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Functional Characterization of Transcription Regulators That Interact with the Electrophile Response Element

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The electrophile response element (EpRE), also referred to as the antioxidant responsive element (ARE), is found in the 5′-regulatory region of a number of genes encoding phase II, drug-metabolizing enzymes. Gene knockout studies have demonstrated the primary regulatory role that an Nrf2:Maf dimer plays by binding to nucleotides within the EpRE consensus sequence. Current models of transcription regulation have also shown the involvement of higher-order transcriptional coactivators, proteins that nucleate around DNA sequence-specific transcription factors, enhancing transcription of the target gene by interacting with components of the basal transcriptional apparatus and by enabling chromatin remodeling. Here, we hypothesized that multiple transcriptional regulators, including: (i) a primary Nrf2-Maf heterodimer, (ii) a proposed secondary, EpRE-specific, p160 family coactivator, ARE-binding protein-1, and (iii) a tertiary coactivator, CBP/p300, nucleate to form a complex at the EpRE that regulates transcription of the dependent gene. To test this hypothesis, we constructed a HepG2 cell line which contains a stably integrated green fluorescent protein (GFP) gene; its inducible expression is regulated by a synthetic TK promoter containing a linked EpRE. To identify the involvement of specific, primary and higher-order transcriptional regulators in the EpRE-mediated regulation of the GFP reporter gene, we microinjected antibodies directed against specific transcription factors into the HepG2/GFP cells and determined their effect upon tBHQ-induced expression of the GFP gene. The results demonstrate that microinjected antibodies directed against Nrf2, MafK, CBP and p300 could each, individually, significantly inhibit tBHQ-induced GFP expression. This directly demonstrates the role that the tertiary regulators, CBP or p300, play in mediating EpRE activation of phase II genes, and also implicates the involvement of secondary, p160 family coactivators. Moreover, we found that the same anti-MafK antibody that blocked induction of the EpRE-regulated GFP gene completely ablated the gel-shift complex that we hypothesize contains an Nrf2:Maf dimer, ARE-binding protein-1, and CBP or p300.

Key Words: **electrophile response element; glutathione *S*-transferase; transcription factors; coactivators; GFP; microinjection.**

An electrophile response element has been identified in the 5′ flanking region of genes encoding phase II enzymes such as glutathione *S*-transferase, NAD(P)H: quinone oxidoreductase-1 (NQO-1) and other antioxidant stress proteins such as γ-glutamylcysteine synthetase (γ-GCS), heme oxygenase-1 (HO-1) and ferritin-L (1–5). These stress-associated proteins are widely recognized to provide protection against the toxicities associated with organic electrophiles and oxygen radicals through various mechanisms and thereby ameliorate the risk of oxidative damage-related diseases including cancer (6, 7).

Induction of these EpRE-regulated genes has been shown to occur at the transcriptional level (8, 9). Although many facets of the EpRE cis-regulatory element have been studied extensively, characterization of the trans-acting factors that interact with it has been limited. Studies have shown that the DNA recognition sequence of the NF-E2 erythroid transcription factor is the same as that found within the EpRE. NF-E2 is a heterodimer composed of a p45 unit and a member of the small Maf family of proteins (10, 11). Several p45-related proteins, also referred to as "CNC family" proteins, which includes Nrf-1/LCR-F1/TCF11, Nrf2, Nrf3, ECH and the Bach family of proteins, have been identified (12–16). Members of the Maf family are

Abbreviations used: ARE, antioxidant responsive element; EpRE, electrophile response element; ARE-BP-1, ARE-binding protein-1; GST, glutathione *S*-transferase; tBHQ, *tert*-butylhydroquinone; GFP, green fluorescent protein; SRC-1, steroid receptor coactivator-1; TIF-2, transcriptional intermediary factor-2; CBP, CREB binding protein; CREB, cyclic AMP response element-binding protein; NF-E2, nuclear factor-erythroid 2; Nrf1, NF-E2-related factor-1; Nrf2, NF-E2-related factor-2; TK, thymidine kinase.

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divided into two subgroups, these include: (i) the large Maf proteins, c-Maf, MafB, NrL and MafA/L-Maf, and (ii) the small Maf proteins, MafK, MafF and MafG (17, 18). Human MafG and MafK have been reported to dimerize specifically with Nrf1 or Nrf2 (19). Itoh *et al.* demonstrated that knockout of the Nrf2 gene in mice resulted in a 70% reduction in the inducibility of the EpRE-regulated GST Ya and NQO1 genes (20). Following this, a number of studies have corroborated the primary role that an Nrf2/MafK dimer plays in regulating expression of genes containing an EpRE element (21–24).

In several current model systems of gene regulation, transcriptional regulation has been shown to involve multiple, interacting factors, acting in a hierarchical manner, to achieve transcriptional control of a specific gene. These regulatory molecules include: (i) primary proteins that recognize specific DNA motifs, such as bZip proteins, (ii) secondary proteins that are recruited to promoters by protein-protein interactions with the DNA-binding proteins; these molecules serve as transcriptional coactivators or corepressors, such as the p160 family of 160 kDa proteins including SRC, p/CIP and TIF-2, (iii) tertiary proteins that interact with secondary proteins as well as with the basal transcription machinery; these include CBP and p300, and (iv) proteins that alter the architecture of chromatin (25, 26).

The p160 family of coactivators consists of SRC-1/NCoA-1, TIF2/GRIP3/NCoA-2 and p/CIP/ACTR/AIB1/RAC3/TRAM-1. Because the molecular mass of each of these factors is ~160 kDa, they are collectively termed the p160 coactivator or steroid receptor coactivator (SRC) family (27, 28).

Tertiary regulators of transcription include two proteins, the CREB binding protein (CBP) and p300; both proteins share considerable sequence homology. They are functionally conserved proteins that have been shown to be essential for the activation of transcription by numerous transcription factors, including CREB, AP-1, p53, NFκB, STATs and nuclear receptors (29–34). Moreover, the CBP/p300 proteins have been shown to interact with p160 coactivator proteins (28, 34), essentially providing higher order transcription regulation.

Previous work from our lab with the GST Ya EpRE, lead to the initial identification of a gel-shift complex formed with an EpRE probe that was named "ARE-BP-1" (35, 36). We showed that, within a family of linker-scanning mutants of the EpRE, the ability to form the high molecular weight ARE-BP-1 complex segregated without exception with the ability of a particular EpRE sequence to support induction. Subsequent UV-cross-linking studies (36) showed that although the dominant cross-linked protein within the ARE-BP-1 complex had a molecular weight of 160 kDa (we named the protein ARE-binding protein-1), the weight of the ARE-BP-1 complex in its native state was far heavier, consistent with the presence of additional proteins, such as an Nrf2:Maf dimer, and possibly a CBP or p300 molecule.

Because the molecular weight of ARE-Binding Protein-1 was found to be 160 kDa, we hypothesized that it is a member of the p160 family of transcription coactivators. Consistent with other current, p160-containing models, one would then predict that multiple transcriptional regulators, including the bZip transcription factors Nrf2 and Maf, a p160 family coactivator, and CBP/p300 form a complex at the EpRE that regulates expression of the EpRE-dependent gene.

To investigate the possible participation of these primary, secondary and tertiary level regulators of EpRE-mediated transcription, we produced a HepG2 cell line that contains an EpRE-regulated green fluorescent protein expression cassette. Microinjection experiments were done in which antibodies against pertinent primary and tertiary regulators were injected into the HepG2 cells; acceptable antibodies against p160 family coactivators were not available for these microinjection studies. Following a 24-h treatment of the injected cells with tBHQ, we then measured the induced level of GFP expression in each microinjected cell. We found that microinjection of specific anti-Nrf2, MafK, CBP and p300 antibodies could efficiently ablate tBHQ-induced GFP expression, which supports the proposition that the EpRE is a nucleation site for primary, secondary and tertiary regulators which together regulate transcription of EpRE-dependent genes.

MATERIALS AND METHODS

Materials. The HepG2 human hepatoma cell line was obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM with high glucose containing 10% fetal bovine serum supplemented with 0.1% gentamicin (Life Technologies Inc., Gaithersburg, MD). The cells were grown at 37°C in a humidified 5% CO_2/95% air atmosphere. The antibodies against MafK and small Maf were gifts from Dr. Yamamoto (University of Tsukuba, Japan) and Dr. Igarashi (Tohoku University, Japan), respectively. Anti-CBP, p300, Nrf1, Nrf2, v-Maf antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The pEGFP expression vector was purchased from Clontech (Palo Alto, CA). tBHQ was purchased from Fluka Chemika (Milwaukee, WI).

Construction of ARE-TK GFP plasmids. A synthesized fragment containing a 41 bp GST Ya EpRE motif (TAGCTTGGAAATGACAT-TGCT AATCGTGACAAAGCAACTTT) and/or a 123 bp thymidine-kinase (TK) promoter fragment were inserted into the multiple cloning site of the green fluorescent protein expression vector pEGFP (Clontech, Palo Alto, CA) generating ARE-TK/pEGFP and TK/pEGFP constructs, respectively. The construct DNAs were purified through Qiagen columns (Qiagen Inc., Santa Clarita, CA) and confirmed by restriction analysis and sequencing.

Transfections and assay. HepG2 cells were seeded at a density of 1×10^5 cells/60 mm plate 24 h prior to transfection. Cells were transfected with 2 μg of either the ARE-TK/pEGFP or TK/pEGFP control plasmids using Lipofectin (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Clones resistant to 1.0 mg/ml G418 (Life Technologies, Grand Island, NY) were iden-

tified. After 2–3 weeks, colonies were picked using a microscope and transferred into wells of a 24-well plate for expansion.

Electrophoretic mobility-shift assays. Gel-shifts were performed according to the method of Costa *et al.* (37). Briefly, 3× reaction buffer [40 mM Hepes, pH 7.9, 80 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 0.2 mM EGTA, 8% Ficoll (M_r = 400,000)], 2 μg nonspecific poly(dI-dC) competitor (Sigma) and 9 μg of nuclear extract were mixed and incubated for 10 min at room temperature. [^{32}P]-internally labeled, gel purified, double-stranded DNA was added, and the mixtures were incubated for an additional 10 min. The samples were electrophoresed through a pre-run 5% native acrylamide gel in 0.5× TBE. The gel was dried, and the positions of retarded protein–DNA complexes were visualized using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Microinjection of antibodies into HepG2 cells. HepG2 cells, containing the stably integrated GFP expression cassette, were seeded on acid-washed glass coverslips and grown in DMEM medium supplemented with 10% fetal bovine serum and 0.1% gentamicin. Before injection, cells were rendered quiescent by incubation in serum-free medium for 24 h. Antibodies at 1.0 mg/ml were injected into the nuclei of cells. Antibodies directed against Nrf1, Nrf2, CBP, p300, v-Maf, MafK or small Maf were coinjected with rhodamine-conjugated dextran (0.1 mg/ml) as a microinjection marker. Control cells were injected with rabbit IgG (1.0 mg/ml) and the rhodamine–dextran conjugate alone. When microinjection was started, the medium was overlaid with a thin layer of sterile 0.9% NaCl-saturated mineral oil to prevent evaporation and retard pH changes. Microinjection was performed using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. Glass capillaries (Sutter Instruments, Novato, CA) were pulled with a vertical pipette puller (Kopf Instruments, Tujunga, CA). The injecting microneedle was attached to an IM 200 microinjector (Narishige, Tokyo) preset to a constant outflow pressure of 0.3–0.4 psi (1 psi = 6.89 kPa) and was back-loaded with antibody solution using microloader pipette tips.

Measurement of green fluorescent protein intensity—GFP intensity in stably transfected cells. 3×10^4 HepG2/GFP cells, which had been stimulated with 90 μM tBHQ or vehicle for 24 h, were transferred into wells of a 96 well black plate with clear well bottoms. The measurement of GFP was performed directly using a fluorescence microplate reader (Molecular Dynamics, Sunnyvale, CA) with excitation/emission at 485 nm/530 nm.

GFP intensity in individually injected cells. Cells that had been injected were identifiable by red fluorescence within the cell from the coinjected dextran-conjugated rhodamine B. One hour following injections, the injected cell dishes received 90 μM tBHQ for the next 24 h. After this 24 h treatment, cells were rinsed three times with PBS, and then fixed with 4% paraformaldehyde for 15 min at room temperature. After three washes with PBS of the fixed cells on coverslips, the coverslips were mounted using SlowFade mounting medium and viewed with a Zeiss Axiophot fluorescence microscope. Images were collected on a laser scanning confocal microscope. The 488- and 568-nm lines of a krypton/argon laser were used for excitation of GFP and rhodamine B, respectively. Acquired fluorescence images of the cells were managed using LaserSharp software and digitized using ScanImage and Adobe Photoshop software.

Statistical analysis. Using ScanImage software, the GFP expression level in single microinjected cells was determined by measuring the number of green pixels (or, total green fluorescence) per unit area of each cell that also showed rhodamine B fluorescence. The mean and SD for green pixel counts per unit cell area were determined from at least 100 microinjected cells. Comparisons of differences in green intensity were made using the Student *t* test. Differences were declared significant if $P < 0.05$.

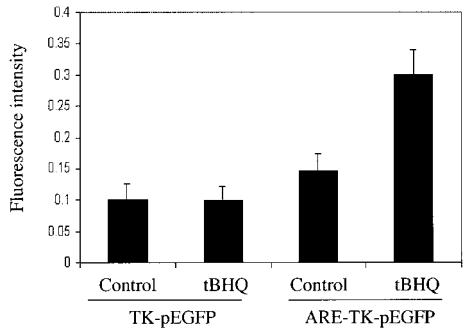

FIG. 1. Induction of GFP expression by tBHQ in stably transfected HepG2 cells. Stably transformed clones of HepG2 cells, carrying either an integrated ARE-TK-pEGFP construct (HepG2/GFP-B clone) or TK-pEGFP construct, were exposed to 90 μM tBHQ in the medium for 24 h. GFP intensity in each well was measured directly using a fluorescence microplate reader.

RESULTS

tBHQ-induced expression of green fluorescent protein. Following transfection with the ARE-TK-GFP expression cassette and selection in G418, individual HepG2 cell clones were screened to identify those clones that had a low fluorescence background, gave the strongest GFP fluorescence signal following tBHQ treatment, and retained these characteristics over time in culture. Cells from one of these isolated clones, HepG2/GFP-B cells, were plated in 96-well plates and treated with 90 μM tBHQ for 24 h. Induced GFP intensity was measured directly in plate wells using a fluorescence microplate reader. tBHQ treatment induced a significant increase (up to 2.5-fold) in the expression level of the GFP reporter gene in HepG2/GFP-B cells, whereas no induced GFP expression was seen in HepG2 cells carrying only the control TK-GFP construct (Fig. 1). As Fig. 1 shows, after tBHQ treatment, there was no induction of GFP expression in cells carrying only the TK-GFP expression cassette; this indicates that the tBHQ-induced expression of GFP in HepG2/GFP-B cells was solely mediated through the EpRE.

Microinjection of antibodies into GFP-expressing cells. To corroborate the regulatory role of Nrf2 and Maf transcription factors, and explore the role of the tertiary coactivators CBP and p300 in EpRE-dependent gene expression in intact cells, we performed a series of single cell microinjection studies (Fig. 2). Solutions of antibodies directed against Nrf1, Nrf2, CBP, p300, v-Maf, MafK and small Maf were individually injected into HepG2/GFP-B cells. For the 24 h following the injections, cells were exposed to 90 μM tBHQ in the growth medium. Following fixation of the cells, and confocal microscopy to record both the red and green fluorescence intensity of each cell (Fig. 2, upper and lower panels), digital images were quantified to determine the level of green fluorescence intensity in in-

jected cells (cells showing rhodamine fluorescence with red activation/emission filters) and in noninjected cells or in cells injected only with a control IgG preparation. Figure 2A, shows no diminution of green fluorescence in cells injected with the control IgG and rhodamine conjugate, but there are clearly discernible decreases in green fluorescence in cells injected with antibodies directed against Nrf2, CBP, p300, and MafK (Figs. 2B–2E, respectively). For each antibody group, the digital images from at least 100 cells were analyzed and the average green fluorescence intensity per cell recorded.

Figure 3 illustrates in summary form the level of green fluorescence per cell for each of the nine microinjection groups. A significant decrease ($P < 0.05$) in green fluorescence was seen in cells injected with several different antibodies that were available against Nrf2, and Maf transcription factors. Essentially all induced expression of GFP was ablated by the Nrf2 and Maf antibodies because the GFP level per injected cell was not significantly different than the level of green fluorescence in cells untreated with tBHQ (i.e., Fig. 3, DMSO bar). Interestingly, an antibody directed against Nrf1, a structural homologue of Nrf2, showed no effect in inhibiting tBHQ-induced GFP expression.

Both Figs. 2C and 2D and 3, CBP and p300 bars, show that injections with antibodies directed against the tertiary coactivators CBP and p300 were as effective ($P < 0.05$) in ablating induced green fluorescence as the antibodies directed against the primary DNA binding molecules, Nrf2 and Maf.

Anti-Maf antibody ablates the ARE-BP-1 gel-shift complex. Because ARE-BP-1 (35, 36) has not yet been isolated, there are no antibodies currently directed against this molecule. Based upon previous work, we believe that the ARE-BP-1 protein, because its molecular weight is 160 kDa (36), serves as a coactivator of the primary Nrf2:Maf heterodimer that binds directly to the ARE consensus sequence, in much the same manner as other 160 kDa coactivators, like SRC, p/CIP and TIF-2, serve as coactivators.

Figure 4 shows a gel retardation study using a 41 bp, ^{32}P-labeled EpRE consensus probe and a nuclear extract prepared from Hep G2 cells. Two specific complexes are formed (lane 1), a high-molecular-weight complex that we previously characterized (35, 36) and named ARE-BP-1, and a lower molecular weight complex (complex B). In lanes 3–5, we see that increasing concentrations of the same anti-MafK antibody that ablated induction when microinjected into cells also disrupts both the ARE-BP-1 complex and the lower molecular weight "B" complex.

DISCUSSION

In this paper, we constructed a reporter system with which to study the regulated transcription of EpRE-controlled genes. Combining this cell-based reporter system with microinjection of antibodies, we: (i) corroborated the important regulatory roles of Nrf2 and Maf transcription factors, (ii) demonstrated the participatory role of the tertiary coactivators CBP and p300, and implied the participatory role of secondary, 160 kDa coactivators; in numerous other models, these molecules are known to bind to both primary bZip dimers and tertiary CBP/p300 coactivators, and (iii) showed that an anti-Maf antibody that could block EpRE inducibility in the HepG2 reporter cells could also ablate formation of the high molecular weight ARE-BP-1 gel-shift complex. This complex is hypothesized to contain an Nrf2:Maf dimer, a 160-kDa coactivator that we call ARE-binding protein-1, and a tertiary coactivator, such as CBP or p300.

The bZIP transcription factor Nrf2, when dimerized with Maf, has been shown to bind to the EpRE motif and mediate transcriptional activation of EpRE-dependent genes. Recent studies demonstrated that Nrf2 is normally bound to Keap1 in the cytoplasm, and upon exposure of cells to an oxidative stress or an electrophilic chemical, it dissociates and translocates to the nucleus (38). Nrf2 then heterodimerizes with a member of the small Maf family of transcription factors and activates gene transcription by binding to the EpRE. In the present study (Figs. 2 and 3), microinjection of either a low (0.1 mg/ml) or a high (1.0 mg/ml) concentration of an anti-Nrf2 antibody blocked nearly 100% of tBHQ-associated induction. The consistency of our "antibody-mediated knockout" data with the earlier Nrf2 gene knockout data from Itoh *et al.* (20, 24) confirm the utility of the antibody microinjection strategy for identifying key participants in the regulation of EpRE-mediated transcription.

In published studies, Nrf2 was shown to be more efficient than Nrf1 in up-regulating hARE-mediated CAT gene expression (39). Toki *et al.* found very strong activation of reporter gene expression by Nrf2 alone in COS cells, but only modest transactivation was observed with p45 and Nrf1 (19). In our experiments, we found that an anti-Nrf1 antibody had no effect upon tBHQ-induced GFP expression (Fig. 3). This result could be explained by differences in binding affinity of Nrf1 and Nrf2 to the EpRE motif, or differences in the abilities of Nrf1 and Nrf2 to heterodimerize with small Maf proteins (39).

Small Maf proteins have been shown to activate gene expression by forming heterodimers with other bZip transcription factors, such as Nrf1, Nrf2, as well as members of the AP-1 family (17, 18). In our present study, we found that two of the three microinjected Maf antibodies that we tried significantly decreased EpRE-regulated GFP expression (Fig. 3). Presumably, the active Maf antibodies blocked the binding of Maf to: (i) Nrf2, (ii) the EpRE DNA motif, or (iii) a putative p160 coactivator.

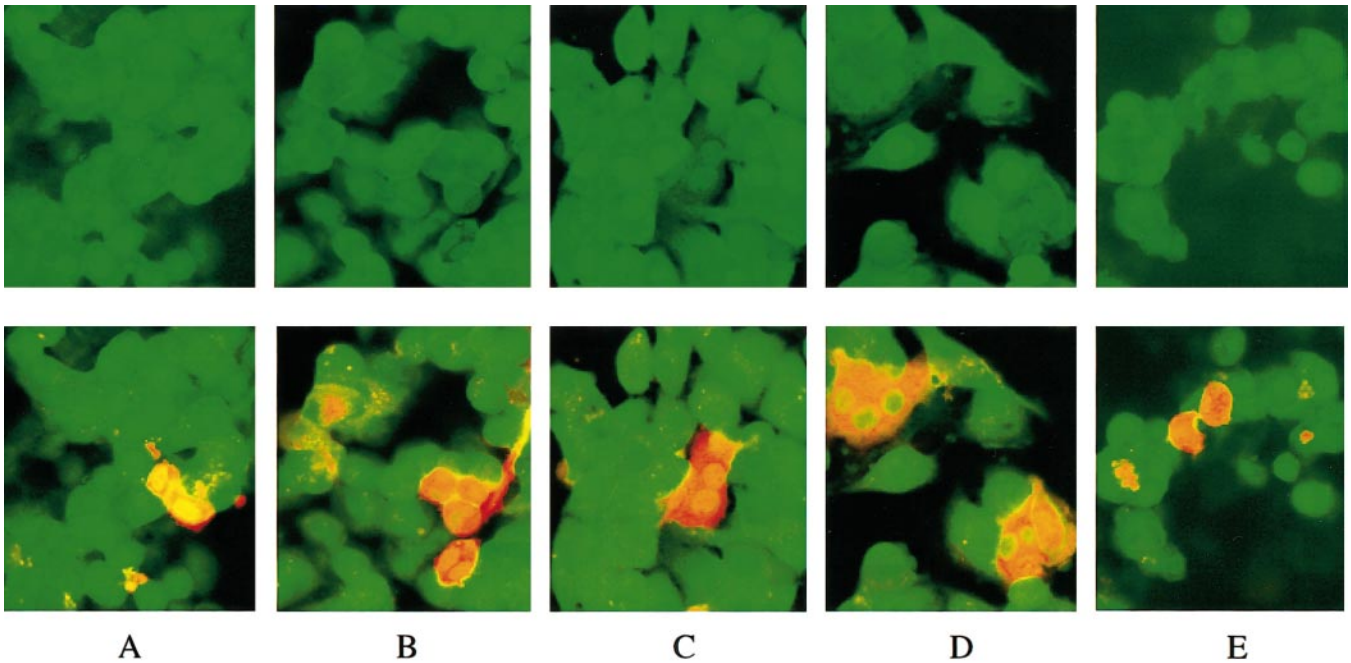

FIG. 2. GFP expression in tBHQ-treated HepG2/GFP-B cells microinjected with antibodies directed against key transcription factors. Successfully microinjected individual cells were identified by the red fluorescence of the dextran-conjugated rhodomine B which was coinjected with antibody solutions (bottom panels). Confocal images of cells were then taken using green fluorescence filters (top panels), and the amount of green fluorescence signal per cell was determined using ScanImage software. Representative images of cells microinjected with either control IgG (A) or antibodies directed against Nrf2, CBP, p300, MafK (B–E, respectively) are shown.

Interestingly, we found that an anti-MafK antibody that could block inducibility when microinjected could also ablate formation of the ARE-BP-1 gel-shift complex (Fig. 4). Earlier work from our lab (35) showed that, within a family of linker-scanning mutants of the EpRE, the ability to form the high molecular weight ARE-BP-1 complex segregated perfectly with the ability of a particular EpRE sequence to support induction. Subsequent UV-cross-linking studies (36) showed that although the dominant cross-linked protein within the complex had a molecular weight of 160 kDa (we named the protein ARE-binding protein-1), the weight of the complex in its native state was far heavier, consistent with the presence of additional proteins, such as an Nrf2:Maf dimmer, and possibly a CBP or p300 molecule. Though indirect, we believe that the ability of the Maf antibody to ablate the ARE-BP-1 complex supports the model for transcription factor assembly at the EpRE shown in Fig. 5. Complex "B" in the Fig. 4 gel-shift is most plausibly explained as an Nrf2:Maf heterodimer bound to the EpRE probe.

It has recently become clear that higher-order p160 and CBP/p300 coactivators play a central role in the transcriptional regulation of a variety of human genes. To date, several distinct but related p160 family members have been characterized, including SRC-1/NCoA-1, TIF2/GRIP3/NCoA-2 and p/CIP/ACTR/AIB1/RAC3/TRAM-1, with each family member having a number of splice variants (27, 28). Although all three p160 family members do possess similar properties in term of enhancement of transcriptional activation, several reports suggest that their activities are not completely overlapping and particularly outline a division between SRC-1 and TIF2/GRIP1 versus p/CIP/ACTR/

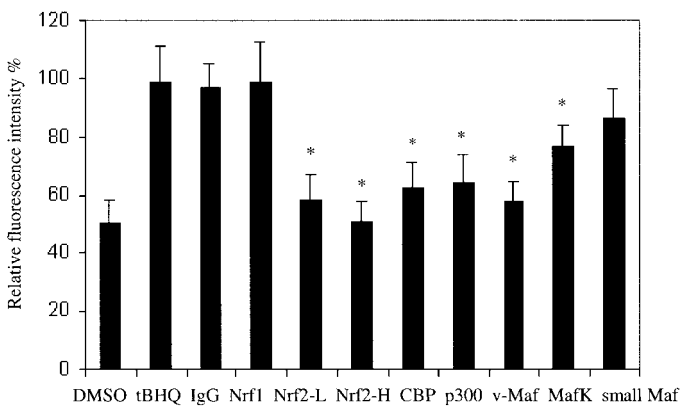

FIG. 3. Quantitative effect of microinjected antibodies on the tBHQ-induced expression of GFP in HepG2/GFP-B cells. Injected antibody solutions were at 1 mg/ml except for the Nrf2-L group which received the Nrf2 antibody at 0.1 mg/ml. After 24 h of stimulation by 90 μM tBHQ, the cells on coverslips were fixed and mounted. Confocal fluorescence images were taken and digitized using ScanImage software. The asterisks indicate those groups that are significantly different from the control IgG group ($P < 0.05$). At least 100 microinjected HepG2 cells were analyzed for each data point.

AIB1/RAC3/ functions. Also, the relative contribution of a particular coactivator may depend on cell or tissue type and/or coactivator levels in these cells (40). Attempts to use anti-SRC or anti-TIF-2 antibodies to ablate the ARE-BP-1 gel-shift complex showed no effect (data not shown). Based upon the aggregate data to date, we propose that ARE-binding protein-1 is a member of the p160 protein family, but that it is sufficiently different in structure to avoid neutralization by antibodies directed against SRC or TIF-2. To determine the relationship between ARE-binding protein-1 and members of the p160 coactivator family, purification and cloning of ARE-binding protein-1 will need to be completed.

In the present study we determined that the transcriptional coactivators CBP and p300 are involved in regulating transcription of EpRE-controlled genes. Transcriptional activation of eukaryotic genes in response to extracellular signals generally requires the recruitment of proteins functioning either as coactivators or corepressors (26, 27). CBP and p300 have been shown to have potent, intrinsic histone acetyltransferase activity, and they can associate with other proteins that also have histone acetyltransferase activity (41, 42). It is thought that acetylation of histones brings about an "open" conformation of chromatin that is more conducive to the interaction of transcription initiation complexes with DNA, and therefore results in activation of transcription. CBP and p300 have also been reported to interact directly with the general transcription factors TFIIB and TBP, with a component of TFIID, and with RNA polymerase II (43–45). In our present studies, microinjection of specific anti-CBP or anti-p300 antibodies blocked over 80% of the tBHQ-induced expression of GFP (Fig. 3), providing direct evidence for the involvement of CBP and p300 in regulation of EpRE-controlled genes. Further studies are needed to determine the precise manner of interaction between the transcription factors Nrf2/MafK, ARE-binding protein-1 or other p160 coactivators, and CBP/p300.

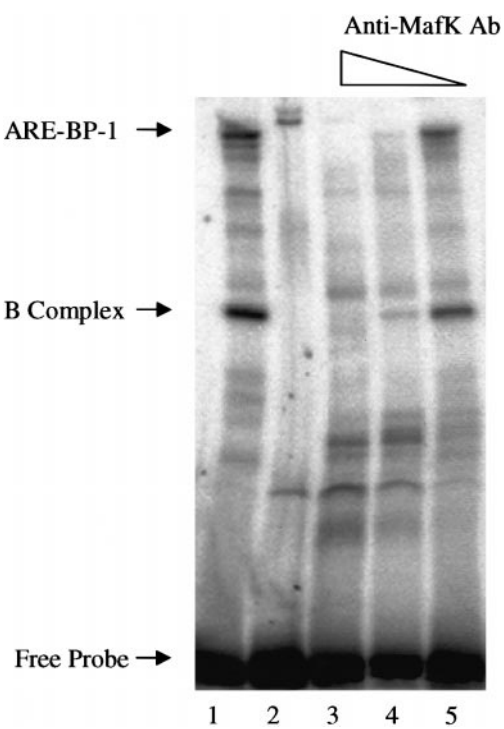

FIG. 4. Disruption of gel-shift complexes by an anti-MafK antibody. Conditions of the assay are described in detail under Materials and Methods. Nuclear extracts were prepared from HepG2 cells, and the 41 bp, ^{32}P-labeled EpRE probe is described under Materials and Methods. Lane 1, standard conditions; lane 2, anti-Mafk antibody without nuclear extract; lane 3 (1.0 μl), lane 4 (0.3 μl), lane 5 (0.1 μl) of anti-MafK antibody. The nuclear extract was incubated with the anti-MafK antibody for 20 min at room temperature, and then the ^{32}P-end-labeled EpRE probe was added and incubated for an additional 10 min.

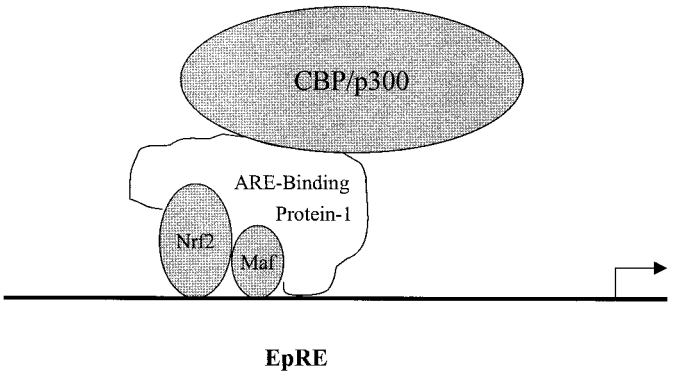

FIG. 5. A hypothetical model showing interaction between primary transcription factors and coactivators at the EpRE. The transcription complex, composed of Nrf2/MafK, ARE-binding protein-1, and CBP or P300, is presumed to regulate EpRE-dependent gene expression. The Nrf2/MafK heterodimer binds directly to the EpRE DNA motif; once bound, it recruits a p160 coactivator binding partner, shown here is the putative p160 ARE-binding protein-1 coactivator; once bound, this coactivator recruits a tertiary regulator, shown here as CBP or p300.

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REFERENCES

1. Friling, R. S., Bensiimon, A., Tichauer, H., and Deniel, V. (1990) Xenobiotic-inducible expression of murine glutathinoe S-transferase Ya subunits gene is controlled by an elctrophile-responsive element. *Proc. Natl. Acad. Sci. USA* **87,** 6258–6262.
2. Favreau, L. V., and Pickett, C. B. (1991) Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Identification of regulatory elements controlling basal level expression and

inducible expression by planar aromatic compounds and phenolic antioxidants. *J. Biol. Chem.* **266,** 4556–4561.

3. Shi, M. M., Kugelman, A., Iwamoto, T., Tian, L., and Forman, H. J. (1994) Quinone-induced oxidative stress elevates glutathione and induces gamma-glutamylcysteine synthetase activity in rat lung epithelial L2 cells. *J. Biol. Chem.* **269,** 26512–26517.
4. Prestera, T., Talalay, P., Alam, J., Ahn, Y. I., Lee, P. J., and Choi, A. M. (1995) Parallel induction of heme oxygenease-1 and chemoprotective phase II enzymes by electrophiles and antioxidants: Regulation by upstream antioxidant-responsive elements (ARE). *Mol. Med.* **1,** 827–837.
5. Tsuji, Y., Ayaki, H., Whitman, S. P., Morrow, C. S., Torti, S. V., and Torti, F. M. (2000) Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress. *Mol. Cell. Biol.* **20,** 5818–5827.
6. Kensler, T. W. (1997) Chemoprevention by inducers of carcinogen detoxification enzymes. *Environ. Health Perspect.* **105,** 965–970.
7. Hayes, J. D., and McLellan, L. I. (1999) Glutathione and glutathione-dependent enzymes represent a coordinately regulated defense against oxidative stress. *Free Radicals Res.* **31,** 273–300.
8. Pearson, W. R., Reinhart, J., Sisk, S. C., Anderson, K. S., and Adler, P. N. (1988) Tissue-specific induction of murine glutathione transferase mRNAs by butylated hydroxyanisole. *J. Biol. Chem.* **263,** 13324–13332.
9. Hayes, J. D., Ellis, E. M., Neal, G. E., Harrison, D. J., and Manson, M. M. (1999) Cellular response to cancer chemopreventive agents: Contribution of the antioxidant responsive element to the adaptive response to oxidative and chemical stress. *Biochem. Soc. Symp.* **64,** 141–168.
10. Andrews, N. C. (1998) The NF-E2 transcription factor. *Int. J. Biochem. Cell Biol.* **30,** 429–432.
11. Igarashi, K., Kataoka, K., Itoh, K., Hayashi, N., Nishizawa, M., and Yamamoto, M. (1994) Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. *Nature* **367,** 568–572.
12. Chan, J. Y., Han, X. L., and Kan, Y. W. (1993) Cloning of Nrf1, an NF-E2-related transcription factor, by genetic selection in yeast. *Proc. Natl. Acad. Sci. USA* **90,** 11371–11375.
13. Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994) Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional adctivitor that binds to the tandem NF-E2/ AP1 repeat of the β-globin locus control region. *Proc. Natl. Acad. Sci. USA* **91,** 9926–9930.
14. Kobayashi, A., Itoh, E., Toki, T., Kogame, K., Takahashi, S., Igarashi, K., Hayashi, N., and Yamamoto, M. (1999) Molecular cloning and functional characterization of a new Cap'n' collar family transcription factor Nrf3. *J. Biol. Chem.* **274,** 6443–6452.
15. Itoh, K., Igarashi, K., Hayashi, N., Nishizawa, M., and Yamamoto, M. (1995) Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. *Mol. Cell. Biol.* **15,** 4184–4193.
16. Oyake, T., Itoh, K., Motohashi, H., Hayashi, N., Hoshino, H., Nishizawa, M., Yamamoto, M., and Igarashi, K. (1996) Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. *Mol. Cell Biol.* **16,** 6083–6095.
17. Blank, V., and Andrews, N. C. (1997) The Maf transcription factors: Regulation of differentiation. *Trends Biochem. Sci.* **22,** 437–441.
18. Motohashi, H., Shavit, J. A., Igarashi, K., Yamamoto, M., and Engel, J. D. (1997) The world according to Maf. *Nucleic Acids Res.* **25,** 2953–2959.
19. Toki, T., Itoh, J., Kitazawa, J., Arai, K., Hatakeyama, K., and Akasaka, J. (1997) Human Maf proteins form heterodimers with CNC family transcription factors and recognize the NF-E2 motif. *Oncogene* **14,** 1901–1910.
20. Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* **236,** 313–322.
21. Nguyen, T., Huang, H. C., and Pickett, C. B. (2000) Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK. *J. Biol. Chem.* **275,** 15466–15473.
22. Dhakshinamoorthy, S., and Jaiswal, A. K. (2000) Small maf (MafG and MafK) proteins negatively regulate antioxidant response element-mediated expression and antioxidant induction of the NAD(P)H:quinone oxidoreductase1 gene. *J. Biol. Chem.* **275,** 40134–40141.
23. Wild, A. C., Moinova, H. R., and Mulcahy, R. T. (1999) Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *J. Biol. Chem.* **274,** 33627–33636.
24. McMahon, M., Itoh, K., Yamamoto, M., Chanas, S. A., Henderson, C. J., McLellan, L. I., Wolf, C. R., Cavin, C., and Hayes, J. D. (2001) The Cap'n'Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. *Cancer Res.* **61,** 3299–3307.
25. Tjian, R., and Maniatis, T. (1994) Transcriptional activation: A complex puzzle with few easy pieces. *Cell* **77,** 5–8.
26. Naar, A. M., Lemon, B. D., and Tjian, R. (2001) Transcriptional coactivator complexes. *Annu. Rev. Biochem.* **70,** 475–501.
27. Torchia, J., Glass, C. K., and Rosenfeld, M. G. (1998) Coactivators and corepressors in the integration of transcriptional responses. *Curr. Opin. Cell Biol.* **10,** 373–383.
28. Leo, C., and Chen, J. D. (2000) The SRC family of nuclear receptor coactivators. *Gene* **245,** 1–11.
29. Kwok, R. P., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G., Green, M. R., and Goodman, R. H. (1994) Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* **370,** 223–226.
30. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85,** 403–414.
31. Grossman, S. R. (2001) p300/CBP/p53 interaction and regulation of the p53 response. *Eur. J. Biochem.* **268,** 2773–2778.
32. Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc. Natl. Acad. Sci. USA* **94,** 2927–2932.
33. Zhang, J. J., Vinkemeier, U., Gu, W., Chakravarti, D., Horvath, C. M., and Darnell, J. E. (1996) Two contact regions between Stat1 and CBP/p300 in interferon gamma signaling. *Proc. Natl. Acad. Sci. USA* **93,** 15092–15096.
34. Lee, J. W., Lee, Y. C., Na, S. Y., Jung, D. J., and Lee, S. K. (2001) Transcriptional coregulators of the nuclear receptor superfamily: Coactivators and corepressors. *Cell. Mol. Life Sci.* **58,** 289–297.
35. Wasserman, W. W., and Fahl, W. E. (1997) Functional antioxidant responsive elements. *Proc. Natl. Acad. Sci. USA* **94,** 5361–5366.
36. Wasserman, W. W., and Fahl, W. E. (1997) Comprehensive anal-

ysis of proteins which interact with the antioxidant responsive element: Correlation of ARE-BP-1 with the chemoprotective induction response. *Arch. Biochem. Biophys.* **344,** 387–396.

37. Costa, R. H., Lai, E., Grayson, D. R., and Darnell, J. E. (1988) The cell-specific enhancer of the mouse transthyretin (prealbumin) gene binds a common factor at one site and a liver-specific factor at two other sites. *Mol. Cell. Biol.* **8,** 81–90.
38. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* **13,** 76–86.
39. Venugopal, R., and Jaiswal, A. (1998) Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene* **17,** 3145–3156.
40. Toechia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) The transcriptional coactivator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387,** 677–684.
41. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferase. *Cell* **87,** 953–959.
42. Yang, X., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996) A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382,** 319–324.
43. Dallas, P. B., Yaciuk, P., and Moran, E. (1997) Characterization of monoclonal antibodies raised against p300: Both p300 and CBP are present in intracellular TBP complexes. *J. Virol.* **71,** 1726–1731.
44. Nakalima, T., Uchida, C., Anderson, S. F., Lee, C., Hurwitz, J., Parvin, J. D., and Montminy, M. (1997) RNA helicase A mediates association of CBP with RNA polymerase II. *Cell* **90,** 1107–1113.
45. Kee, B. L., Arias, J., and Montminy, M. R. (1996) Adaptor-mediated recruitment of RNA polymerase II to a signal-dependent activator. *J. Biol. Chem.* **271,** 2373–2375.