrafish may serve as an excellent model system for addressing issues of toxicology and carcinogenesis, especially the molecular and genetic basis of Nrf2 activation. We know that GST genes can be induced by *trans*-stilbene oxide in flatfish (Leaver *et al.* 1993). Apart from that, however, little is known regarding the transcriptional regulation of phase II enzymes and GSH biosynthetic proteins in fish.

We assumed that fish also possess the Nrf2-Keap1 system for regulating enzymes that are cytoprotective against toxic electrophiles. In order to ascertain whether this regulatory mechanism does actually exist, we isolated zebrafish GSTπ, NQO1 and γGCS-h genes. We found these genes to be highly conserved in the zebrafish and its mammalian counterparts, indicating the importance of this defence mechanism for animal life. We also successfully isolated the zebrafish homologue genes of Nrf2 and Keap1. To our expectation, treatment with electrophiles and/or artificial Nrf2 expression induced *gstp*, *nqo1* and *γgcsh* gene activation in zebrafish, but concomitant over-expression of Keap1 repressed Nrf2 from activating these genes. The important finding here is that the repressive function of Keap1 was diminished by a point mutation in Keap1 that abrogates its interaction with Nrf2. These results therefore unequivocally demonstrate that the Nrf2-Keap1 regulatory system of the phase II and anti-oxidant enzyme genes is highly conserved in vertebrates, from fish to mammals. The zebrafish system seems to be particularly beneficial for molecular mechanistic studies into the underlying toxicology and carcinogenesis *in vivo*.

Results

Induction of detoxifying and anti-oxidant enzyme genes by electrophiles in zebrafish

Recent progress in zebrafish Expressed Sequence Tags (EST) projects led us to hypothesize that most of the detoxifying and anti-oxidant genes known in mammals may also exist in zebrafish. These genes include GST (α, μ, π and θ), NQO1, UDP-glucuronosyltransferase, and microsomal epoxide hydrolase. This knowledge further suggests that the regulatory mechanisms governing the expression of detoxifying and anti-oxidant genes may also be conserved between mammals and fish.

To address this important issue experimentally, we attempted a molecular cloning of several cDNAs encoding detoxifying and anti-oxidant enzymes in zebrafish. We first isolated *gstp*, a π-class GST gene of zebrafish, by exploiting EST database information to polymerase chain reaction (PCR)-amplify a zebrafish cDNA library. The percentage identity of deduced amino acid sequences was higher between zebrafish GSTπ and rat GSTπ (58%) than between zebrafish GSTπ and other classes of rat GST proteins (α, 29%; μ, 29%; σ, 27%; θ, 19%).

We carried out a *gstp* expression analysis in zebrafish larvae, either with or without electrophile treatment, by the whole mount *in situ* hybridization method. Albino fish were used in this analysis, because of their transparency during early larval development. A strong induction in *gstp* expression was observed in 7-day-old zebrafish larvae treated for 6 h with 30 μM of *tert*-butylhydroquinone (tBHQ), a synthetic anti-oxidant metabolized to an electrophilic quinone in cells (Fig. 1A, right panel). In contrast, no induction was observed in control larvae treated with vehicle alone (Fig. 1A, left panel). Induction in *gstp* was also observed in larvae at 4 days of age, but not in 24-h embryos (data not shown). Similarly, RNA blotting analyses indicated that *gstp* expression in larvae at 4 days old is markedly induced by tBHQ in a dose-dependent (Fig. 1B) and time-dependent (Fig. 1C)

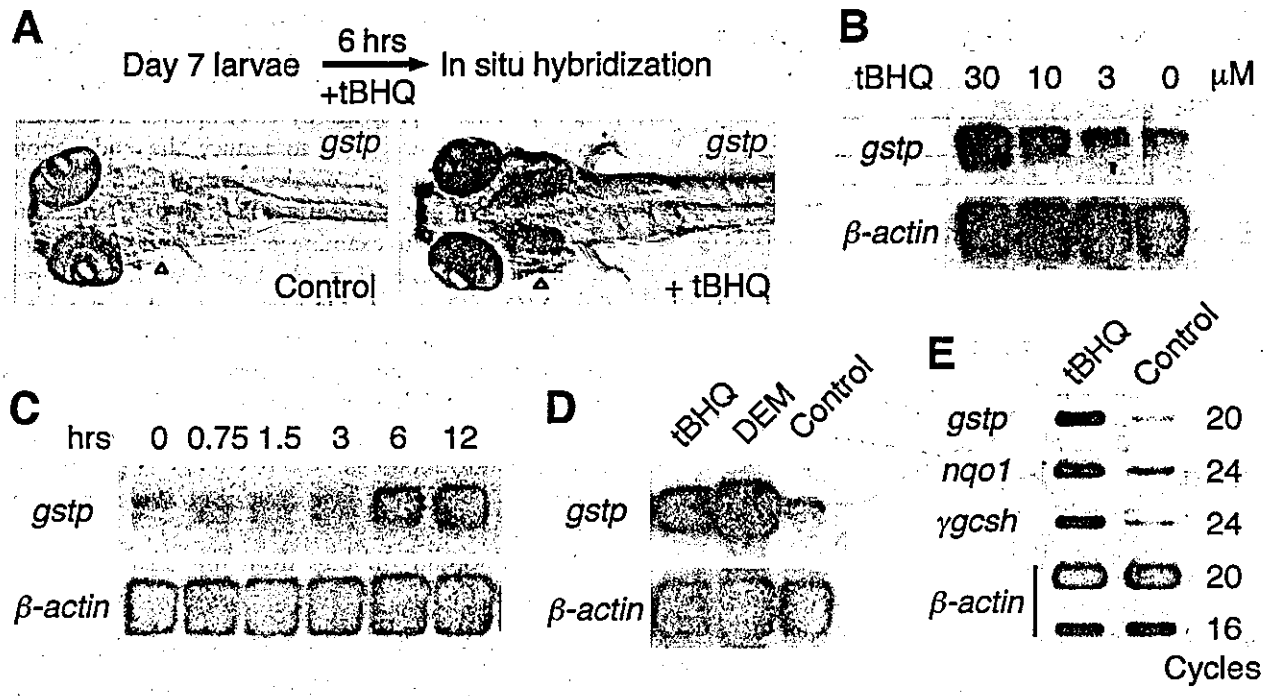

Figure 1 Expression of phase II enzyme genes in electrophile-treated zebrafish larvae. (A) Ventral views of *gstp* expression in 7-day-old larvae after 6 h treatment with 30 μM tBHQ (+ tBHQ) or vehicle DMSO (Control, 0.3%). Induced expression of *gstp* was observed in tBHQ-treated larvae, especially in the gill (open triangles). (B) RNA blot analysis using *gstp* and β-*actin* probes of total RNA from 4-day-old larvae, which were treated with the indicated concentration of tBHQ for 6 h. Expression of *gstp* was induced at a concentration of tBHQ above 10 μM in a dose-dependent manner. (C) RNA blot analysis using *gstp* and β-*actin* probes of total RNA from 4 day-old larvae which were treated with 30 μM tBHQ for the indicated times. Induction became apparent after 6 h. (D) RNA blot analysis using *gstp* and β-*actin* probes of total RNA from 4-day-old larvae which were treated with 30 μM tBHQ or 100 μM DEM for 6 h. (E) RT-PCR analysis using specific primers for *gstp*, *nqo1*, *γgcsh* and β-*actin* of total RNA from 4-day-old larvae which were treated with 30 μM tBHQ or vehicle for 6 h. The numbers indicate reaction cycles performed in the PCR.

manner. These results, therefore indicate that *gstp* gene expression is inducible by tBHQ in zebrafish.

To test whether other electrophilic agents can induce *gstp* expression, we treated zebrafish larvae with diethylmaleate (DEM) 100 μM, and analysed the gene expression by RNA blot analysis. As expected, the GSTπ mRNA level was increased by DEM (Fig. 1D), suggesting that detoxifying and anti-oxidant enzymes in fish can be induced in response to a wide variety of electrophilic agents.

We also examined whether tBHQ can induce other detoxifying and anti-oxidant enzymes, including GSH biosynthetic enzymes. For this purpose, we isolated two additional zebrafish cDNAs encoding NQO1 and γGCS-h by a similar strategy used to isolate GSTπ cDNA. These zebrafish cDNAs showed a high amino acid sequence identity to rat NQO1 protein (51%) and the catalytic subunit of γGCS (71%). The expression of *nqo1* and *γgcsh* genes was markedly induced by tBHQ in zebrafish larvae when examined by RT-PCR (Fig. 1E). These results further support the notion that the regulatory system responding to electrophilic agents by inducing the expression of detoxifying and anti-oxidant enzymes is common among vertebrates.

Identification of the zebrafish Nrf2

The induction of detoxifying and anti-oxidant genes by tBHQ and DEM in zebrafish implies that the Nrf2-Keap1 regulatory system exists in fish. Indeed, we found a cDNA related to Nrf2 in the EST database. To clarify this point therefore, we set about and successfully isolated full-length cDNA clones corresponding to the zebrafish EST clone. The deduced amino acid sequence identity between the zebrafish Nrf2-related clone and mouse Nrf2 is only 46.7%. Two highly conserved domains are present in the human and chicken Nrf2 proteins: the Neh2 domain (Nrf2-ECH homology) (Itoh *et al.* 1999b) and the CNC-type bZip (or Neh1) domain. We found the sequence identity between the Neh2 domains of

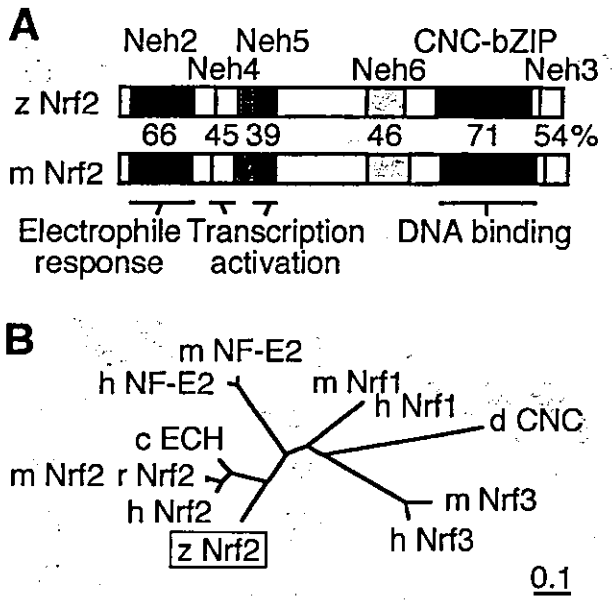

Figure 2 Comparison of zebrafish and mouse Nrf2 proteins, as predicted from their cDNA sequences. (A) Percentages of amino acid sequence identities in the Neh domains between zebrafish and mouse Nrf2. Nucleotide sequence data of zebrafish *nrf2* have been deposited in the DDBJ/EMBL/GenBank databases with the accession number AB081314. (B) Phylogenetic tree of CNC family proteins. Amino acid sequences in the CNC-bZip proteins were analysed. The tree was constructed by the NJ method (Saitou & Nei 1987) using the Clustal W program (Thompson *et al.* 1994). Abbreviations: c, chicken; d, *Drosophila*; h, human; m, mouse; z, zebrafish. Scale bar, genetic distance.

zebrafish and mouse Nrf2 proteins to be 66% and that of the CNC-type bZip (Neh1) domains to be 71% (Fig. 2A). From this high sequence identity between the zebrafish and mouse Nrf2 domains, we concluded that the gene we isolated encodes zebrafish Nrf2 and we will refer to this gene as zebrafish *nrf2*. The functional analyses conducted in this study further supported this conclusion (below).

Phylogenetic trees based on comparisons among the CNC-bZip domain structures unequivocally indicated that zebrafish Nrf2 belongs to the Nrf2 subfamily rather than to the other CNC subfamilies, such as NF-E2 p45, Nrf1 or Nrf3 (Fig. 2B). Intriguingly, zebrafish Nrf2 contains a Neh4 domain (Fig. 2A), a domain conserved only among the Nrf2 subfamily, with 45% identity to the mouse Neh4 sequence (Katoh *et al.* 2001).

Nrf2 is essential for *gstp* induction by tBHQ

Recently, an *in vivo* gene targeting strategy using morpholino phosphorodiamidate oligonucleotide (MO) was established and has been successfully utilized in zebrafish (Ekker & Larson 2001). To examine whether Nrf2 is responsible for the inducible expression of detoxifying and anti-oxidant enzymes by electrophiles in zebrafish larvae, we exploited MO to reduce the endogenous expression level of Nrf2. We designed a specific MO for zebrafish Nrf2 mRNA (MO-nrf2) and injected it into zebrafish embryos to examine its effect on the expression of Nrf2-target genes (Fig. 3A). Day 4 larvae developed from MO-nrf2 or mock-injected embryos were treated with tBHQ (30 μM) or vehicle alone for 6 h and after the tBHQ treatment, the *gstp* expression in the larvae was analysed by RNA blotting analysis (Fig. 3B) or by *in situ* hybridization (Fig. 3C). In both cases, *gstp* expression was efficiently abolished by the MO-nrf2 treatment of embryos. These results indicated that Nrf2 is essential for the inducible expression of *gstp* by tBHQ.

The important findings from this analysis can be summarized into three points. Firstly, based on both structural and functional criteria, zebrafish Nrf2 is an authentic homologue of mammalian Nrf2. Secondly, Nrf2 regulation of detoxifying and anti-oxidant genes appears to be highly conserved among vertebrates. Thirdly, Nrf2 might not be crucial for early morphogenesis in zebrafish, as most of the embryos injected with MO-nrf2 executed normal development (Fig. 3C and data not shown). This observation is consistent with studies in rodents, in which Nrf2-deficient mice develop normally and are fertile (Itoh *et al.* 1997; Chan *et al.* 1996).

Nrf2 is a transcriptional activator in zebrafish embryos

We examined the transactivation activity of zebrafish Nrf2 by co-injection of firefly luciferase (Luc) reporter DNA and synthetic capped RNA providing zebrafish Nrf2 expression. We used pRBGP2 as a testing reporter, which contains three copies of ARE (or NF-E2 binding sequence) tandemly upstream of the rabbit β-globin basal promoter (Igarashi *et al.* 1994). After co-injecting pRBGP2 and Nrf2 mRNA into zebrafish embryos at the one-cell stage, the Luc activities of the whole cell extracts were measured at mid-gastrula. Luc expression was dramatically enhanced in embryos over-expressing Nrf2 compared to that in embryos injected with the reporter gene alone (approximately 75-fold; Fig. 4). This activation was not observed when we used pRBGP3, a reporter construct lacking the ARE sequences.

We recently showed that the Neh4 and Neh5 domains (see Fig. 2A) of the mouse Nrf2 protein bind cooperatively to the transcriptional co-activator CBP (CREB binding protein) and act synergistically to attain a

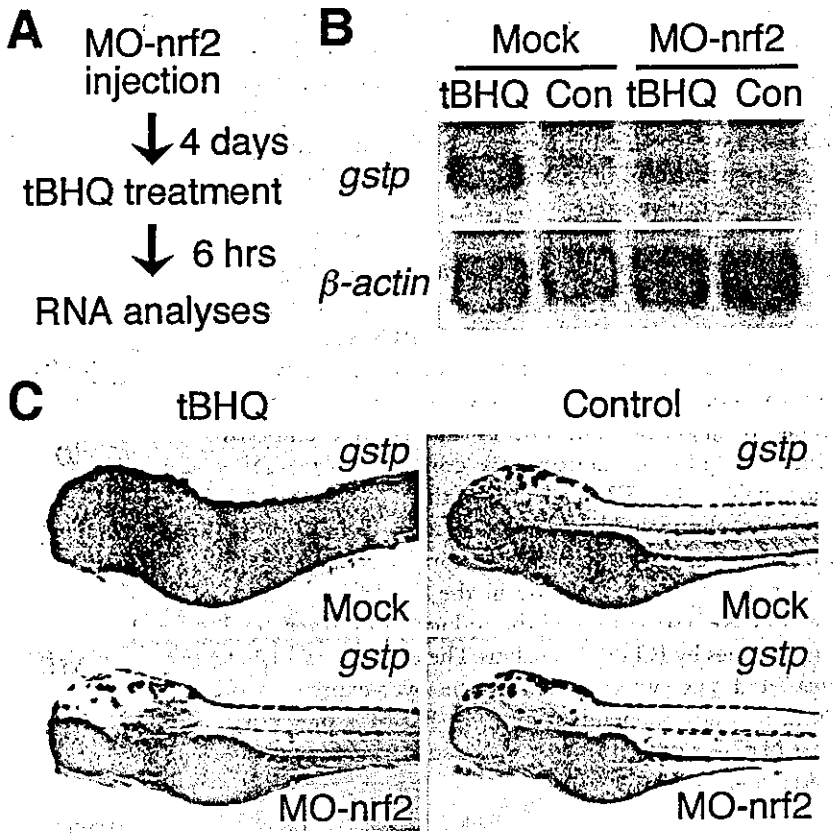

Figure 3 Effect of MO-nrf2 on the tBHQ-induced *gstp* expression. (A) Scheme of experiment. MO-nrf2 was injected into embryos at the one-cell stage. After 4 days, embryos were treated with either tBHQ or DMSO (Control or Con) for 6 h. (B) RNA blot analysis using *gstp* or β-*actin* probes of total RNA from MO-nrf2 (4.5 ng) or mock injected larvae. (C) *In situ* hybridization analysis of MO-nrf2 (9 ng) or mock injected larvae using a *gstp* probe. The same expression pattern was observed in all tested larvae ($n = 10$ for each condition).

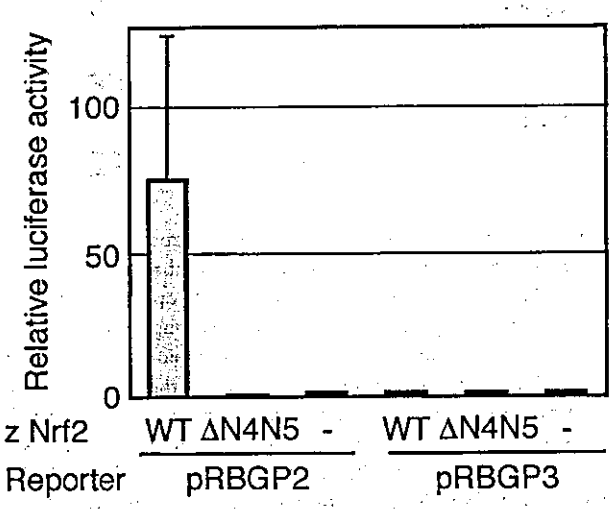

Figure 4 Effect of Nrf2-over-expression on ARE-regulated gene expression in zebrafish embryos. 50 pg of reporter constructs were co-injected with 100 pg of wild-type or mutant Nrf2 mRNA into yolk at the one-cell stage. Luc activity of pRBGP2 or pRBGP3 in the absence of Nrf2 derivatives (denoted as -) was set at 1. Standard deviation values are shown by bars.

maximum transcriptional activation in mouse hepatoma cells (Katoh *et al.* 2001). Since zebrafish Nrf2 possess both Neh4 and Neh5 domains (data not shown), the latter of which includes the FXE/DXXXL sequence known as a CBP binding motif in E1A protein (O'Connor *et al.* 1999), it is quite plausible that these two domains also play a role in transactivation in zebrafish. The contribution of the Neh4 and Neh5 domains to the transactivation activity in zebrafish embryos was assessed using synthetic mutant mRNA for zebrafish Nrf2 in which the Neh4 and Neh5 domains were deleted (ΔN4N5). Over-expression of ΔN4N5 resulted in insignificant transactivation of the pRBGP2 reporter gene (Fig. 4), indicating that the Neh4 and Neh5 domains of zebrafish Nrf2 are fundamentally important for transcriptional activation.

Nrf2 over-expression induces detoxifying and anti-oxidant enzymes in zebrafish embryos

It has been difficult to monitor the transactivation of endogenous target genes by Nrf2 in cultured cell lines (unpublished observations). One possible explanation is