The Antioxidant Responsive Element

ACTIVATION BY OXIDATIVE STRESS AND IDENTIFICATION OF THE DNA CONSENSUS SEQUENCE REQUIRED FOR FUNCTIONAL Activity*

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We have characterized further the antioxidant responsive element (ARE) identified in the 5'-flanking region of the rat glutathione S-transferase Ya subunit gene and the NAD(P)H:quinone reductase gene by mutational and deletion analyses. Our data suggest that the sequence,

5' - puGTACNNNGC - 3'
3' - pyCAGTGNNCG - 5'

where N is any nucleotide, represents the core sequence of the ARE required for transcriptional activation by phenolic antioxidants and metabolizable planar aromatic compounds (e.g. β-naphthoflavone and 3-methylcholanthrene). We also have found that the ARE is responsive to hydrogen peroxide and phenolic antioxidants that undergo redox cycling. These latter data suggest that the ARE is responsive to reactive oxygen species and thus may represent part of a signal transduction pathway that allow eukaryotic cells to sense and respond to oxidative stress.

In previous work from our laboratory, we have described two regions in the 5'-flanking region of the rat glutathione S-transferase Ya subunit gene involved in the regulation of gene expression by planar aromatic compounds (1) and phenolic antioxidants (2). One of the regulatory regions contained a single copy of the xenobiotic responsive element (XRE)1 core sequence found in multiple copies in the 5'-flanking region of the cytochrome P-450 IA1 (CYP1A1) gene (3-7). The second region contained a responsive element, the antioxidant responsive element (ARE), shown previously to be responsive to metabolites of planar aromatic compounds and phenolic antioxidants (1, 2). The ARE also contributes significantly to basal level (constitutive) expression of the Ya subunit gene (1). The identification of the ARE and its response to phenolic antioxidants in the absence of a functional Ah receptor (2) confirm observations by Talalay et al. (8-11), who had previously suggested that induction of several phase I1 drug-metabolizing enzymes can occur through an Ah receptor-independent mechanism. The ARE has also recently been identified in the 5'-flanking region of the rat NAD(P)H:quinone reductase gene (12).

In this paper, we have characterized further the ARE by defining regions of the sequence required for basal and xenobiotic inducible expression. We have shown these activities to be separate yet related through a common DNA binding region. These studies have allowed for the elucidation of a consensus sequence required for inducible expression by phenolic antioxidants. We also demonstrate that the ARE responds to hydrogen peroxide as well as di- and trihydroxybenzenes, which can undergo one- or two-electron reduction and redox cycle to generate reactive oxygen species (13, 14). Our studies suggest that the ARE represents a cis-acting regulatory element which is responsive to oxidative stress.

MATERIALS AND METHODS

Chemicals—β-Naphthoflavone, α-naphthoflavone, 3,5-di-tert-butylcatechol, and tert-butyldihydroquinone were obtained from Aldrich. Hydroquinone, catechol, resorcinol, 1,2,3-trihydroxybenzene, and 1,3,5-trihydroxybenzenes were obtained from Sigma. H2O2 (30% solution) was obtained from Mallinkrodt Chemical Co. H2O2 was also obtained from Sigma as a stabilized 30% solution and from American Chemicals Ltd. (Montreal, Canada) as well as Aldrich. These latter solutions were 30% H2O2 solutions. [14C]Chloramphenicol was obtained from Amersham Corp. Aetyl-CoA was from Pharmacia LKB Biotechnology Inc. All other chemicals were of the highest grade available. The β-galactosidase expression plasmid, pCH110, was obtained from Pharmacia.

Tissue Culture—The tissue culture cells were maintained in minimal essential medium plus l-glutamine (Hazleton Laboratory, Lenoexa, KS) in a humidified incubator maintained at 37 °C and an atmosphere of 7% CO2/93% air. All medium additives were obtained from Hazleton Laboratory unless otherwise specified.

Human hepatoma cells, HepG2, were obtained from the ATCC tissue culture cell collection. The HepG2 cells were maintained in Eagle’s minimal essential medium plus l-glutamine with nonessential amino acids, sodium pyruvate, 10% heat-inactivated fetal bovine serum (GIBCO), penicillin (10 units/ml), and streptomycin (10 units/ml).

Transfection of Hepatoma Cells—The cells were transfected at 80% confluency by a modified calcium phosphate precipitation method routinely used in our laboratory (1). Briefly, the HepG2 cells were grown to 90% confluency, trypsinized for 2 min at room temperature, and plated in 25-cm² plastic flasks (Corning) at a density of 1-2 x 10⁵ cells/flask. The cells were allowed to recover overnight.

The medium was changed, and 3 h later 10 µg of test plasmid coprecipitated with 10 µg of control plasmid pCH110 were added to each flask. Plasmid pCH110 is a β-galactosidase reporter gene linked to the SV40 early promoter. The cells were incubated at 37 °C with the precipitated DNA for 5 h. A fine grainy layer of precipitated (complexed) DNA was visible by microscopic examination on the cells and flasks after a 5-h incubation.

Following exposure to the DNA, the cells were washed with 1 × phosphate-buffered saline, exposed to a glycerol shock solution for 2 min, rinsed with fresh medium (medium preheated to 37 °C), and returned to the incubator to recover overnight. The cells were then treated with the appropriate xenobiotic for 24 h as described in the figure legends. The xenobiotics were prepared as 1000-fold concen-
trated solutions in dimethyl sulfoxide just prior to use. An equal volume of dimethyl sulfoxide was added to the control flasks. The various xenobiotics were evaluated for cytotoxicity over a concentration range from 5-400 μM by monitoring the leakage of lactate dehydrogenase from the cells over 24 h of exposure (15). For H₂O₂, cytotoxicity was evaluated from 100 μM to 1 mM H₂O₂.

**Subcloning of Synthetic ARE Deletions and Point Mutations**—Oligonucleotides complimentary to the ARE sequence, 5' and 3' deletions of the ARE sequence, and point mutations within the ARE sequence were synthesized and purified as described above. The sequence of each deletion is shown in Fig. 5, and the site of each point mutation is described in Fig. 6. Complementary oligonucleotides were phosphorylated, annealed, and ligated into the Ndel site of clone -164CAT as previously described (2). Briefly, clone -164CAT, which contains the CAT structural gene and glutathione S-transferase Ya subunit proximal promoter (base pairs -164 to +6), was linearized by digestion with restriction enzyme Ndel. The 5' overhangs were blunt-ended with Klenow fragment of DNA polymerase I and the terminal phosphate group removed with bacterial alkaline phosphatase (22). The oligonucleotides were then ligated into the Ndel site by overnight reaction with T4 DNA ligase (22). An aliquot of the ligation mix was used to transform competent E. coli SCS-1 cells (Stratagene). The resulting colonies were screened by hybridization and the positive clones purified (20, 21). All clones were sequenced to verify number and orientation of the inserts.

**RESULTS**

**Effect of Di- and Trihydroxybenzenes and Hydrogen Peroxide on Transcriptional Regulation of the Glutathione S-Transferase Ya Subunit Gene**—In previous studies from our laboratory we have shown that the ARE sequence is required for transcriptional activation of the glutathione S-transferase Ya subunit and quinone reductase genes by tert-butylhydroquinone and 3,5-di-tert-butylcatechol (2, 12). In order to further define the structure-activity relationship of phenolic antioxidants, a stable HepG2 cell line containing the ARE sequence inserted in the XRE-CAT construct, was transfected with single copies of the XRE gene or the XRE gene with each of the following deletions: deletions Δ22CAT, Δ22CATΔ3, Δ22CATΔ2, Δ22CATΔ3Δ2, Δ3CATΔ2, Δ2CATΔ3, Δ3CATΔ2Δ3, Δ2CATΔ3Δ2Δ3, Δ3CATΔ2Δ3Δ2, Δ2CATΔ3Δ2Δ3Δ2, and Δ3CATΔ2Δ3Δ2Δ3Δ2. The cells were exposed to a number of different di- and trihydroxybenzenes. When the stable cell lines were exposed to catechol (25-400 μM), hydroquinone (25-400 μM), or p-benzoquinone (10-50 μM), CAT activity was induced in a dose-dependent manner (Fig. 2). Exposure of the stable HepG2 cells to resorcinol, a 1,3-diphenol, did not result in an increase in CAT activity at any concentration used in Fig. 2. These data are consistent with previous work by Talalay et al. (8-11), who found that quinone reductase enzymatic activity was inducible by 1,2-diphenols (catechol) and 1,4-diphenols (hydroquinone) but not by resorcinol (a 1,3-diphenol).

We also examined whether 1,2,3-trihydroxybenzene or 1,3,5-trihydroxybenzene induced CAT activity in the stable cell line. Exposure of the cells to 1,2,3-trihydroxybenzene induced CAT activity in a dose-dependent manner, whereas 1,3,5-trihydroxybenzene had no effect on CAT activity (Fig. 2). These data are consistent with our studies on the diphenols.

Since the stable HepG2 cell line used in the previous studies contained the entire 5'-flanking region of the glutathione S-transferase Ya subunit gene, which has an XRE and ARE sequence, we examined the contribution of each responsive element to the transcriptional activation of the glutathione S-transferase Ya subunit gene by the di- and trihydroxybenzenes. In these experiments transient assays were done using HepG2 cells transfected with single copies of the XRE or the ARE sequence ligated to the glutathione S-transferase Ya minimal promoter-CAT construct as described previously (2). Exposure of HepG2 cells transfected with the ARE-CAT construct to the various xenobiotics resulted in an induction pattern of CAT activity similar to that described for the stable cell line (Fig. 3A). All of the compounds except resorcinol and 1,3,5-trihydroxybenzene induced CAT activity through the ARE sequence (Fig. 3A). Exposure of the HepG2 cells transfected with the XRE-CAT construct to the xenobiotics gave
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FIG. 1. Polymerase change reaction primers for 5' and 3' deletions of the ARE sequence. The nucleotides in boldface type represent nucleotides in the ARE sequence.

![Polymerase change reaction primers](image)

a very different pattern of induction (Fig. 3B). Hydroquinone, t-butylhydroquinone, catechol, resorcinol, and 1,3,5-trihydroxybenzene had no effect on CAT activity (Fig. 3B). Therefore, t-butylhydroquinone, hydroquinone, and catechol all induce CAT activity through the ARE sequence. It is interesting that 1,2,3-trihydroxybenzene and p-benzoquinone did elevate CAT activity 2-fold in cells transfected with the XRE-CAT construct. These two compounds may have some weak interaction with the Ah receptor, or they may polymerize to produce compounds that are capable of interacting with the Ah receptor. α-Naphthoflavone, which is an inhibitor of CYP1A1 in vitro (24), can transcriptionally activate the Ya subunit gene via the XRE or ARE (Fig. 3).

A common feature of all the phenolic antioxidants which transcriptionally activate the glutathione S-transferase Ya subunit gene via the ARE sequence is their ability to undergo redox cycling with the potential to form superoxide anion radicals and hydrogen peroxide (13, 14). Therefore we examined whether hydrogen peroxide might transcriptionally activate the glutathione S-transferase Ya subunit gene via the ARE sequence. Exposure of the stable cell line or cells transfected with the ARE-CAT construct to hydrogen peroxide (100 µM to 1.0 mM) resulted in a dose-dependent increase in CAT activity (Fig. 4). Both "stabilized" and "unstabilized" hydrogen peroxide were utilized in these studies, and no difference in the extent of CAT induction was seen with

![Graph showing Acetylation of [14C] Chloramphenicol](image)
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Unstabilized versus stabilized solutions. Hydrogen peroxide had no effect on CAT activity in cells transfected with the XRE-CAT construct (Fig. 4). These data suggest that the ARE sequence is responsive to reactive oxygen species and thus responds to oxidative stress.

Deletion Analysis of the Antioxidant Responsive Element: Determination of the Minimum Sequence Required for Basal and Inducible Activity—To more precisely define those nucleotides of the ARE sequence required for both basal and inducible activity, a series of 5' and 3' deletion mutants of the ARE sequence (nucleotides -722 to -682 of the glutathione S-transferase Ya subunit gene flanking region) were prepared using PCR. A deletion procedure utilizing PCR was chosen because it provided the greatest control over the extent of each deletion compared to procedures employing nuclease digestion. Clone -722CAT and -663CAT (1) were used as templates in the amplification reactions.

The 5'-flanking region of the glutathione S-transferase Ya subunit gene in clone -722CAT was deleted every 3-7 nucleotides through the entire ARE sequence in order to determine the 5' boundary for basal and inducible activities. The data from these deletions are presented in Fig. 5.

The first construct prepared using the PCR technique contained the entire Ya subunit promoter starting at nucleotide -722. The clone containing this PCR fragment was designated -722CAT. This construct was made as a control to insure that the deletions prepared by the PCR technique had activities similar to those produced previously with Bal31 (1). A comparison of the expression data presented in Fig. 5 for the PCR -722CAT construct was identical with that previously reported for the -722CAT produced by digestion with Bal31 (1).

When the 5' sequence in clone -722CAT was deleted from nucleotide -722 to nucleotide -714, the basal level and inducible CAT activity were identical to that seen with the -722CAT construct. However, deletion of 14 nucleotides from clone -722CAT to nucleotide -708, construct -708CAT, reduced the basal level CAT activity to 17.7 ± 2.5 pmol/h/µg, approximately 50% of that seen with the -722CAT construct. Nevertheless, exposure of HepG2 cells transfected with clone -708CAT to β-NF still resulted in inducible CAT activity (Fig. 5). Additional deletions of the 5' sequence in clone -722CAT to nucleotide -702 did not affect basal or inducible CAT activity.

Deletion of the 5' sequence in clone -722CAT to nucleotide -699, construct -699CAT, had a significant effect on both the basal and inducible CAT activities. The basal CAT activity in HepG2 cells transfected with clone -699CAT was 10.4 ± 2.1 pmol/h/µg. This level of basal CAT activity was approximately 58% of the basal level activity in cells transfected with clones -708CAT to -702CAT (17.7 ± 2.5 and 18.0 ± 2.3 pmol/h/µg, respectively) and 33% of the basal level activity in cells transfected with clones -722CAT and -714CAT (31.9 ± 2.8 and 28.8 ± 3.6 pmol/h/µg, respectively). Nevertheless, exposure of HepG2 cells transfected with clone -699CAT to β-NF increased CAT activity from the basal level of 10.4 ± 2.1 pmol/h/µg to 27.7 ± 3.9 pmol/h/µg, a 2.7-fold increase.
Deletion of the 5′ sequence in clone −722CAT to nucleotide −697 had no further effect on the basal or inducible CAT activity. When the 5′ sequence in clone −722CAT was deleted to nucleotide −696 (clone −696CAT), basal CAT activity of the construct was identical to −697CAT. However, exposure of HepG2 cells transfected with clone −696CAT to β-NF failed to increase the level of CAT activity above basal level. Therefore removal of nucleotide −697 abolished inducible activity and defined the 5′ boundary of the ARE sequence essential for xenobiotic induction. Deletions to nucleotides −698, −690, and −687 resulted in constructs with basal and inducible CAT activities similar to clone −696CAT.

A second series of deletion mutants were constructed to define the 3′ boundary of the ARE sequence essential for basal and xenobiotic inducible activities. Deletions from the 3′ end of the ARE sequence, starting with nucleotide −682, were prepared using the same PCR technique. A Bal31 deletion clone, −663CAT (1), that contained none of the ARE sequence was used as the template for these deletions. Deletion of 3 nucleotides from the 3′ end of the ARE sequence, nucleotides −682 to −684, produced a clone, −722(−685)CAT, that, when transfected into HepG2 cells, expressed the same basal and inducible CAT activity as clone −722CAT (Fig. 5). Removal of 3 additional nucleotides from the 3′ end of the ARE sequence to nucleotide −685, clone −722(−688)CAT, had no effect on the basal or inducible CAT activities when transfected into HepG2 cells, compared with clone −722(−685)CAT.

When 10 nucleotides from the 3′ end of the ARE sequence were removed (clone −722(−697)CAT) basal level and inducible CAT activity were abolished. This construct produced CAT activities identical to the −696CAT construct described previously. Further 3′ deletion to the nucleotide −704 clone −722(−704)CAT had no additional effect on the basal or inducible CAT activities when transfected into HepG2 cells, compared with clone −722(−685)CAT.
proximal promoter produced a construct, clone AREOCAT, that, when transfected in HepG2 cells, expressed both basal and inducible CAT activity identical to that seen with deletion clone -722CAT.

Insertion of a single copy of an oligonucleotide containing the ARE sequence between nucleotides -708 to -682 in front of the Ya subunit proximal promoter produced a clone, ARE1CAT, that, when transfected into HepG2 cells, expressed both basal and inducible CAT activities, 17.1 ± 2.9 and 43.4 ± 4.9 pmol/h/μg, respectively, identical to constructs -708 through -702CAT.

To verify the position of the 3' boundary of the ARE identified during the deletion studies, we synthesized and inserted in front of Ya subunit proximal promoter a series of 3' deletion mutants of the ARE sequence in clone ARE1CAT. The clones were transfected into HepG2 cells and monitored for basal and inducible CAT activity. When a single copy of the deleted ARE sequence, nucleotide -708 to -680 or ARE2CAT, was inserted in front of the Ya subunit proximal promoter and transfected into HepG2 cells, basal and inducible activity was identical to the 5' deletion clone -708CAT. However, when a single copy of the deleted ARE sequence (nucleotides -708 to -692 or ARE3CAT) was inserted in front of the Ya subunit proximal promoter and transfected into HepG2 cells, inducible CAT activity was abolished. Basal CAT activity was comparable with the 5' deletion construct -708CAT. These data indicated that the 3' boundary of the ARE sequence required for inducible activity had been passed.

Interestingly when a single copy of the deleted ARE sequence (nucleotides -708 to -695 or ARE4CAT) was inserted in front of the Ya subunit proximal promoter, and transfected into HepG2 cells, basal level CAT activity was significantly lower than that reported for any other clone, 4.1 ± 3.1 pmol/h/μg. Exposure of HepG2 cells transfected with clone ARE4CAT to β-NF also failed to increase the level of CAT activity above the basal level, 4.0 ± 2.8 pmol/h/μg.

Again, to verify the position of the 3' boundary of the ARE identified during the deletion studies, we synthesized and inserted in front of the Ya subunit promoter a 3' deletion construct of the ARE (ARE6CAT). The clone was transfected into HepG2 cells and monitored for basal and inducible CAT activity. This construct resulted in basal and inducible CAT activity that was identical to the 5' deletion construct -703CAT. A longer construct, ARE5CAT, gave identical results to the ARE6CAT construct. Therefore, based on the data generated from the 5' and 3' deletions as well as from the synthetic oligonucleotides, xenobiotoic inducible activity could be localized between -697 and -688. In addition, nucleotides important for basal level expression also appear to reside between -697 and -688 as well as 5' of -697.

Mutational Analysis of the ARE Sequence Determination of Essential Nucleotides Required for Xenobiotoic Inducible and Basal Level Expression—We described in the previous section the 5' and 3' deletion analysis of the ARE sequence. The results suggested that the 5' and 3' boundary defining the xenobiotoic reducible region was localized between nucleotides -697 and -688. The identification of the 5' and 3' boundary required for basal level expression was less clear. In order to determine which nucleotides were essential for the inducible activity, we synthesized a series of oligonucleotides encompassing the entire ARE sequence, with single base mutations at unique sites (Fig. 6). Each mutant clone contained the sequence shown plus the remaining 5'-ARE sequence, nucleotides -722 to -705. Each nucleotide pair between nucleotides -704 to -687, inclusive, was changed (A and T to G; G and C to A) except nucleotides -701 and -699. The oligonucleotides were cloned into the NdeI site of clone -164CAT as described in "Materials and Methods" and co-transfected with pCH110 into human HepG2 cells. Clones AREOCAT and -164CAT were used as positive and negative controls for basal and inducible CAT activity, respectively. The results of the transfection of point mutants of the ARE into HepG2 cells are presented in Fig. 6.

Successive single point mutations of the ARE sequence from nucleotides -704 to -697 had no effect on either the basal or inducible CAT activities, as compared with the wild type sequence, AREOCAT. No point mutation was made at nucleotides -701 and -699. These residues, as shown in Fig. 6, correspond to nucleotides in the ARE consensus sequence, predicted from the sequence identity between the ARE in the 5'-flanking region of the quinone reductase gene and glutathione S-transferase Ya subunit gene (12). Activities shown in Fig. 6 for these positions were obtained using the wild type glutathione S-transferase Ya-ARE sequence, clone AREOCAT (Fig. 5).

A mutation at nucleotide -696, a T to G change, abolished both the basal level and inducible CAT activities, 11.9 ± 1.0 and 9.4 ± 0.9, respectively, as compared with the positive and negative controls. Point mutations at nucleotides -695 (G to A), -694 (A to G) and -693 (C to A) gave similar results to those described for the mutation at nucleotide -696 (see Fig. 6). Point mutations in the nucleotides from -692 through -690 had no effect on either the basal level or inducible CAT activities as compared to the controls. Mutation of nucleotide -689, a G to A change, had no significant effect on the basal level CAT activity, but the mutation abolished inducibility by xenobiotoic. Mutation of nucleotide -688, a C to A change, gave identical results to those described for the mutation at nucleotide -689. Mutation of nucleotide -687 had no effect on either the basal level or inducible CAT activities as compared with the controls. These data suggest that nucleotides -689 and -688 are required for inducible activity but not basal level expression, whereas nucleotides -696 to -693 are required for both basal and inducible activity. It is interesting that these nucleotides are also conserved in the ARE identified in the rat quinone reductase gene. The data presented in the point mutation studies are in good agreement with the 5' and 3' deletion studies, which suggests that nucleotides spanning -697 to -688 are important for xenobiotoic inducible expression.

DISCUSSION

In the present study, we have characterized further the ARE identified previously in the rat glutathione S-transferase Ya subunit gene (1, 2) and the NAD(P)H quinone reductase gene (12). Using 5' and 3' deletion analysis as well as mutational analysis of the ARE, we have been able to define a core sequence required for xenobiotoic induction. A single copy of the core sequence,

5' - pUGTGACNNNGC - 3'
3' - pyCAGCTGNNNGG - 5'

where N is any nucleotide, is found in the ARE sequence that has been described for the glutathione S-transferase Ya subunit and the quinone reductase genes. The ARE core sequence shares no identity to the XRE core sequence

5' - TNGCGTG - 3'
3' - ANCGCAC - 5'

found in multiple copies in the CYPIA1 gene (3-7) as well as in a single copy in the 5'-flanking region of the rat glutathione...
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Fig. 6. Point mutations within the ARE and their respective CAT activities upon transfection into HepG2 cells. Oligonucleotides corresponding to the ARE sequence (nucleotides -722 to -682) and single nucleotide mutations within this sequence were synthesized, purified, and subcloned in front of expression clone -164CAT as described under "Materials and Methods" and previously (2). The various clones were transfected, or co-transfected with the β-galactosidase expression plasmid CH110, into HepG2 cells and left untreated or exposed to β-NF as described in Fig. 1. CAT activity and β-galactosidase activity were monitored as described. The experimental value for CAT activity is expressed as the relative activity, CAT activity divided by β-galactosidase activity, and is the mean ± S.D. of three separate experiments. All assays were done in duplicate. The subcloned oligonucleotides all contained nucleotides -722 to -682; only nucleotides -704 to -687 were mutated and shown in the figure. Wild represents the 41-base pair ARE sequence. QR-ARE, ARE in 5'-flanking region of the quinone reductase gene.

S-transferase Ya subunit gene and quinone reductase gene (1, 2, 12). The lack of sequence identity between these two core sequences is consistent with their functional activity (2). The ARE regulates transcription of the glutathione S-transferase Ya subunit and quinone reductase genes by metabolizable planar aromatic compounds, phenolic antioxidants, and hydrogen peroxide, whereas the XRE is responsive only to planar aromatic compounds (e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin and 3-methylcholanthrene) (1, 2, 12). Therefore, the ARE represents a novel xenobiotic responsive element which can mediate induction of at least two different genes encoding drug metabolizing enzymes.

The deletion analysis presented in this manuscript as well as our previous work (1, 2, 12) also demonstrates that the ARE contributes to the basal level expression of the glutathione S-transferase Ya subunit and quinone reductase genes. Although we have not completely defined the nucleotides responsible for basal level expression, it does appear that the same nucleotides responsible for inducible expression also are important for basal level expression. This can best be seen with the point mutations at position -696, -695, -694, and -693, which eliminate both basal and inducible expression. However, point mutations at position -689 and -688 abolish inducible activity without affecting basal level expression. Therefore, proteins involved in basal level and inducible expression share overlapping but not identical DNA recognition motifs. These data are consistent with our deletion analysis, which shows that nucleotides upstream from -697 are also required for maximum basal level expression but not inducible expression.

Although methylation interference and protection experiments as well as gel mobility shift experiments show DNA-protein interactions in the 5'-flanking region of the Ya gene containing the ARE (1, 25), it is unclear how many DNA-protein or protein-protein interactions occur to regulate basal and inducible expression via the ARE sequence. Clearly, these interactions are complex and will require additional studies to delineate.

In the present study, we have also found that phenolic antioxidants, which can undergo one- or two-electron reductions as well as redox cycling to form reactive oxygen species (13, 14) and oxidants such as hydrogen peroxide, activate expression of the glutathione S-transferase Ya subunit gene as well as the quinone reductase gene through the ARE. These data suggest that the ARE represents a cis-acting regulatory element which activates genes encoding proteins (e.g., glutathione S-transferase and quinone reductase) that protect eukaryotic cells against oxidative stress. Interestingly, a similar
mechanism has recently been described in bacteria that respond to oxidative stress by inducing catalase and alkyl hydroperoxide reductase. Tartaglia et al. (26) and Storz et al. (27) have found cis-acting regulatory elements in the 5′-flanking region of the E. coli catalase and alkyl hydroperoxide genes which are recognized by a trans-activator named oxyR. The oxyR protein activates these genes in cells treated with hydrogen peroxide, but it is not induced by oxidative stress (i.e., hydrogen peroxide). Storz et al. (27) have demonstrated that the reduced and oxidized forms of oxyR bind to the catalase promoter but only the oxidized form activates transcription. The DNase I footprints of the reduced and oxidized forms of oxyR are different, suggesting that a conformational change in the oxyR protein is associated with its transition from an inactive to active form (27). Although it is unclear from our studies whether a similar mechanism is responsible for activating gene transcription via the ARE, our gel mobility shift data reported previously suggest that a protein from nuclear extracts of untreated cells binds to the ARE (1).

Interestingly, treatment of the cells with xenobiotic inducers shifts data reported previously suggest that a protein from nuclear extracts of untreated cells binds to the ARE (1). Hence the ARE and its DNA binding protein(s) are part of a signal transduction pathway that enables eukaryotic cells to respond to oxidative stress. The identification of the core recognition motif described in this manuscript should facilitate the isolation and characterization of the protein(s) which interact with the ARE and activate gene expression during oxidative stress.

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