Parallel Induction of Heme Oxygenase–1 and Chemoprotective Phase 2 Enzymes by Electrophiles and Antioxidants: Regulation by Upstream Antioxidant-Responsive Elements (ARE)

Tory Prestera,* Paul Talalay,* Jawed Alam,† Young I. Ahn,† Patty J. Lee,‡ and Augustine M. K. Choi‡

*Department of Pharmacology and Molecular Sciences, and ‡Division of Pulmonary and Critical Care Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland, U.S.A.
†Department of Molecular Genetics, Alton Ochsner Medical Foundation, and Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, Louisiana, U.S.A.

ABSTRACT

Background: Heme oxygenase (HO; EC 1.14.99.3) catalyzes the conversion of heme to biliverdin, which is reduced enzymatically to bilirubin. Since bilirubin is a potent antioxidant and heme a pro-oxidant, HO may protect cells against oxidative damage. HO-1 is highly inducible by diverse chemical agents, resembling those evoking induction of phase 2 enzymes (i.e., Michael reaction acceptors, heavy metals, trivalent arsenicals, and sulfhydryl reagents). Phase 2 enzymes (glutathione transferases; NAD(P)H:quinone reductase; glucuronosyltransferases) are regulated by antioxidant-responsive elements (ARE), and their induction protects against chemical carcinogenesis. Is HO-1 regulated by chemical agents and enhancer elements similar to those controlling phase 2 enzymes?

Materials and Methods: Induction of HO-1 by phorbol ester and heavy metals is transcriptionally controlled through a 268-bp SX2 fragment, containing two phorbol ester–responsive (TRE) sites (TGA$^{C}/_{G}$T $^{C}/_{A}$A) which overlap ARE consensus sequences (TGACNNNGC). Therefore, mutations of the SX2 element designed to distinguish ARE from TRE were inserted into chloramphenicol acetyltransferase (CAT) reporter plasmids, and the response of the CAT activity of murine hepatoma cells stably transfected with these constructs was examined with a wide range of inducers of phase 2 enzymes.

Results: All compounds raised HO-1 mRNA and CAT expression constructs containing wild-type SX2. When the SX2 region was mutated to alter TRE consensus sequences without destroying the ARE consensus, full inducibility was preserved. Conversely, when the ARE consensus was disturbed, inducibility was abolished.

Conclusion: Induction of heme oxygenase–1 is regulated by several chemically distinct classes of inducers (mostly electrophiles), which also induce phase 2 enzymes, and these inductions are mediated by similar AREs. These findings support the importance of HO-1 as a protector against oxidative damage and suggest that HO-1 induction is part of a more generalized protective cellular response that involves phase 2 enzymes.

INTRODUCTION

This paper provides new insight into the chemical specificity and molecular mechanisms of the regulation of heme oxygenase–1 (HO-1; heme, hydrogen-donor:oxygen oxidoreductase [α-methene-oxidizing, hydroxylating], EC 1.14.99.3) (see review in Ref. 1). The primary function of this widely distributed microsomal protein is to convert heme into biliverdin, which is further metabolized to bilirubin. Since the latter compounds are powerful antioxidants and heme is a pro-oxidant, it has been suggested that HO provides cellular protection against oxidative dam-

Address correspondence and reprint requests to: Augustine M. K. Choi, Division of Pulmonary and Critical Care Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD, 21205, U.S.A.

Copyright © 1995, *Molecular Medicine*, 1076-1551/95/$10.50/0

age (2,3). It is therefore significant that (i) inducers of HO-1 include phenolic antioxidants, Michael reaction acceptors, isothiocyanates, 1,2-dithiole-3-thione, trivalent arsenicals, mercuric chloride, and 2,3-dimercapto-1-propanol; and (ii) control of HO-1 by these agents involves specific enhancer elements known as antioxidant-responsive elements (ARE) or electrophile-responsive elements (EpRE) (which have the consensus sequence TGACNNNGC).[1] This is intriguing because the very same chemical agents, by utilizing similar enhancer elements, also stimulate the synthesis of glutathione transferases, NAD(P)H:quinone reductase (QR; EC 1.6.99.2), glucuronosyltransferases, and other enzymes, collectively known as phase 2 enzymes,[2] which are widely recognized protectors against electrophile and oxidative toxicity. Induction of HO-1 therefore appears to be part of a generalized cellular response that protects cells against the toxicity of electrophiles and reactive oxygen species, and thereby ameliorates the risk of cancer.

Heme oxygenase exists in two distinct isoforms, HO-1 and HO-2. Although both forms are present in a wide variety of tissues, HO-2 is constitutively expressed, while HO-1 is highly inducible (see Ref. 1). Prior work has shown that induction of HO-1 is mediated by physical factors such as heat shock and ultraviolet irradiation, and two main types of chemical stresses: (i) those generating reactive oxygen species such as hydrogen peroxide and phorbol esters (e.g., 12-*O*-tetradecanoylphorbol-13-acetate [TPA]); and (ii) thiol reagents and modulators of glutathione levels such as such as arsenite, Cd^{2+} and Hg^{2+}, buthionine sulfoximine, and diamide (1,15). It has been proposed that HO-1 may exert protection by reducing levels of the potentially prooxidant heme pool, and by increasing local levels of the antioxidants biliverdin and bilirubin (15–17). HO-1 induction protects human skin fibroblasts against UVA produced membrane damage (16) and confers resistance to arsenic in human lung adenocarcinoma cells (17). Thus, the hypothesis that induction of HO-1 protects cells against oxidant stress (15) seems well founded.

Damage of critical centers of DNA by electrophiles or reactive oxygen species can lead to malignant transformation, and cells have evolved multiple elaborate mechanisms to cope with such toxicities. Thus, exposure of cells to low but tolerated levels of electrophiles results in the coordinate induction of a number of phase 2 enzymes that detoxify electrophiles and reactive oxygen species (8,14), as well as elevations of intracellular glutathione (see Ref. 8). This inductive response, termed the Electrophile Counterattack Response (8), reduces the vulnerability of cells to higher concentrations of the same or other toxic electrophiles and consequently may protect cells against genotoxicity and neoplasia (18,19).

Understanding of the molecular mechanisms of regulation of phase 2 enzymes is therefore important for devising strategies for protection against neoplasia. The mouse (4,20) and rat (5) glutathione transferase Ya genes and the human (21) and rat (12) quinone reductase genes contain similar control elements present in the 5′-upstream regions of the genes for these enzymes. These elements have been designated ARE (5). Although these elements are "AP-1–like" in their sequences (AP-1 is a family of transcriptional activator DNA-binding proteins that bind to the TPA-responsive element [TRE] consensus sequence [TGA$^{C}/_{G}$T $^{C}/_{A}$A]), recent evidence indicates (9,22,24) that the transcription factors that activate ARE sequences are quite distinct from those that activate the consensus TRE.

[1]The electrophile-responsive element (EpRE) was so named because it was shown to be activated by dimethyl fumarate, *trans*-4-phenylbut-3-en-2-one, and *tert*-butylhydroquinone (4). These compounds all have (or can easily acquire) Michael reaction acceptor groups, which are reactive electrophiles. In addition to its electrophilic potential, *tert*-butylhydroquinone is a well-known antioxidant and can engage in redox cycling reactions. The antioxidant responsive element (ARE) (5) was so named because it was responsive to redox-active quinone precursors (catechol, hydroquinone) or 1,4-benzoquinone itself, but failed to respond to a redox-inactive diphenol (resorcinol). Redox-active quinones, however, share a common property with most other monofunctional phase 2 enzyme inducers, in that they are also Michael reaction acceptors and thus are powerful electrophiles (6). Consequently, we prefer the term electrophile-responsive element (EpRE), as we have previously shown (6–8) that the common chemical characteristic of nearly all monofunctional phase 2 enzyme inducers is that they are electrophiles. Nevertheless, we respect the originally proposed nomenclature and have elsewhere referred to the enhancer element as ARE/EpRE (9). In this paper, it is less cumbersome to designate this element as ARE.

[2]Two classes of enzymes metabolize xenobiotics: (i) phase 1 enzymes (mostly cytochromes P450), which functionalize molecules by introducing hydroxyl or epoxide groups; and (ii) phase 2 enzymes (10), which detoxify by either conjugating these functionalized molecules with endogenous ligands (e.g., glutathione), thus facilitating their excretion, or by destroying their reactive centers (e.g., hydrolysis of epoxides by epoxide hydrolase, or reduction of quinones by QR). Reasons for considering QR a phase 2 enzyme are presented elsewhere (11–13).
Inducers of enzymes of xenobiotic metabolism belong to two families (14): (i) Bifunctional inducers, which bind to the *A*ryl *h*ydrocarbon (A*h*) receptor and induce both certain phase 1 enzymes and phase 2 enzymes; and (ii) Monofunctional inducers, which induce phase 2 enzymes independently of the *Ah* receptor.

The molecular mechanisms underlying the transcriptional induction of HO-1 have also been extensively investigated. Recently, a 268-bp DNA fragment, designated SX2, was located approximately 4 kb upstream of the start site of the mouse HO-1 gene. This element acts both as a basal level enhancer, and mediates induction by TPA, heme, and several heavy metals (25–27). In addition to two CCAAT/enhancer-binding protein (C/EBP) sequences, this fragment contains two potential TRE and/or ARE binding sites. Furthermore, it has been demonstrated that the "TRE/ARE-like" sites are necessary and sufficient for both heavy metal and TPA induction (25,26).

In the present study we demonstrate that all known classes of monofunctional phase 2 enzyme inducers also dramatically induce HO-1 mRNA in a mouse hepatoma cell line (Hepa 1c1c7). Indeed, these compounds represent some of the most potent and effective HO-1 inducers so far discovered. We show that these compounds induce transcription of the HO-1 gene and that the presence of ARE (not TRE) consensus enhancer sequences is required for these inductions.

MATERIALS AND METHODS

Compounds

Most of the inducers were obtained commercially and were of the highest purity available. Synthetic racemic 1-isothiocyanato-4-(methylsulfinyl)butane (CH_3-SO-$[CH_2]_4$-NCS) (sulforaphane) was a gift of C.-G. Cho and G. H. Posner (Department of Chemistry, The Johns Hopkins University) (28). 1,2-Dithiole-3-thione was a gift from T. W. Kensler (Department of Environmental Health Sciences, The Johns Hopkins University School of Hygiene and Public Health).

Cell Culture and Treatment with Compounds

Hepa 1c1c7 cells were grown in alpha minimal essential medium supplemented with 10% untreated fetal calf serum in a humidified atmosphere of 5–7% CO_2 at 37°C. Cell lines were free from mycoplasma. For all assays, cells were plated at a density of 2.5–5 $\times$ 10^4 cells/cm^2 in 10-cm plates 24 hr before treatment with inducers. All inducers were added in dimethyl sulfoxide (DMSO), except $HgCl_2$ and sodium arsenite, which were added in water. The final concentration of DMSO in the medium was 0.1% by volume in all inducer experiments.

RNA Extraction and Northern Blot Analysis

Total RNA was isolated by the STAT-60 RNAzol method with direct lysis in STAT-60 RNAzol buffer followed by chloroform extractions (Tel-Test "B" Inc., Friendswood, TX, U.S.A.). Ten micrograms of total RNA were subjected to electrophoresis in a 1% agarose gel and then transferred to nylon membranes by capillary action. The nylon membranes were then incubated in hybridization buffer (1% bovine serum albumin, 7% SDS, 1.0 mM EDTA, 0.5 M sodium phosphate buffer, pH 7.0) at 65°C for 2 hr, followed by hybridization at 65°C for 24 hr in the same buffer containing ^{32}P-labeled rat HO-1 cDNA. A full-length rat HO-1 cDNA was generously provided by Dr. S. Shibahara, Sendai University, Japan. It was subcloned into a pBluescript vector and a *Hind*III/*Eco*RI digestion was performed to excise the 0.9-kb HO-1 cDNA from the vector. Nylon membranes were then washed in buffer A (0.5% bovine serum albumin, 5% SDS, 1 mM EDTA, 40 mM sodium phosphate buffer, pH 7.0) for two 15-min periods at 65°C, followed by washes in buffer B (1% SDS, 40 mM sodium phosphate buffer, pH 7.0, 1.0 mM EDTA) for four 15-min periods at 65°C (29). Autoradiogram signals were quantitated by densitometric scanning (Molecular Dynamics, Sunnyvale, CA, U.S.A.). To control for variation in either the amount of RNA in different samples or loading errors, blots were hybridized with an oligonucleotide probe corresponding to the 18S rRNA. A 24-bp oligonucleotide (5′-ACG GTA TCT GAT CGT CTT CGA ACC-3′) complementary to the 18S rRNA was synthesized using a DNA synthesizer (Applied Biosystems, Foster City, CA, U.S.A.). The 18S oligonucleotide was labeled with [α-^{32}P]ATP at the 3′-end with terminal deoxynucleotidyl transferase (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.). All densitometric values obtained for the HO-1 mRNA transcript (1.8 kb) were normalized to values for 18S rRNA obtained on the same blot. Steady-state HO-1 mRNA levels of cells were measured in densitometric absorbance units, and the inductions were expressed as a ratios of inducer-treated cells to untreated controls.

Chloramphenicol Acetyltransferase Assay

Cellular protein extracts were prepared within 24 hr after termination of chemical treatment. Cells from 10-cm plates were washed with ice-cold phosphate-buffered saline, resuspended in 1.0 ml of 0.125 M Tris-HCl (pH 7.5), and then lysed by three cycles of freezing and thawing. Cell debris was then removed by centrifugation for 10 min at 14,000 rpm in a microcentrifuge. Protein concentrations of the supernatant fluids were determined by Coomassie Blue dye-binding assay (30). Reaction mixtures containing, in a final volume of 150 μl, 20 mM acetyl-CoA, 0.3 μCi [^{14}C]chloramphenicol (50 μCi/μmol; Amersham, Arlington Heights, IL, U.S.A.), and 100 μg of protein were incubated for 4 hr at 37°C. The amount of acetylation was then determined by measuring the radioactivity of the acetylated and nonacetylated forms of chloramphenicol, which were separated by ascending thin layer chromatography. Percentages of chloramphenicol acetylation were obtained over the linear range of the assay (up to 30% conversion) for each sample and normalized for the protein content of the extract. Induction is expressed as the ratio of relative chloramphenicol acetyltransferase (CAT) activities of inducer-treated cells to control cells, corrected for protein concentration.

The relative basal CAT activities of the extracts of Hepa 1c1c7 cells transfected with the various plasmids were as follows: untransfected, 1.00 ± 0.07%; pMHO1cat, 1.20 ± 0.07%; pMHO9cat, 6.67 ± 0.14%; pMHOSX2cat, 3.00 ± 0.13%; pMHOSX2M2cat, 2.20 ± 0.10%; pMHOSX2M4cat, 6.47 ± 0.12%; pMHOSX2M789cat, 2.33 ± 0.09%; pMHOSX2M239cat, 1.67 ± 0.08%.

Plasmid Constructs and Mutations

In plasmid pMHO1cat (26), expression of the CAT reporter gene is under control of the 1.3 kb minimal promoter sequence from the 5′-upstream region of the mouse HO-1 gene (bp −1287 to +73). All other plasmids were derived from this construct. A 9.0-kb *Bam*H1/*Bam*H1 fragment from the 5′-upstream region of the human HO-1 gene (−12.5 to −3.5 kb) was cloned into the *Bam*H1 site of pMHO1cat to produce pMHO9cat (27). The 268-bp SX2 enhancer fragment or the corresponding mutants (see below) were cloned between the *Sac*I and *Xba*I sites of pMO1cat (25,26). Oligonucleotide-mediated site-directed mutagenesis of the SX2 fragment was carried out by the procedure of Deng and Nickoloff (31) as previously described (25) (see Fig. 7 below). Mutants SX2M2 and SX2M4 have been described (25). In SX2M2, 2-bp substitutions were introduced into both of the TRE/ARE composite elements such that TRE sites were mutated, but the ARE sequences remained intact. SX2M4 contains 3-bp mutations within both of the C/EBP binding sites. Both TRE (SX2M2) and C/EBP (SX2M4) mutations abolish protein binding at these sites, as shown by DNase I footprint analyses (25). Mutant SX2M239 contains extended base substitutions within both of the TRE sites, altering both the TRE consensus binding site and the ARE consensus site in each composite element. In SX2M789, the three nucleotide residues directly upstream of the TRE consensus site were altered such that the ARE consensus sequence is mutated, leaving the TRE site intact. The oligonucleotides sequences used to generate the SX2M239 mutation were 111-cttttatgcGTAgATCtggttgggaggg and 163-agatttt gcGTagATCccctctgttccctc, and those used to generate SX2M789 were 63-ggctggaaGCTtgagttgtgatt, 109-tgcttttaAAAtgtgtcatggtt, and 161-acagatttAAA tgagtcaccctc. The numbers correspond to the nucleotide within the SX2 sequence (see Fig. 7) and the nucleotide substitutions are capitalized. In addition to alterations within the previously characterized TRE/ARE composite elements, mutant SX2M789 also contains substitutions at residues 70–72 of the SX2 sequence. This region contains an TRE/ARE composite element that deviates from the consensus TRE binding site and the consensus ARE by two residues. This composite element does not by itself mediate activation of the CAT reporter gene in response to either heme or Cd^{2+} (data not shown). All mutations were confirmed by DNA sequence analysis.

Transfections

Hepa 1c1c7 cells were maintained in Dulbecco's modified Eagle's medium containing 0.4% glucose and supplemented with 10% fetal bovine serum. Transfections were carried out by the calcium phosphate precipitation technique (32). Briefly, Hepa 1c1c7 cells (1–2 × 10^6/10-cm plate) were exposed for 16 hr to a DNA mixture containing 10 μg of the CAT reporter plasmid and 1 μg of pSV2neo (33). The precipitate was removed and the cells were cultured in complete medium. Geneticin (G418 sulfate) was added 24 hr later to a final concentration of 400 μg/ml, and resistant colonies were selected over a 3- to 4-week period. Resistant colonies (>50/plate)

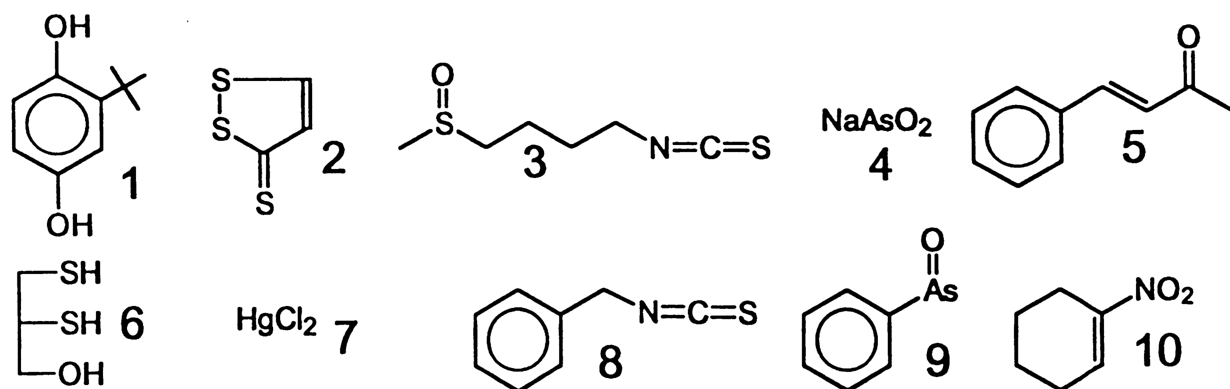

FIG. 1. Structures of inducers used in this study

Tert-butylhydroquinone (Compound 1), 1,2-dithiole-3-thione (Compound 2), sulforaphane (Compound 3), sodium arsenite (Compound 4), *trans*-4-phenylbut-3-en-2-one (Compound 5), 2,3-dimercapto-1-propanol (Compound 6), mercuric chloride (Compound 7), benzyl isothiocyanate (Compound 8), phenylarsine oxide (Compound 9), and 1-nitro-1-cyclohexene (Compound 10).

were pooled and propagated in the presence of Geneticin.

In all transfection experiments we used a control DNA sample that does not contain pSV2neo. If resistant colonies were obtained on the control plate, then all plates were discarded. In more than 300 independent transfection experiments, this problem has occurred only once. The 10:1 ratio of CAT plasmid to pSV2neo practically guarantees that the G418-resistant colonies will also contain the CAT gene. Even if individual G418-resistant/CAT minus colonies did arise, the influence of these clones is effectively eliminated by using pooled clonal populations for the induction experiments. Moreover, basal CAT activity in extracts from all HO-1/CAT transfectants is greater than that observed in extracts from untransfected Hepa cells or cells transfected with the promoterless construct pSKcat (34). Therefore, it would be highly unlikely that the cells utilized in this study lacked the HO-1/CAT fusion constructs.

RESULTS AND DISCUSSION

Induction of HO-1 mRNA by Monofunctional Phase 2 Enzyme Inducers: Time Course and Concentration Dependence

We have previously shown that phase 2 detoxication enzymes are induced by several classes of structurally diverse chemical agents, most of which are electrophiles, and all of which react with thiol groups (6–9). Figure 1 shows the structures of representative examples of these classes of compounds that were used in this study. This group of agents includes: oxidizable diphenols (e.g., *tert*-butylhydroquinone [Compound 1]); Michael reaction acceptors (e.g., *trans*-4-phenylbut-3-en-2-one [Compound 5] and 1-nitro-1-cyclohexene [Compound 10]); isothiocyanates (e.g., sulforaphane [Compound 3] and benzyl isothiocyanate [Compound 8]); heavy metals (e.g., mercuric chloride [Compound 7]); trivalent arsenicals (e.g., sodium arsenite [Compound 4] and phenylarsine oxide [Compound 9]); and vicinal dimercaptans (e.g., 2,3-dimercapto-1-propanol [Compound 6]; and 1,2-dithiole-3-thione [Compound 2]) (6–8,35,36).

We first established whether HO-1 was inducible by the whole range of known monofunctional phase 2 enzyme inducers, by treating Hepa 1c1c7 murine hepatoma cells with representative compounds and measuring steady state HO-1 mRNA levels by Northern blot analyses (Fig. 2). A more detailed quantitative analysis of the time course of induction was then carried out with *tert*-butylhydroquinone, *trans*-4-phenylbut-3-en-2-one, 1,2-dithiole-3-thione, sodium arsenite, $HgCl_2$, 2,3-dimercapto-1-propanol, and benzyl isothiocyanate, as shown in Fig. 3. All compounds produced significant HO-1 mRNA induction, with notable increases by 2 hr, maximum levels at 4 to 6 hr, and a modest decrease by 10 hr. At the concentrations used in this experiment, increases in mRNA levels varied from

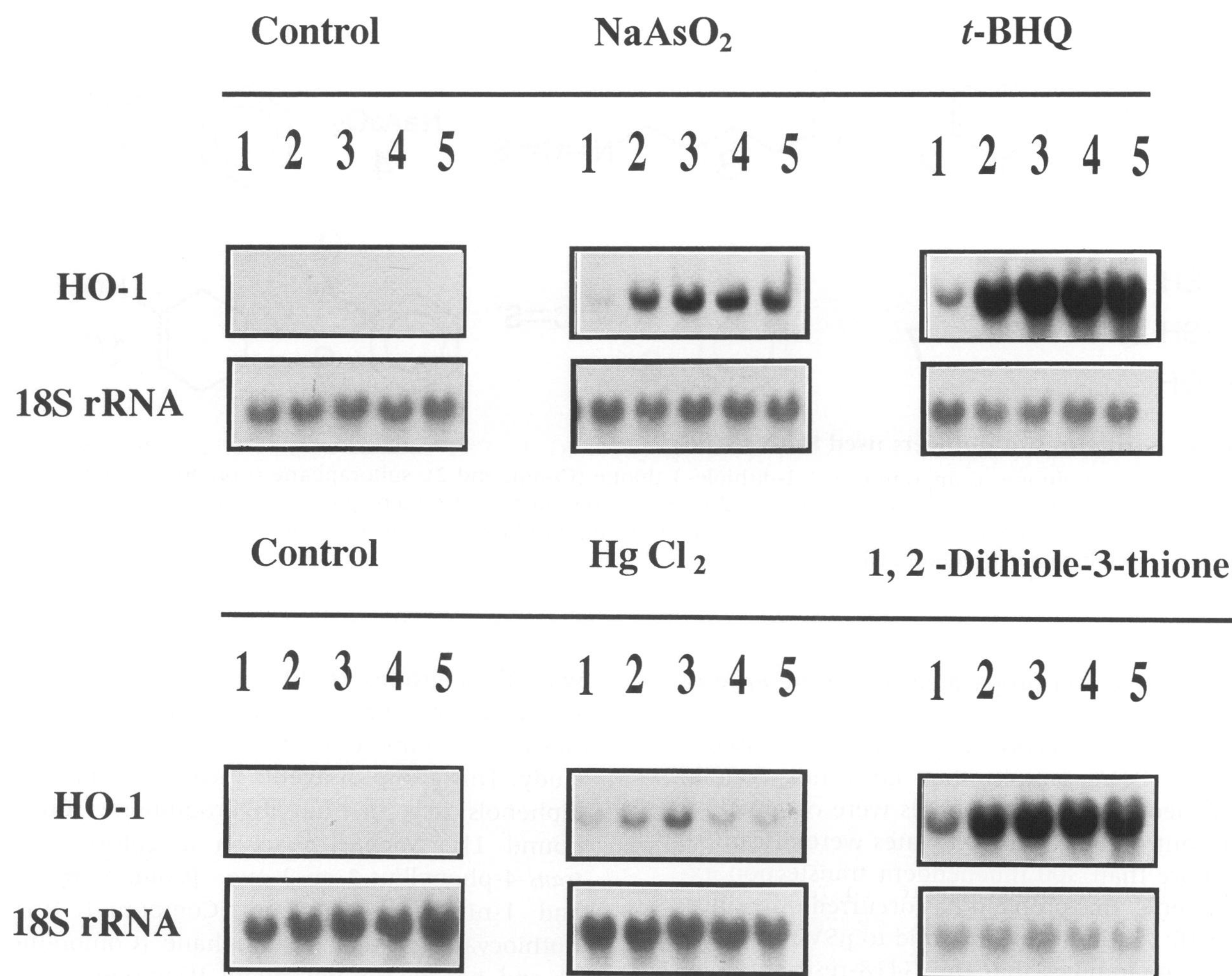

FIG. 2. Northern blot analyses of the time course of the induction of HO-1 mRNA in Hepa 1c1c7 cells
The cells were treated with 2.0 μM sodium arsenite ($NaAsO_2$), 10 μM *tert*-butylhydroquinone (*t*-BHQ), 10 μM $HgCl_2$, and 150 μM 1,2-dithiole-3-thione for 2, 4, 6, 8, and 10 hr (Lanes 1–5, respectively). Blots of 18S rRNA are shown for comparison.

about 8-fold for 2,3-dimercapto-1-propanol to about 180-fold for *tert*-butylhydroquinone.

After establishing the time course of induction of a selection of compounds, we assessed the inducer potencies of Compounds 1–9 in inducing HO-1 mRNA. As shown in Fig. 4, all compounds were capable of dramatic, dose-dependent induction of HO-1 mRNA. At the maximum concentrations tested, nearly all compounds produced relative increases in mRNA levels of at least 10-fold.

Transcriptional Regulation of HO-1 Induction by Monofunctional Phase 2 Enzyme Inducers

In order to determine whether increased transcription of the HO-1 gene was mediated by its 5′-upstream region, Hepa1c1c7 cells were stably transfected with a construct containing 9 kb of this region and the CAT reporter gene (pMHO9cat). Cells were treated with Compounds 1, 2, and 4–9, and assayed for CAT activity after 24 hr (Fig. 5). All compounds produced highly significant increases in CAT activity driven by this 5′-upstream region. Relative increases in mRNA levels ranged from 2-fold ($HgCl_2$) to 12-fold (phenylarsine oxide). In sharp contrast, when the plasmid containing only the 1.3-kb promoter from the 5′-upstream region (pMHO1cat) was treated with these compounds, no inductions occurred. Clearly induction of HO-1 mRNA by these compounds occurs via increased transcription, mediated through enhancer elements contained in the 9-kb region.

Since the SX2 region contains two consensus

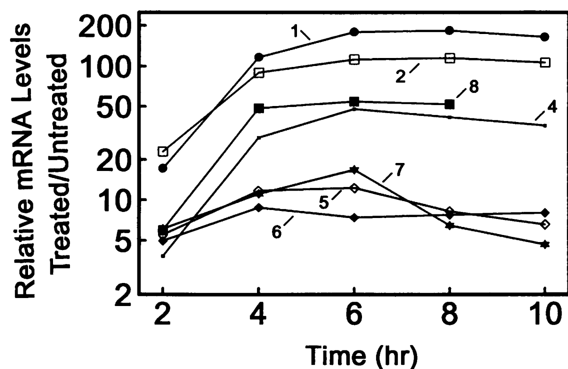

FIG. 3. Time course of the induction of HO-1 mRNA in Hepa 1c1c7 cells

Cells were treated with the following inducers for 2, 4, 6, 8, and 10 hr: 100 μM *tert*-butylhydroquinone (1); 150 μM 1,2-dithiole-3-thione (2); 2.0 μM sodium arsenite (4); 100 μM *trans*-4-phenylbut-3-en-2-one (5); 100 μM 2,3-dimercapto-1-propanol (6); and 10 μM $HgCl_2$ (7); 10 μM benzyl isothiocyanate (8). The mRNA was quantitated by Northern blot analysis, and the values were normalized to the 18S rRNA amounts on the same blots. Note the logarithmic scale of the relative RNA ratios.

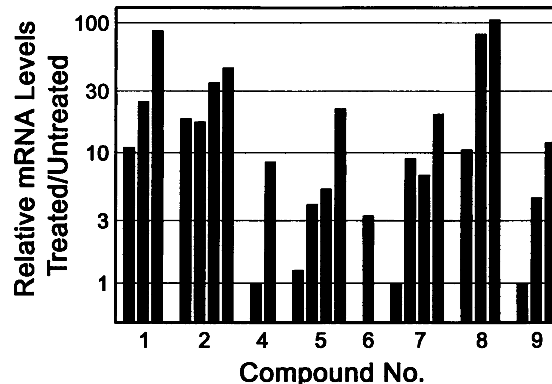

FIG. 4. Effect of concentration of inducers on HO-1 mRNA induction in Hepa 1c1c7 cells after 6-hr exposure

Compounds used were 13, 25, or 100 μM *tert*-butylhydroquinone (Compound 1); 19, 38, 75, or 150 μM 1,2-dithiole-3-thione (Compound 2); 0.25, or 1.0 μM sodium arsenite (Compound 4); 12.5, 25, 50, or 100 μM *trans*-4-phenylbut-3-en-2-one (Compound 5); 100 μM 2,3-dimercapto-1-propanol (Compound 6); 1.3, 2.5, 5, or 10 μM $HgCl_2$ (Compound 7); 1.25, 5, or 10 μM benzyl isothiocyanate (Compound 8); 25, 50, or 125 nM phenylarsine oxide (Compound 9). The concentrations refer to the bars from left to right, respectively. The mRNA was quantitated by Northern blots, and the values were normalized to the 18S rRNA amounts on the same blots. Note the logarithmic scale of the relative RNA ratios.

ARE sites, and we had previously shown that monofunctional inducers increased transcription of phase 2 enzymes through ARE elements (9), we reasoned that induction of HO-1 by these agents was likely to be mediated by the SX2 region. Therefore, we tested a CAT reporter construct containing the HO-1 minimal promoter linked to the 268-bp SX2 region (pMHOSX2cat), which had been stably transfected into Hepa1c1c7 cells. Cells were treated with Compounds 1–10 and assayed for CAT reporter activity 24 hr later. As shown in Fig. 6, significant induction by all of these compounds is mediated through this 268-bp region. Furthermore, the magnitudes of induction were similar to those obtained when the entire 9-kb 5′-upstream region was used (cf. Fig. 5), indicating that the SX2 region mediates most of the inducibility of mouse HO-1 by these compounds.

Mutational Analysis of the SX2 Region

The SX2 region of mouse HO-1 contains two ARE consensus elements (Fig. 7) which overlap consensus TRE sites. The SX2 region also contains two identical consensus C/EBP elements (37). In order to determine which of these elements was involved in induction, we mutated both of the TRE/ARE sites, either changing the TRE consensus bases while retaining the ARE consensus sequence, or changing the ARE bases while retaining the consensus TRE sequence. The following mutations of the SX2 region were prepared (Fig. 7B): (i) in SX2M2, the sequences making up the two consensus TRE sites (TGACT CAGCA and TGACACAGCA) were changed to TGACTACGCA, which leaves the ARE consensus sequence intact, but mutates the TRE consensus sequence; (ii) in SX2M4, the two C/EBP sequences (TGAGGAAAT) were changed to TGAG GCCCT; (iii) in SX2M789, the consensus ARE sites (TGACTCAGCA and TGACACAGCA) were changed to TGACTCATTT and TGACACATTT, respectively, which leaves the TRE consensus intact, but eliminates the terminal GC bases, which have been shown to be crucial for ARE inducibility; and (iv) in SX2M239, the bases TGACT CAGCA and TGACACAGCA were changed to GATCTACGCA in both the consensus ARE and TRE sites. Apart from these mutations, the constructs were identical to pHO1SX2cat.

All of the constructs were then tested for

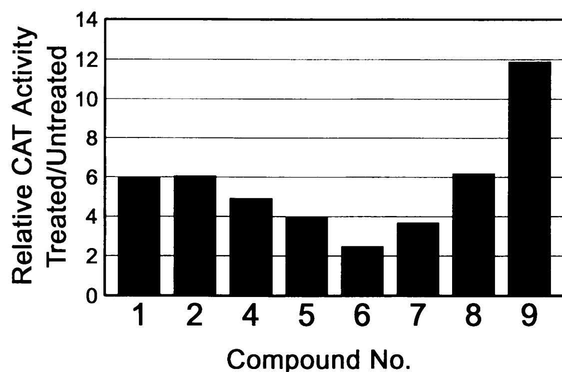

FIG. 5. Response of the 9-kb upstream region of mouse HO-1 gene to inducers

Hepa 1c1c7 cells that had been stably transfected with the construct pMHO9cat, containing 9-kb of the 5′-upstream region of the HO-1 gene linked to the CAT reporter gene were treated with the following inducers: 25 μM *tert*-butylhydroquinone (Compound 1); 25 μM 1,2-dithiole-3-thione (Compound 2); 1.0 μM sodium arsenite (Compound 4); 50 μM *trans*-4-phenylbut-3-en-2-one (Compound 5); 12.5 μM 2,3-dimercapto-1-propanol (Compound 6); 2.5 μM $HgCl_2$ (Compound 7); 2.5 μM benzyl isothiocyanate (Compound 8); and 100 nM phenylarsine oxide (Compound 9). Cells were harvested after 24 hr of treatment and CAT activity was determined. Results are presented as the relative CAT activities of treated to untreated cells, and were corrected for protein concentrations.

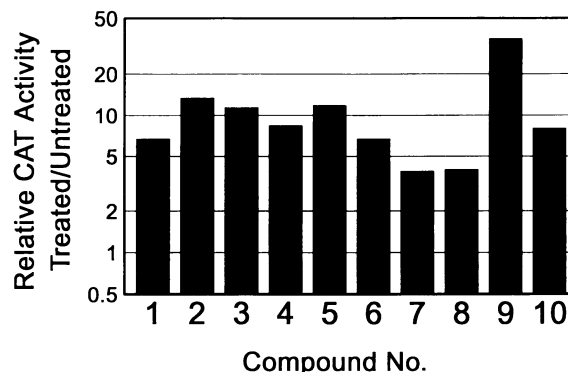

FIG. 6. Response of the SX2 upstream region of mouse HO-1 gene to inducers

Hepa 1c1c7 cells that had been stably transfected with the construct pMHOSX2cat, which contains the 268-bp SX2 fragment linked to the HO-1 minimal promoter, were treated with the following inducers: 25 μM *tert*-butylhydroquinone (Compound 1); 50 μM 1,2-dithiole-3-thione (Compound 2); 5 μM sulforaphane (Compound 3); 1.0 μM sodium arsenite (Compound 4); 50 μM *trans*-4-phenylbut-3-en-2-one (Compound 5); 30 μM 2,3-dimercapto-1-propanol (Compound 6); 3.0 μM $HgCl_2$ (Compound 7); 2.5 μM benzyl isothiocyanate (Compound 8); 100 nM phenylarsine oxide (Compound 9); 2.5 μM 1-nitro-1-cyclohexene (Compound 10). Cells were harvested after 24 hr of treatment and CAT activities were determined. Results are presented as the relative CAT activities of treated to untreated cells, and were corrected for protein concentrations. Note the logarithmic scale of the relative CAT activities.

inducibility by a panel of inducer compounds. Figure 8A shows that with the SX2M2 mutation, changing the sequences that make up the consensus TRE element had no significant effect upon the inducibility of the SX2 region by a variety of inducers. This strongly suggests that the remaining ARE consensus sequence is mediating this induction, as was shown for the glutathione transferase Ya gene (9). Figure 8B shows the effect of mutating the C/EBP sequences (SX2M4). All of the compounds still induced this mutated SX2 element, but the degree of induction was diminished. This result is similar to that previously observed for Cd^{2+} induction of this region (25). Mutations that eliminated the consensus ARE sequences (SX2M239 and SX2M789) completely abolished inducibility by these compounds. Thus, we conclude that induction of HO-1 is mediated by the ARE sites, and is facilitated by the C/EBP sites, but that only the ARE sites are necessary (and sufficient) for induction of HO-1 by monofunctional phase 2 enzyme inducers.

CONCLUSIONS

In this study, we demonstrate that all known classes of monofunctional phase 2 enzyme inducers are also highly potent and effective inducers of HO-1. In addition, we show that induction proceeds via increased gene transcription and is mediated entirely through a 268-bp region that is located approximately 4 kb upstream of the transcription start site. Furthermore, induction of HO-1 depends on the presence of consensus ARE sequences and is entirely independent of the presence of the consensus TRE sequence. The induction by these compounds is greatly enhanced by the adjacent C/EBP sites. Together with other recent studies (9,22,23), the present experiments establish that ARE and TRE elements are not functionally equivalent, but represent specific elements with differential responsiveness, and presumably bind different transcription factors.

Interestingly, it was possible to predict the presence of ARE enhancers in the gene for HO-1

A

```
-268
 TCTAGAACTT TCACTTCCTT CTGCCTGAAG TTAAAGCCGT TCCGGAACCT TTTACCAACT
-208                                      TRE/ARE
 ATTCCTAGCT GAGGCAGAGG GAACAGAGGG TGACTCAGCA AAATCTGTCT TCCCTTTGTC
-148                          TRE/ARE                    C/EBP
 TGCTAATCAC CCCTCCCAAC CATGACACAG CATAAAAGCA GCTGAGGAAA TGAGCAGTGA
-88  C/EBP      TRE/ARE like
 GGAAATCACA ACTCAGCATT CCAGCCGCTG CGGGAAAAAC AAAGTTCAGC TCCTGTGGGG
```

B

MUTANTS — ENHANCER REGIONS

	-179 TRE/ARE	-127 TRE/ARE	-106 C/EBP	-91 C/EBP	-81 TRE/ARE like
SX2	GTGACTCAGCA	ATGACACAGCA	TGAGGAAA	TGAGGAAAT	ACAACTCAGCA
SX2M2	AC	AC			
SX2M4			CCC	CCC	
SX2M789	TTT	TTT			AGC
SX2M239	GATCTAC	GATCTAC			

Consensus TRE: $TGA^{C}/_{G}T^{C}/_{A}A$

Consensus ARE: TGACNNNGC

FIG. 7. Sequences of the SX2 region and its mutations

(A) Sequence of the SX2 element of the 5′-upstream region of the mouse HO-1 gene showing the region of −268 to −29 bp from the origin of replication. The locations of three potential TRE/ARE and two C/EBP regulatory regions are shown. (B) The five above-mentioned regulatory regions present in the SX2 sequence are shown together with the base changes that were introduced to provide the mutants designated SX2M2, SX2M4, SX2M789, and SX2M239. The consensus TRE and ARE sequences are shown for comparison.

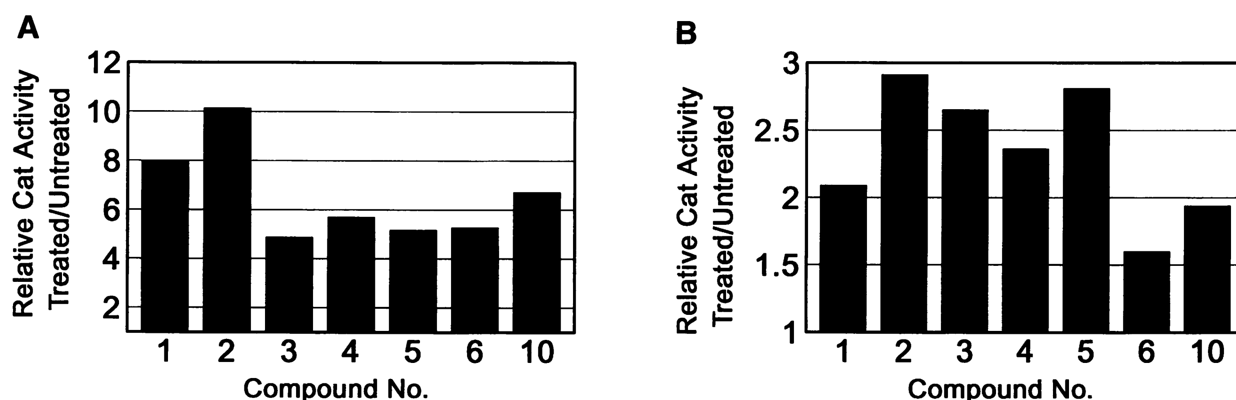

FIG. 8. Response of M2 (A) or M4 (B) SX2 mutants to inducers

Hepa 1c1c7 cells that had been stably transfected with the constructs pMHOSX2M2cat (A) or pMHOSX2M4cat (B) were treated with the following inducers: 25 μM *tert*-butylhydroquinone (Compound 1); 50 μM 1,2-dithiole-3-thione (Compound 2); 5 μM sulforaphane (Compound 3); 1.0 μM sodium arsenite (Compound 4); 50 μM *trans*-4-phenylbut-3-en-2-one (Compound 5); 30 μM 2,3-dimercapto-1-propanol (Compound 6); 2.5 μM 1-nitro-1-cyclohexene (Compound 10). Cells were harvested after 24 hr of treatment and CAT activities were determined. Results are presented as the relative CAT activities of treated to untreated cells, and were corrected for protein concentrations.

by observing the response of this enzyme to known monofunctional phase 2 enzyme inducers. As supported by preliminary evidence from several groups (see Introduction), this strongly implies that HO-1 is an important enzyme in protecting cells against oxidative and electrophile stress.

ACKNOWLEDGMENTS

The studies by TP and PT were supported by a grant from the National Cancer Institute, Department of Health and Human Services (PO1 CA44530). AMKC was supported by a Physician Scientist award (NIH/NIA K11 AG00516) and a research grant from the American Lung Association. JA was supported by a National Institutes of Health grant (RO1 DK43135). TP was supported by a fellowship from the National Cancer Institute (T32 CA09243), and PJL was supported by a Multidisciplinary Training Grant (NHLBI 07534). We thank Gale Doremus for preparing the manuscript and illustrations.

REFERENCES

1. Maines MD. (1992) *Heme Oxygenase. Clinical Applications and Functions.* CRC Press, Boca Raton, FL.
2. Stocker R, Glazer AN, Ames BN. (1987) Antioxidant activity of albumin-bound bilirubin. *Proc. Natl. Acad. Sci. U.S.A.* **84:** 5918–5922.
3. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. (1987) Bilirubin is an antioxidant of possible physiological importance. *Science* **235:** 1043–1046.
4. Friling RS, Bensimon A, Tichauer Y, Daniel V. (1990) Xenobiotic-inducible expression of murine glutathione *S*-transferase Ya subunit gene is controlled by an electrophile responsive element. *Proc. Natl. Acad. Sci. U.S.A.* **87:** 6258–6262.
5. Rushmore TH, Morton MR, Pickett CB. (1991) The antioxidant response element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.* **266:** 11632–11639.
6. Talalay P, De Long MJ, Prochaska HJ. (1988) Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **85:** 8261–8265.
7. Prestera T, Holtzclaw WD, Zhang Y, Talalay P. (1993) Chemical and molecular regulation of enzymes that detoxify carcinogens. *Proc. Natl. Acad. Sci. U.S.A.* **90:** 2965–2969.
8. Prestera T, Spencer SR, Wilczak C, Zhang Y, Talalay P. (1993) The electrophile counterattack response: Protection against neoplasia and toxicity. *Adv. Enzyme Regul.* **33:** 281–296.
9. Prestera T, Talalay P. (1995) Electrophile and antioxidant regulation of enzymes that detoxify carcinogens. *Proc. Natl. Acad. Sci. U.S.A.* **92:** 8965–8969.
10. Jakoby WB, Ziegler DM. (1990) The enzymes of detoxication. *J. Biol. Chem.* **265:** 20715–20718.
11. Prochaska HJ, Talalay P. (1991) The role of NAD(P)H:quinone reductase in protection against the toxicity of quinones and related agents. In: Sies H (ed). *Oxidative Stress. Oxidants and Antioxidants.* Academic Press, London, pp. 195–211.
12. Favreau LV, Pickett CB. (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.
13. Jaiswal AK. (1991) Human NAD(P)H:quinone oxidoreductase (NQO) gene structure and induction by dioxin. *Biochemistry* **30:** 10647–10653.
14. Prochaska HJ, Talalay P. (1988) Regulatory mechanisms of monofunctional and bifunctional anticarcinogenic enzyme inducers in murine liver. *Cancer Res.* **48:** 4776–4782.
15. Applegate LA, Luscher P, Tyrell RM. (1991) Induction of heme oxygenase: A general response to oxidant stress in cultured mammalian cells. *Cancer Res.* **51:** 974–978.
16. Vile GF, Basu-Modak S, Waltner C, Tyrrell RM. (1994) Heme oxygenase 1 mediates an adaptive response to oxidative stress in human skin fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 2607–2610.
17. Lee T-C, Ho I-C. (1994) Expression of heme oxygenase in arsenic-resistant human lung adenocarcinoma cells. *Cancer Res.* **54:** 1660–1664.
18. Talalay P, De Long MJ, Prochaska HJ. (1987) Molecular mechanisms in protection against carcinogenesis. In: Cory JG, Szentivanyi A

(eds). *Cancer Biology and Therapeutics.* Plenum Press, New York, pp. 197–216.

19. Talalay P. (1989) Mechanisms of induction of enzymes that protect against chemical carcinogenesis. *Adv. Enzyme Regul.* **28:** 237–250.
20. Friling RS, Bergelson S, Daniel V. (1992) Two adjacent AP-1-like binding sites from the electrophile-responsive element of the murine glutathione *S*-transferase Ya subunit gene. *Proc. Natl. Acad. Sci. U.S.A.* **89:** 668–672.
21. Li Y, Jaiswal AK. (1994) Human antioxidant-response-element-mediated regulation of type 1 NAD(P)H:quinone oxidoreductase gene expression. Effect of sulfhydryl modifying agents. *Eur. J. Biochem.* **226:** 31–39.
22. Nguyen T, Rushmore TH, Pickett CB. (1994) Transcriptional regulation of a rat liver glutathione *S*-transferase Ya subunit gene. *J. Biol. Chem.* **269:** 13656–13662.
23. Yoshioka K, Deng T, Cavigelli M, Karin M. (1995) Antitumor promotion by phenolic antioxidants: Inhibition of AP-1 activity through induction of Fra expression. *Proc. Natl. Acad. Sci. U.S.A.* **92:** 4972–4976.
24. Wang B, Williamson G. (1994) Detection of a nuclear protein which binds specifically to the antioxidant responsive element (ARE) of the human NAD(P)H:quinone oxidoreductase gene. *Biochim. Biophys. Acta* **1219:** 645–652.
25. Alam J. (1994) Multiple elements within the 5′ distal enhancer of the mouse heme oxygenase-1 gene mediate induction by heavy metals. *J. Biol. Chem.* **269:** 25049–25056.
26. Alam J, Zhining D. (1992) Distal AP-1 binding sites mediate basal level enhancement and TPA induction of the mouse heme oxygenase-1 gene. *J. Biol. Chem.* **267:** 21894–21900.
27. Alam J, Cai J, Smith A. (1994) Isolation and characterization of the mouse heme oxygenase-1 gene. Distal 5′ sequences are required for induction by heme or heavy metals. *J. Biol. Chem.* **269:** 1001–1009.
28. Zhang Y, Talalay P, Cho C-G, Posner GH. (1992) A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure. *Proc. Natl. Acad. Sci. U.S.A.* **89:** 2399–2403.
29. Church GM, Gilbert W. (1984) Genomic sequencing. *Proc. Natl. Acad. Sci. U.S.A.* **81:** 1991–1995.
30. Bradford MM. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
31. Deng WP, Nickoloff JA. (1992) Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal. Biochem.* **200:** 81–88.
32. Graham FL, van der Eb AJ. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52:** 456–467.
33. Southern PJ, Berg P. (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Gen.* **1:** 327–341.
34. Egner PA, Kensler TW, Prestera T, et al. (1994) Regulation of phase 2 enzyme induction by oltipraz and other dithiolethiones. *Carcinogenesis* **15:** 177–181.
35. Alam J, Yu N, Irias S, Cook JL, Vig E. (1991) Reduced chloramphenicol acetyltransferase activity observed with vectors containing an upstream *Sph*I recognition sequence. *BioTechniques* **10:** 423–425.
36. Putzer RR, Zhang Y, Prestera T, Holtzclaw WD, Wade KL, Talalay P. (1995) Mercurials and dimercaptans: Synergism in the induction of chemoprotective enzymes. *Chem. Res. Toxicol.* **8:** 103–110.
37. Landschulz WH, Johnson PF, McKnight SL. (1988) The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. *Science* **240:** 1759–1764.

Contributed by Paul Talalay on August 25, 1995.