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# Enhanced Expression of the Transcription Factor Nrf2 by Cancer Chemopreventive Agents: Role of Antioxidant Response Element-Like Sequences in the *nrf2* Promoter

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**Induction of phase 2 enzymes, which neutralize reactive electrophiles and act as indirect antioxidants, is an important mechanism for protection against carcinogenesis. The transcription factor Nrf2, which binds to the antioxidant response element (ARE) found in the upstream regulatory region of many phase 2 genes, is essential for the induction of these enzymes. We have investigated the effect of the potent enzyme inducer and anticarcinogen 3*H*-1,2-dithiole-3-thione (D3T) on the fate of Nrf2 in murine keratinocytes. Both total and nuclear Nrf2 levels increased rapidly and persistently after treatment with D3T but could be blocked by cotreatment with cycloheximide. Nrf2 mRNA levels increased ~2-fold 6 h after D3T treatment. To examine the transcriptional activation of Nrf2 by D3T, the proximal region (1 kb) of the *nrf2* promoter was isolated. Deletion and mutagenesis analyses demonstrated that *nrf2* promoter-luciferase reporter activity was enhanced by treatment with D3T and that ARE-like sequences were required for this activation. Gel shift assays with nuclear extracts from PE cells indicated that common factors bind to typical AREs and the ARE-like sequences of the *nrf2* promoter. Direct binding of Nrf2 to its own promoter was demonstrated by chromatin immunoprecipitation assay. Overexpression of Nrf2 increased the activity of the *nrf2* promoter-luciferase reporter, while expression of mutant Nrf2 protein repressed activity. Thus, Nrf2 appears to autoregulate its own expression through an ARE-like element located in the proximal region of its promoter, leading to persistent nuclear accumulation of Nrf2 and protracted induction of phase 2 genes in response to chemopreventive agents.**

Inducers of phase 2 and antioxidative enzymes are known to enhance the detoxication of environmental carcinogens in animals, often leading to protection against neoplasia (14, 24, 26). Use of enzyme inducers as cancer chemopreventive agents in humans is currently under clinical investigation (37, 41). Regulation of both basal and inducible expression of protective enzymes is mediated in part by the antioxidant response element (ARE), a *cis*-acting sequence found in the 5′-flanking region of the genes encoding many phase 2 enzymes such as mouse glutathione *S*-transferase (GST) Ya, human NAD(P)H quinone oxidoreductase (NQO1), and human γ-glutamylcysteine ligase. The core sequence of the ARE has been identified as TGACnnnGC, and several transcription factors are known to bind to the ARE (19, 28, 31, 35, 39). Among these transcription factors, members of basic leucine zipper (bZIP) NF-E2 family such as Nrf1 and Nrf2, which heterodimerize with small Maf family proteins, may be particularly important. Overexpression of Nrf1 and Nrf2 in human hepatoma cells enhanced the basal and inducible transcriptional activation of an ARE reporter gene (39). Recent studies with *nrf2*-disrupted mice indicated that Nrf2 was essential for induction of GST and NQO1 activities in vivo by many different classes of chemopreventive agents, including phenolic antioxidants, 3*H*-1,2-dithiole-3-thione (D3T), and isothiocyanates (19, 27, 30). Moreover, these knockout mice were considerably more sensitive to the toxicities of acetaminophen, butylated hydroxytoluene, and hyperoxia (4, 6, 11) and the carcinogenicity of benzopyrene (34). Collectively, these reports demonstrated a central role for Nrf2 in the regulation of basal and inducible expression of genes that defend against environmental stresses.

Nuclear levels of Nrf2 are increased when peritoneal macrophages are treated with oxidative stressors such as diethylmaleate and paraquat (18). Increased nuclear accumulation of Nrf2 has also been observed in the liver of mice treated with D3T and β-naphthoflavone (26, 27). Initially, this accumulation results from translocation of Nrf2 protein from the cytoplasm. Itoh et al. (20) have identified Keap1, a protein located in the cytoplasm that sequesters Nrf2 by specific binding to the amino-terminal regulatory domain of Nrf2. Administration of sulfhydryl reactive reagents such as diethylmaleate (which are also phase 2 inducers) abolished Keap1 repression of Nrf2 activity in cells and facilitated the nuclear accumulation of Nrf2 (20). Huang et al. (16) have recently proposed that protein kinase C-mediated phosphorylation of Nrf2 could be a critical event for the nuclear translocation of this protein. Involvement of ERK and p38 mitogen-activated protein kinase pathways in the nuclear binding of Nrf2 to the ARE have been proposed by others (45).

We have previously observed that treatment of mice with dithiolethiones led to increased steady-state mRNA levels for Nrf2 in several tissues (27, 34). In this study with a murine cell

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culture system in which phase 2 enzymes were readily inducible, we observed that Nrf2 accumulated in nuclei and in total cellular homogenate after treatment with D3T. mRNA levels for Nrf2 were also increased after treatment. Reporter constructs containing either −35 to −1065 of the murine *nrf2* promoter or nested deletion fragments indicated that intact promoter activity was increased twofold after incubation with D3T and that this activation was blunted when the ARE-like sequences in the promoter were deleted or mutated. Overexpression of Nrf2 doubled the activity of the reporter promoter and coexpression of MafK in the cells further enhanced the promoter activity of Nrf2. However, the mutated ARE-like promoter showed no activation when Nrf2 was overexpressed. Chromatin immunoprecipitation (ChIP) with Nrf2 antibody also indicated association of Nrf2 with its promoter. Collectively, these results suggest that rapid accumulation of Nrf2 within nuclei after treatment with D3T may upregulate its own expression through ARE-like sequences in its promoter. This autoregulation of Nrf2 expression can, in turn, lead to a more sustained signaling of phase 2 gene expression.

## MATERIALS AND METHODS

**Reagents.** All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.), and D3T was provided by Thomas Curphey (Dartmouth Medical School, Hanover, N.H.) (10). [$\gamma$-$^{32}$P]ATP (4,500 Ci/mmol) was purchased from ICN (Costa Mesa, Calif.). The luciferase reporter vector pGLbasic and the assay kit were purchased from Promega (Madison, Wis.) and pTATALuc+ from the American Type Culture Collection (Manassas, Va.).

**Cell culture.** Murine keratinocyte PE cells were established from 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)-induced murine skin papillomas as described previously (46). The cells were maintained in Eagle minimal essential medium containing 10% heat-inactivated and Chelex-treated fetal bovine serum (Life Technologies, Inc., Grand Island, N.Y.), 2 mM $CaCl_2$, and antimycotics or antibiotics (Life Technologies).

**Plasmids.** The sequence of the promoter region of *nrf2* (−1065 to −35) has been published (5), and this promoter was isolated by PCR amplification from hepatic genomic DNA of ICR mice. The isolated PCR product was ligated into pCR2.1 (Invitrogen, Carsbad, Calif.) and a *Sac*I-*Xho*I fragment from this construct was recloned into the luciferase reporter vector pGLbasic (pGLNRP −1063/−35). Deleted sequences of the *nrf2* promoter (−599 to −35 and −429 to −35) were produced by PCR from the full-length promoter and ligated into pGLbasic vector (pGLNRP−599/−35, pGLNRP−429/−35). Sequences containing ARE-like 1 (−574 to −403) and ARE-like 2 (−848 to −684) were also produced by PCR and ligated into the enhancer reporter vector pTATALuc+ (pTATA AREL1, AREL2). Plasmids for overexpression of murine Nrf2 and MafK were made by ligating cDNAs generated by PCR from mouse brain cDNA (Clontech, Palo Alto, Calif.) into pcDNA3 (Promega) (5, 17). A mutant Nrf2 construct, in which the N-terminal region (amino acids 1 to 368), including the transactivation domain, was excluded, was generated as described previously (1). All plasmid sequences were confirmed by sequencing analysis by the DNA Analysis Facility of the Johns Hopkins University (Baltimore, Md.).

**Site-directed mutagenesis.** Mutated ARE-like sequence-containing promoters of *nrf2* were generated by site-directed mutagenesis. Primers containing mutated AREL1 (ACCGTCCTCCGCCAT) or AREL2 (GGCGTCCTGTGGCGC) were used for PCR amplification of mutated *nrf2* promoter, and PCR products were digested with *Dpn*I for 1 h to cleave the wild-type promoter. The sequence of each promoter was verified.

**DNA transfection and luciferase activity.** Cells were transfected at 50% confluency by using Lipofectamine Plus reagent (Life Technologies). Briefly, cells were seeded in 12-well plates at a density of $3 \times 10^4$ to $4 \times 10^4$ cells/well. Cells were grown overnight, and the transfection complex containing 1 μg of plasmid DNA, 0.2 μg of pRLtk plasmid (Promega), and transfection reagent was added to each well in the absence of fetal bovine serum. Medium containing 20% fetal bovine serum was added 3 h after transfection was begun, and cells were incubated for another 16 to 18 h. Cells recovered for 6 h in normal media after removal of the transfection reagents. After incubations with inducers, cells were lysed and *Renilla* and firefly luciferase activities were measured by using the dual luciferase assay kit (Promega) with a luminometer (EG&G Wallac, Inc., Gaithersburg, Md.). Luciferase activities were normalized relative to *Renilla* luciferase activities, the internal control. For overexpression studies, pcDNA3 and pcDNA3–wild-type Nrf2, -mutant Nrf2, or -MafK constructs were cotransfected with the pGL-Nrf2 promoter. Reported luciferase activities are from three to five different transfections.

**Preparation of cell extracts.** Nuclear extracts from PE cells were prepared as described previously (9), and supernatant resulting from isolation of nuclei was centrifuged at $100{,}000 \times g$ for 1 h to obtain the cytosolic fraction. Total homogenate from PE cells was prepared by disrupting cells with a glass-Teflon homogenizer in an extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 10% glycerol, 0.2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined by the modified Lowry method (Bio-Rad, Hercules, Calif.).

**Electrophoretic mobility shift assays (EMSAs).** *Nrf2* ARE-like sequence 1 (GCCCACCTGACTCCGCCATGCCC) or *Nrf2* ARE-like sequence 2 (AACT GGCGCCACAGTCAGCCGGT) was end labeled with [$\gamma$-$^{32}$P]ATP and incubated with 5 g of nuclear extracts from PE cells for 30 min at room temperature in a reaction mixture containing 10 mM HEPES (pH 7.9), 60 mM KCl, 0.5 mM EDTA, 4% Ficoll, 1 mM phenylmethylsulfonyl fluoride, and 0.2 g of poly(dI-dC). For competition binding, a 200-fold excess of cold AREL1 or AREL2, human *NQO1* ARE (GCAGTCACAGTGACTCAGCAGAATCT), NF-E2 (TGGGGA ACCTGTGCTGAGTCACTGGT), and AP-1 (TATCGATAAGCTATGACTC ATCCGGG) consensus binding sequences were incubated with radiolabeled AREL1 and AREL2. For immunodepletion studies, nuclear extracts from D3T-treated cells preincubated with 1 μg of Nrf2 antibody for 2 h. After incubation, loading buffer was added and the reaction products were analyzed on a 4% acrylamide gel (80:1 [acrylamide-bisacrylamide]) and exposed to X-ray film for 16 h.

**SDS-PAGE and Western blotting.** Total cellular homogenate (20 μg), cytosol (35 μg) and nuclear extract (12 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 6% polyacrylamide gel. Gels were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.) at 50 V for 3 h, and immunoblotting was carried out with Nrf2 antibodies reacting with the N-terminal of murine Nrf2 (27). Immunoblotted membranes were developed by using the ECL Western blotting system (Amersham Pharmacia Biotech) as described in the manufacturer's instructions.

**Isolation of RNA and Northern blot hybridization.** Total RNA was isolated by the procedure of Chomczynski and Sacchi (7), and RNA samples were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde and then transferred to nylon membranes (Schleicher and Schuell, Keene, N.H.). cDNA for mouse Nrf2 was labeled with [$\gamma$-$^{32}$P]dCTP by using a random primer labeling kit (Amersham Pharmacia Biotech), hybridized, and washed as described previously (27). After being washed, the membranes were exposed to X-ray film (Eastman Kodak, Rochester, N.Y.) and developed with a Konica (Tokyo, Japan) film processor. Labeled membranes were stripped and reprobed with oligonucleotides for β-actin for loading control.

**ChIP assay.** Formaldehyde cross-linking and immunoprecipitations were carried out as described previously (3, 44) by using an acetyl-histone H4 ChIP assay kit (Upstate Biotechnology, Lake Placid, N.Y.). Briefly, 37% formaldehyde was directly added to cell culture medium at a final concentration of 1%. Cells were incubated for 10 min at 37°C and washed twice with ice-cold phosphate-buffered saline containing protease inhibitor cocktail (Sigma). Nuclei were isolated after Dounce homogenization and resuspended in sonication buffer (1% SDS; 10 mM EDTA; 50 mM Tris-HCl, pH 8.1; protease inhibitor cocktail). Samples were sonicated on ice to an average length of 500 to 1,000 bp by using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, Pa.) and centrifuged at 12,000 rpm. The chromatin solution was diluted 10-fold with dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris-HCl, pH 8.1; 167 mM NaCl); one-third was reserved as a total input of chromatin. Diluted chromatin solution was precleared with salmon sperm DNA–protein A-agarose for 1 h and incubated with either anti-Nrf2 antibody, anti-GATA-1 antibody (Santa Cruz, Santa Cruz, Calif.), nonspecific immunoglobulin, or no antibody for 18 h at 4°C with rotation. Immunoprecipitation, washing, and elution were carried out according to the manufacturer's instructions. Cross-linked immunoprecipitates and total chromatin input were reversed, and samples were treated with proteinase K (Sigma) and extracted with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated with ethanol and resuspended with 30 μl of water. Then, 1 μl of DNA was used for 32 to 36 cycles of PCR amplification with the following primers: *gst Ya* ARE (5′-ACTTGGCAGGAAGGATCAGT-3′ and 5′-TGCTC TAGGTCTCAGTGCAG-3′), *nrf2* AREL-2 (5′-GGCAGTTGGCCTCTTGCA AA-3′ and 5′-CCTGCAGAACCTTGCCCGCT-3′), promoter of β-*actin* (5′-C CGGTCGAGTCGC-GTCCACC-3′ and 5′-GGCGAACTGGTGGC-GGGTGT-

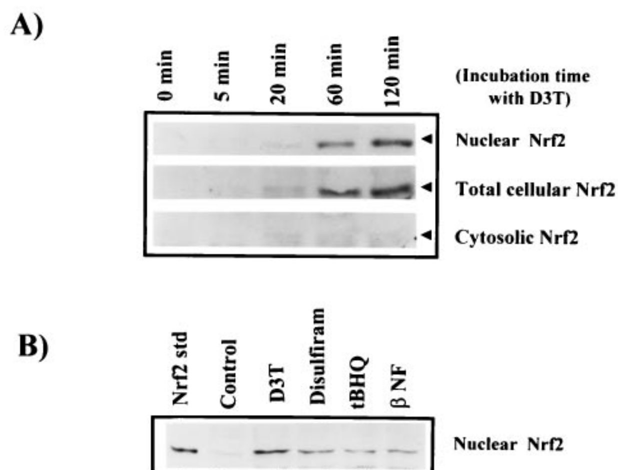


FIG. 1. Effects of D3T on levels of Nrf2 in PE cells. (A) Nrf2 levels were examined in nuclear extracts, total cellular homogenates, and cytosolic fractions after treatment with D3T for different incubation times. Each lane contains three pooled samples. (B) Nuclear extracts were isolated from dimethyl sulfoxide (control)-, D3T (10 μM)-, disulfiram (50 μM)-, *tert*-butylhydroxyquinone (tBHQ) (50 μM)-, or β-naphthofalvone (β NF) (20 μM)-treated cells 6 h after treatment. Immunoblot analyses were performed with Nrf2 antibody. A positive standard for the immunoblot was prepared from Nrf2-overexpressing cells (Nrf2 std).

3′), and hematopoietic enhancer of *GATA-1* (5′-GGATCCAAGGAAGAGAG GAC-3′ and 5′-TTCCTGGAGGTGACAAAGGG-3′).

## RESULTS

**Nrf2 levels are increased in nuclei and total cellular homogenates by enzyme inducers.** Murine keratinocyte PE cells are very responsive to induction of GST and NQO1 activities. For example, NQO1 activity increased fivefold after treatment with 10 μM D3T for 18 h compared to vehicle-treated cells. Therefore, these cells were used to investigate changes in the distribution of Nrf2 after treatment with enzyme inducers by Western blot analysis. Time course studies (Fig. 1A) indicated that Nrf2 levels increased within 20 min of addition of D3T, both in nuclei and total cellular homogenates. At least sixfold increases in Nrf2 were seen after 2-h incubations in nuclear and total cellular homogenate. Basal levels of cytosolic Nrf2 were difficult to detect but appeared to increase slightly after treatment of cells with D3T. Nuclear Nrf2 levels remained elevated for at least 6 h (Fig. 1B). Moreover, treatment with different phase 2 enzyme inducers (D3T [10 μM], disulfiram [50 μM], *tert*-butylhydroquinone [50 μM], and β-naphthoflavone [20 μM]) led to substantial increases in nuclear Nrf2 content compared to vehicle-treated PE cells. Thus, overall levels of the ARE-binding transcription factor Nrf2 increase rapidly and persistently after treatment of PE cells with enzyme inducers.

**Inhibitors of protein synthesis and proteasome function affect the accumulation of Nrf2.** Accumulation of Nrf2 in PE cells was very sensitive to treatment with the protein synthesis inhibitor cycloheximide (CHX), since the cotreatment of 1 μg of CHX/ml with D3T for 3 h completely blocked both the nuclear and total cellular accumulation of Nrf2 triggered by D3T (Fig. 2A). Treatment with CHX for 1 h prior to addition of D3T also blocked the induction of NQO1 and heme oxygenase-1 activities; products of genes known to be regulated through Nrf2-ARE interactions (data not shown). As shown in Fig. 2A, washout experiments, in which the rate of disappearance of Nrf2 from nuclei was monitored, indicated that the addition of CHX significantly enhanced the loss of nuclear Nrf2. Thus, while continued incubation of PE cells with D3T from 3 to 4.5 h maintained a constant elevation of nuclear Nrf2, removal of D3T from cells at 3 h led to a 50% reduction in nuclear levels of Nrf2 50 min later. Removal of D3T at 3 h coupled with addition of CHX led to a 50% reduction in 30 min and a complete loss of Nrf2 in nuclei within 90 min. Similar results were seen when total cellular levels of Nrf2 monitored. These observations suggest that maintenance of elevated nuclear Nrf2 depends upon de novo synthesis of Nrf2 after treatment with D3T.

Protein degradation also appears to play a role in the regulation of Nrf2. Addition of the 26S proteasome inhibitor, MG132 (15 μM) increased the level of total cellular Nrf2 after incubation of PE cells for 3 h but did not increase the nuclear level of Nrf2 (Fig. 2B). Apparently, Nrf2 is constantly degraded by the 26S proteasome in uninduced cells, such that inhibition of proteasomes causes accumulation of Nrf2 within cells. Presumably, however, there is an excess of Keap1, the cytoplasmic tether for Nrf2, in the cell to keep Nrf2 sequestered in the cytoplasm rather than allowing for nuclear accumulation as its concentration is increased. However, accumulation of both total and nuclear levels of Nrf2 was greatly increased by coincubation of cells with MG132 and D3T, indicating that D3T increases Nrf2 levels in part through a proteasome-independent pathway. Collectively, the effects of CHX and MG132 suggest that levels of Nrf2 protein are regulated through a balance between rapid protein synthesis and constant degradation. D3T treatment may elevate the level of Nrf2 not only by enhanced translocation but also by enhanced protein synthesis.

**Levels of Nrf2 mRNA are increased by D3T.** The sensitivity of Nrf2 nuclear accumulation to CHX and the additive effect of D3T and the proteasome inhibitor on the accumulation of Nrf2 suggested that transcriptional activation of *nrf2* leading to enhanced synthesis of Nrf2 protein may contribute to the increased level of this transcription factor after D3T treatment. Steady-state mRNA levels of Nrf2 increased ~2-fold 6 h after treatment with D3T (Fig. 2C). mRNA levels returned to basal levels at 24 h, a pattern also observed with nuclear levels of Nrf2 in the liver of mice treated with D3T (27).

**Promoter activity of *nrf2* is increased by D3T.** The promoter activity of *nrf2* was measured by using a luciferase reporter system to further test the hypothesis that enzyme inducers may enhance transcriptional activation of *nrf2* in PE cells. Chan et al. (5) previously described a 1-kb proximal promoter region for *nrf2*, so this region was isolated by PCR amplification from genomic DNA prepared from the livers of ICR mice. The sequence of the isolated promoter determined here matched the sequence reported earlier. This promoter is very GC-rich and contains several putative AP-2 and SP-1 binding sites. Of particular interest, there are two ARE-like sequences at −754 (AREL2; GCCACAGTCA) and −492 (AREL1; TGACTCC GC) from the transcription start site (Fig. 3A). A full-length promoter construct (pGLNRP−1065/−35) was transiently

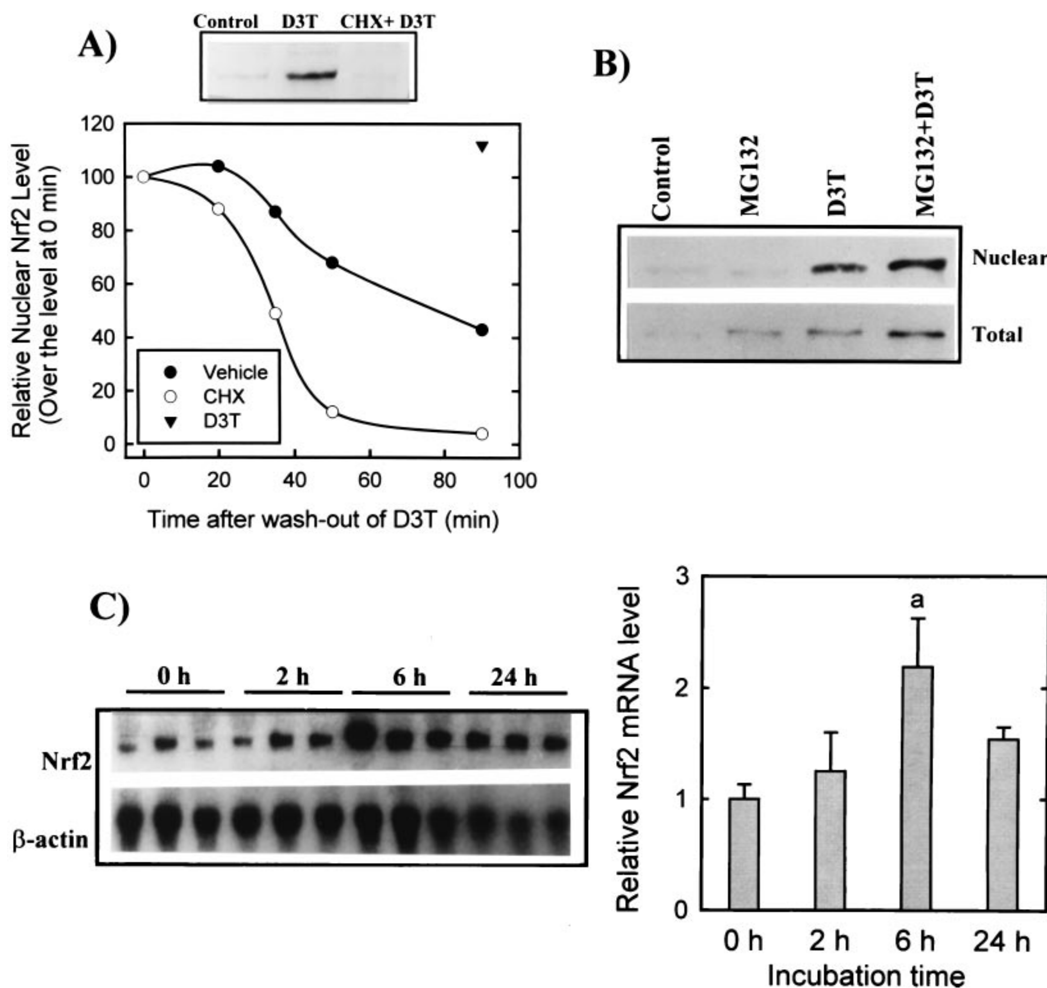


FIG. 2. Effects of protein synthesis inhibitor, CHX, and proteasome inhibitor, MG132, on the accumulation of Nrf2 and effect of D3T on levels of Nrf2 mRNA. (A) In the upper panel, cells were treated with vehicle, D3T (10 μM), or D3T plus CHX (1 μg/ml) for 3 h. Immunoblotting with Nrf2 antibody was carried 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 (TGACTGTGGC → 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-

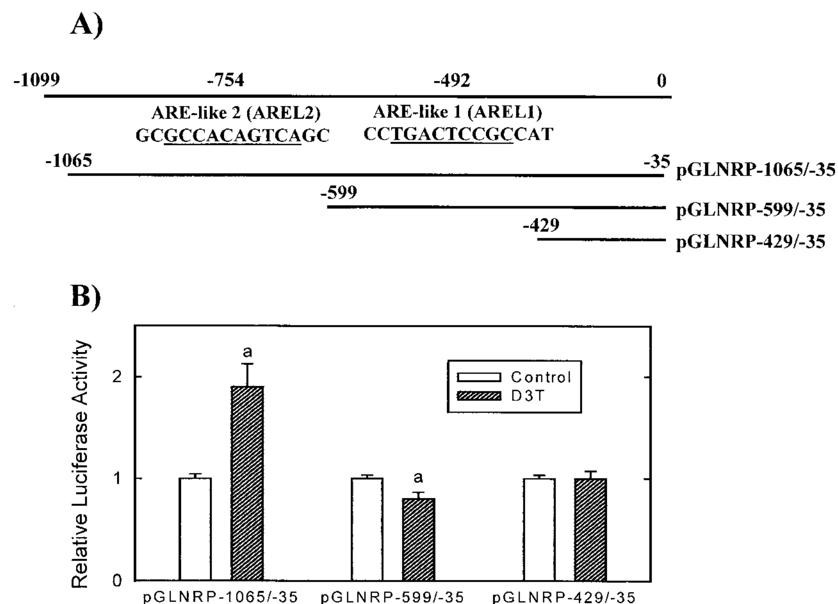


FIG. 3. Effect of D3T on *nrf2* promoter activity in PE cells. (A) A 1-kb portion of the promoter of *nrf2* was isolated from murine liver and ligated into a luciferase reporter vector (pGLNRP−1065/−35) to monitor the activity of this promoter. ARE-like sequences (AREL1; −492, AREL2; −754) are found 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.

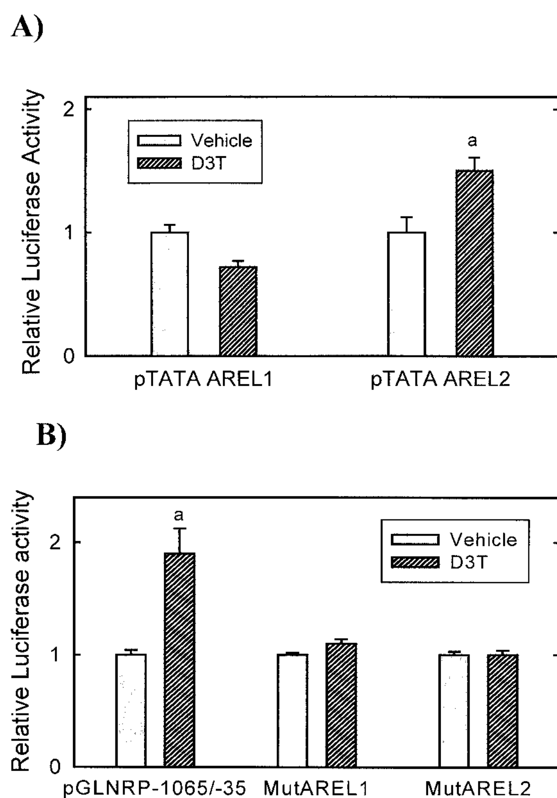


FIG. 4. Effects of D3T on ARE-like-mediated luciferase activity and site-directed mutagenesis studies of the *nrf2* promoter. (A) Luciferase plasmids containing ARE-like sequences from *nrf2* promoter (pTATA AREL1 and pTATA AREL2) were transfected, and the luciferase activities were measured after D3T 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

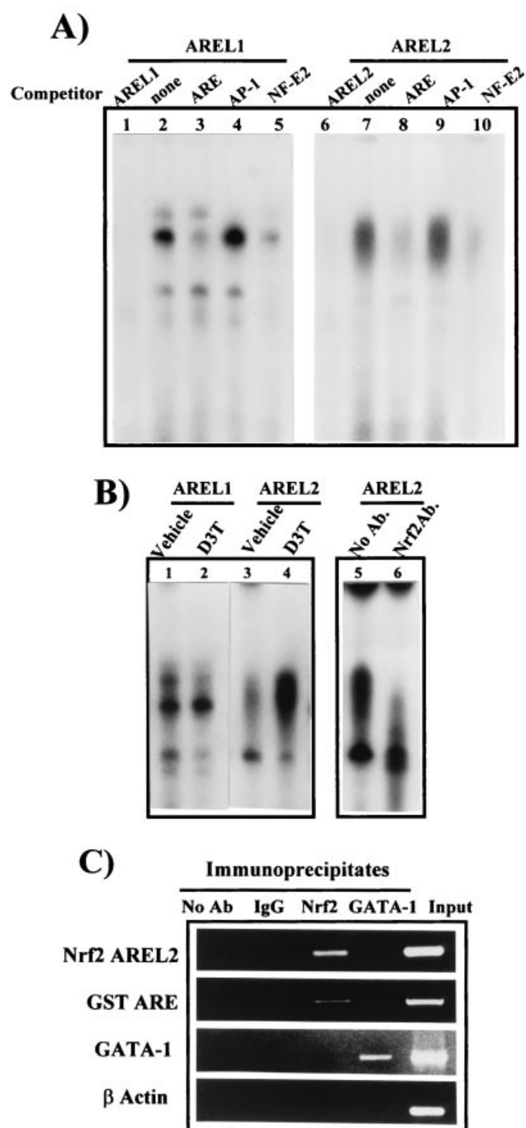


FIG. 5. EMSA analyses with AREL1 and AREL2 sequences and ChIP assay. (A) Competitive binding of AREL1 (lanes 1 to 5) and AREL2 (lanes 6 to 10) with consensus sequences of human *NQO1* ARE, AP-1, or NF-E2 with nuclear extracts from D3T-treated cells. (B) Total binding of nuclear extracts from vehicle- 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.

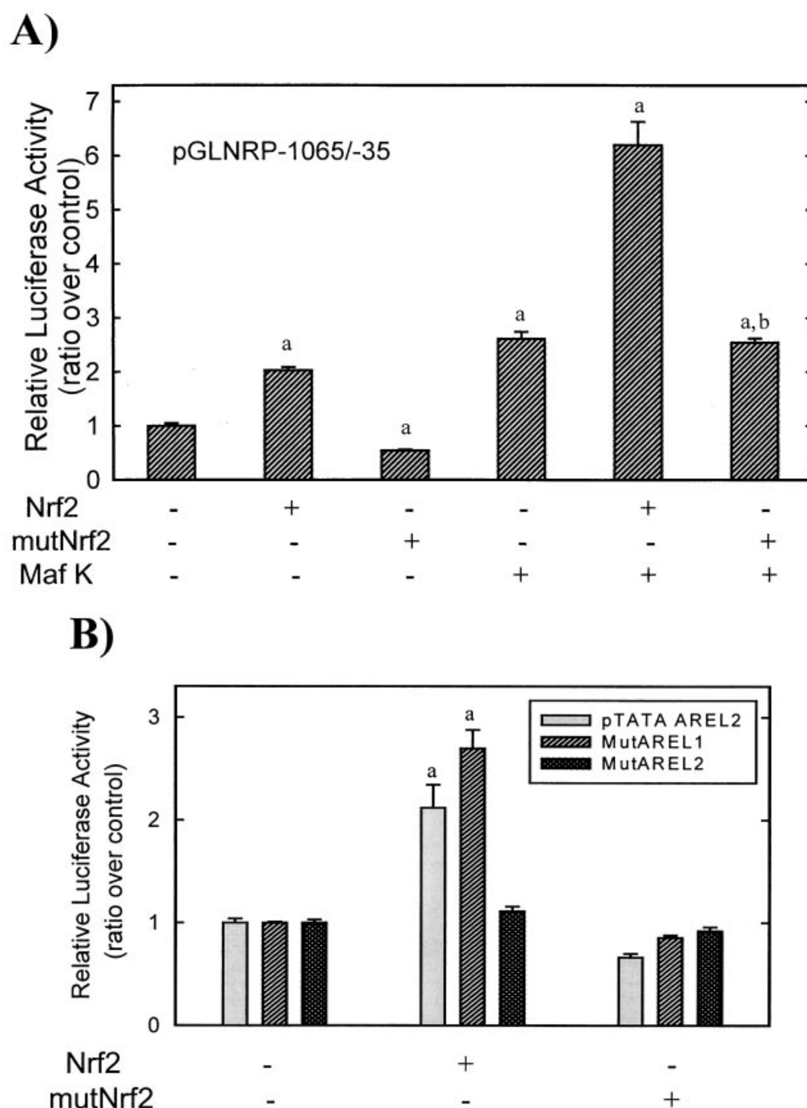


FIG. 6. Effect of overexpression of Nrf2 on the activity of the *nrf2* promoter. (A) *Nrf2* promoter luciferase activity was measured after transfection of wild-type or mutant Nrf2 with or without MafK. Values are means ± the SE from four different experiments. a, $P < 0.05$ compared to blank plasmid-transfected 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 (TG ACTCA) 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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