The antioxidant response element (ARE) found in the 5'-flanking region of the rat quinone reductase gene has been further characterized by mutational and deletion analysis. The results indicate that the 31-base pair ARE, which contains a 13-base pair palindromic sequence, can be further separated into three regions, all three of which are required for elevated basal level gene expression. These three regions include the proximal and distal half-sites as well as a 3'-flanking region consisting of 4 adenine nucleotides. Neither the proximal nor the distal half-site alone mediates transcriptional activation by β-naphthoflavone. However, when placed together the two half-sites restore responsiveness to the inducer. Interestingly, the presence of only 1 of the 4 adenine nucleotides in the 3'-flanking region of the proximal half-site is required for responsiveness to the inducer. Point mutations within the ARE indicate that several nucleotides in both the proximal and distal half-sites are required for basal level gene expression. Electrophoretic mobility shift analysis using the ARE as the probe indicates that enhancers found in the glutathione S-transferase Ya and P genes recognize a similar trans-acting factor(s) found in crude nuclear extracts from human Hep G2 cells. Further, this complex can be detected in nuclear extracts from rat liver and rat hepatoma cells but not in mouse Hepa 1c1c7 cells or in human HeLa cells. The ARE-nucleoprotein complex can also be detected in F9 cells which lack significant levels of un/Fos proteins. Although the rat ARE resembles the human quinone reductase ARE which contains a consensus TRE, the 2-nucleotide change in the core sequence (TGACTCA versus TGACTTG) eliminates the high affinity TRE motif in the rat ARE. The rat ARE forms a nucleoprotein complex in Hep G2 and other cells with different properties than AP-1.

NAD(P)H:quinone oxidoreductase (DT-diaphorase, quinone reductase) is a phase II drug-metabolizing enzyme found in liver and other tissues and is inducible by a wide variety of compounds including phenolic antioxidants, planar aromatic compounds and TCDD (1–3). In order to further understand the molecular mechanisms underlying the constitutive and inducible expression of this gene, two response elements found in the 5'-flanking region of the rat gene have been identified (4). One of these elements is the xenobiotic response element (XRE) found in the CYP1A1 and rat glutathione S-transferase Ya subunit genes (5, 6).

The second element is the antioxidant response element (ARE) which is responsible for basal and inducible expression of the gene (4). We have demonstrated that diverse xenobiotics such as β-naphthoflavone (β-NF), tert-butylhydroquinone (t-BHQ), hydrogen peroxide, and 12-O-tetradecanoylphorbol 13-acetate (TPA) can transcriptionally activate gene expression through the ARE (4, 7). A high affinity DNA-protein binding complex exists in crude nuclear extracts of Hep G2 cells based on DNase I footprinting, methylation interference and protection assays, and electrophoretic mobility shift analyses (7).

The ARE for the human quinone reductase gene has been identified and shown to contain a consensus TRE sequence within the ARE (8, 9). However, the rat ARE does not contain a high affinity binding site for in vitro synthesized c-jun and c-Fos, nor does the ARE bind jun/Fos proteins found in crude nuclear extracts from HeLa cells (7).

Similar studies have been performed for the rat glutathione S-transferase Ya subunit gene, and ARE and XRE motifs have also been identified and characterized as well (6, 10, 11). Thus, the coordinate induction of these two genes may be at least partially explained by the presence of these two elements.

The mouse glutathione S-transferase Ya subunit gene is regulated by an electrophile responsive element (EpRE) which resembles the rat Ya ARE in 39 of 41 nucleotides (12). A second glutathione S-transferase, glutathione transferase P, has also been identified which is induced by phenolic antioxidants (13). A response element (GPEI, glutathione transferase P enhancer I) in the 5'-flanking region of this gene mediates constitutive expression in hepatoma cells (14, 15). Analysis of this enhancer reveals a significant degree of sequence identity with segments of the AREs found in the rat quinone reductase and the rat and mouse glutathione S-transferase Ya subunit genes (4, 6, 12, 15).

In this communication, we determine the specific nucleotides within the rat quinone reductase ARE sequence that are required for basal level expression and xenobiotic responsiveness. We demonstrate that a high affinity DNA-binding protein complex is present in nuclear extracts of Hep G2 cells, rat liver, a rat hepatoma cell line (H4IIEC3), and mouse F9 cells, but not in extracts from HeLa or Hepa 1c1c7 mouse hepatoma cell lines. Finally, we demonstrate that the GPEI response element; XRE, xenobiotic response element; CAT, bacterial chloramphenicol acetyltransferase; t-BHQ, tert-butylhydroquinone; β-NF, β-naphthoflavone; GPEI, glutathione transferase P enhancer I.
enhancer of the rat glutathione S-transferase P gene and the rat quinine reductase ARE recognize a similar nucleoprotein(s), as judged by electrophoretic mobility shift assays, found in nuclear extracts from rat liver, Hep G2, rat H4IIEC3, and mouse F9 cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Preparation of Nuclear Extracts, and Transfections—Hep G2 cells were grown in Eagle's minimum essential medium (J RH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, penicillin, and streptomycin as described previously (4). HeLa and F9 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The F9 cells were plated on plastic surfaces treated with 0.1% gelatin overnight and rinsed twice with phosphate-buffered saline. Mouse hepatoma Hepa 1c1c7 cells (obtained from Oliver Hankinson, UCLA) were grown in minimum essential medium α modification supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Rat hepatoma H4IIEC3 cells were grown as described for mouse 1c1c7 cells.

Nuclear extracts from all five cell lines were prepared according to the procedure of Dignam et al. (16). After dialysis for 4–5 h, the extracts were clarified by centrifugation and frozen at −80°C. The extracts were stable for at least 6 months when stored under these conditions.

Transfection of Hep G2 cells was performed by a modification of the calcium phosphate-glycerol shock procedure (4, 17). To correct for transfection efficiency, the plasmid pTK β-galactosidase assays were performed as described previously (7). Synthesis of Oligonucleotides and Preparation of Mutant ARE Constructs—Synthetic oligonucleotides were prepared using an Applied Biosystems 380B DNA synthesizer as described previously (4). Coding and noncoding strand oligonucleotides containing multiple or single nucleotide mutations were gel-purified, kinased with T4 polynucleotide kinase, annealed, and subcloned into the NdeI site of p-164CAT as described previously (4). p-164CAT contains the proximal promoter of the glutathione S-transferase Ya subunit gene fused to the bacterial chloramphenicol acetyltransferase structural gene (6). This construct was previously used to prepare pARE-CAT which contains the 31-nucleotide wild type ARE sequence found at positions −434 to −404 relative to the start of transcription in the rat quinine reductase gene (4). Plasmids containing a single copy of the insert in the correct orientation were confirmed by sequencing (Sequenase, U. S. Biochemical Corp.), amplified, and purified by two rounds of centrifugation in cesium chloride. The GPE1 enhancer (15), CAAAGATGTAGTCAGTCACGCATGGCA-3′ and its complement were also synthesized and inserted into p-164CAT. The Ya ARE-CAT construct containing the ARE sequence for the glutathione S-transferase Ya subunit gene has been described (6, 10). The sequences for the human collagenase TRE and a random oligonucleotide have been described (7). A mutant “ARE/TRE” was prepared by converting 2 nucleotides within the quinine reductase ARE to a consensus TRE, TCTAGAATCTGAGTCATAAACATCTGAG.

Electrophoretic Mobility Shift Analysis—Gels shifts were performed in a 6% polyacrylamide nondenaturing system as described previously (7). Where indicated, a 150-fold molar excess of unlabeled double-stranded oligonucleotides used as competitors was preincubated with nuclear extract (prepared from untreated cells) for 15 min at room temperature. The radiolabeled probe was then added, and the mixture was incubated for another 15 min before electrophoresis.

RESULTS

Analysis of the Quinone Reductase ARE: Half-site Requirements for Basal and Inducible Expression—We have previously shown that a palindromic sequence, 5′-…AGTCACAG-TGACTGAG…-3′, exists within the 31-nucleotide ARE (6). The complete proximal and distal half-site sequences found within the ARE are 5′-GTGACTGGCA-3′ and 5′-GTGACTctaga-3′, respectively. However, electrophoretic mobility shift assays indicate that only an intact proximal half-site is required to compete for binding to a nucleoprotein complex found in Hep G2 cells (7). Upon transfaction of mutant ARE CAT constructs into Hep G2 cells, neither half-site alone produces an elevation in CAT activity much above the level seen for p-164CAT (Fig. 1A). When the proximal half-site is mutated, β-naphthoflavone responsiveness is completely lost (M1, Fig. 1A). When the distal half-site is mutated, inducer responsiveness is attenuated (M2, Fig. 1A). Mutation of the 5′-GC-3′ in the proximal half-site results in a construct which lacks both high basal level and inducible responsiveness to β-NF (Fig. 1A).

Deletion mutants in the 3′-terminus of the ARE were prepared in order to determine the minimum sequence required for functional activity. Deletion of 5 nucleotides at the 3′-end of the pARE-CAT construct (i.e. pARE26) results in a construct which retains high basal and inducible CAT expression (Fig. 1B). Deletion of one, two, or three additional adenine nucleotides at the 3′-end (pARE25, pARE24, and pARE23) results in constructs which progressively lose basal level gene expression. However, all three constructs retain inducible activity. Deletion of the final adenine nucleotide results in a construct (pARE22) that is only slightly responsive to β-NF (L3-1.5-fold). Thus, the terminal adenine in the sequence 5′-GTGACTGGCA-3′ represents the 3′ boundary of the minimal sequence required for inducible activity. In contrast, the highest basal level expression occurs when all 4 adenines are present. pARE23 is 5′-7-fold inducible with β-NF while the other constructs (pARE24, pARE25, and pARE26) are only about 3-fold inducible. However, the absolute level of inducible activity in cells transfected with pARE23 is lower than that observed in cells transfected with the wild type pARE-CAT construct.

A deletion analysis of the 5′-end of the ARE was performed using the minimum sequence required for inducible activity as the 3′-end. Deletion of 2 or 5 nucleotides in the 5′-end results in constructs (pARE21 and pARE18) which retain inducible expression of CAT activity (Fig. 1C). Deletion of 8 or 9 nucleotides in the 5′-end yields constructs containing only the complete downstream half-site. When these constructs (pARE15 and pARE14) are transfected into Hep G2 cells no increase in basal level or inducible activity is detected. Therefore, β-NF responsiveness requires the presence of several residues found in the 5′-region flanking the proximal ARE half-site.

Since a single copy of the proximal half-site was not responsive to β-NF, a construct containing two copies of this sequence in a head to tail orientation was made in order to determine if β-NF responsiveness could be restored. When this plasmid (pARE-2X15) is transfected into Hep G2 cells, no increase in basal level expression of CAT activity is found (Fig. 1D). However, transfected cells treated with β-NF have a 2.5-3.0-fold higher level of CAT activity. Thus, the presence of two copies of the proximal ARE half-sites restores β-NF responsiveness. Interestingly, the level of induction is lower for this construct than for pARE23 (i.e. 2.5-3.0-fold compared to 5′-7-fold induction). Since the wild type ARE exists as a palindromic, additional constructs were made which contained two copies of either the distal or proximal half-sites arranged in a palindromic orientation. The distal half-site arranged as a palindrome does not mediate an increase in basal level or inducible gene expression (Fig. 1D). However, a palindromic orientation of the proximal half-site results in an increase in basal level CAT expression (6′-10-fold) as well as β-NF responsiveness (5′-7-fold).

Analysis of Point Mutations in the ARE—Since mutation of the “TGAC” residues in the half-sites on both the coding and noncoding strands abolished basal level expression, selected individual point mutations were made in order to assess their contribution to the functional activity of the ARE. Mutations in the upstream half-site (G-5′, C-3′ and C-1′) result in decreased basal level expression (Fig. 2). The effect is more pronounced the closer the mutation is to the center of the palindrome. Mutants G-5′ and C-3′ retain the ability to respond to β-naphthoflavone, while mutant C-1′ is only slightly
responsive to the inducer. In contrast, several mutations made in the downstream half-site abolish basal level expression and \(\beta\)-naphthoflavone responsiveness (Fig. 2). When nucleotides at positions T2, G3, or A4 are mutated, both basal level and inducible expression is completely abolished. Mutations at positions C5 and C10 result in constructs which also have decreased basal activity relative to the minimal promoter (22 and 13% of the wild type ARE activity). However, both mutants C5 and C10 are responsive to \(\beta\)-naphthoflavone (2.5- and 2.0-fold, respectively). Additional mutations made at positions G1, G8, G9, A11, and C16 result in constructs with varying degrees of basal level expression all of which retain responsiveness to the inducer (Fig. 2). Although both half-sites are required for full basal level gene expression, only 3 residues in the downstream half-site appear to be absolutely required for high basal and inducible expression, 5'-TGA-3'.

Electrophoretic Mobility Shift Analysis of Mutant ARE Sequences—We have previously shown that the downstream half-site is sufficient for binding of the ARE-nucleoprotein complex from crude Hep G2 nuclear extract in vitro (7). Consistent with this observation, mutations in the upstream half-site do not interfere with the ability to compete with the wild type ARE sequence in the gel shift assay (Fig. 3). Only 4 point mutants made in the downstream half-site have lost the ability to compete with the wild type ARE sequence, i.e. mutants T2, G3, A4,
and C\textsuperscript{10}. Three of these 4 nucleotides are those that are required for both basal and inducible expression, T\textsuperscript{2}, G\textsuperscript{3}, and A\textsuperscript{4} (Fig. 2). The fourth nucleotide, C\textsuperscript{10}, when mutated is only 13% as active as the wild type ARE. Comparison of the Rat Quinone Reductase ARE with the Rat Glutathione Transferase P Enhancer—Previously, we showed that the rat glutathione S-transferase Ya subunit ARE could compete with the quinone reductase ARE in the gel mobility shift assay using natural crude nuclear extracts from Hep G2 cells (7). Excess GPEI also competes effectively for the quinone reductase ARE-binding protein (Fig. 4A). Electrophoretic mobility shift assays performed with either the radiolabeled GPEI or a probe containing the Ya sequence yielded a complex with the same mobility as the complex formed with the quinone reductase ARE (data not shown). Therefore, based on gel shift experiments all three of these enhancers recognize a similar binding protein complex found in nuclear extracts from Hep G2 cells. When a construct containing the GPEI enhancer is transfected into Hep G2 cells which are then treated with T\textsuperscript{BHQ} or TPA, a 2.5–3.0-fold elevation in CAT activity above the levels seen in untreated cells results (Fig. 4B). This level of induction is similar to that seen for the rat quinone reductase ARE and the rat glutathione S-transferase Ya subunit gene ARE.

Identification of ARE-Nucleoprotein Complexes in Other Cell Lines—Since the initial characterization of the quinone reductase ARE-nucleoprotein complex had been performed only in nuclear extracts from human Hep G2 cells, we tested a number of other cell lines to determine if the complex is present in hepatoma and non-hepatoma cells. Crude nuclear extracts from the rat hepatoma cell line H4IIEC3, but not the mouse hepatoma cell line Hepa 1c1c7, form a complex with the same mobility as the complex identified in Hep G2 cells (Fig. 5A). In contrast, a complex is formed in Hepa 1c1c7 cells which has a different electrophoretic mobility than the complex found in Hep G2 cells (Fig. 5A). The different mobility of this complex suggests that it is formed by a different protein or combination of proteins. However, only a weak signal can be detected with 40 \(\mu\)g of nuclear extract. Two non-hepatoma cell lines, F9 and HeLa cells, were also tested. F9 cells form a complex with the same mobility and specificity as the ARE complex found in Hep G2 and H4IIEC3 cells (Fig. 5, A and D). Only weak binding complexes can be detected in nuclear extracts from HeLa cells. Competition by oligonucleotides containing the rat and human quinone reductase ARE, the rat glutathione S-transferase Ya ARE and the glutathione transferase P enhancer (GPEI) indicate that high affinity specific complexes exist in the H4IIEC3, Hepa 1c1c7, and F9 extracts but not the HeLa extract (Fig. 5, B–E). As seen in Hep G2 cells, a mutation in the distal half-site of the ARE does not interfere with the ability to compete with the wild type sequence in any of the cell lines. When nuclear extracts from rat liver were tested, a specific complex with properties similar to those detected in Hep G2, H4IIEC3, and F9 cells was observed (Fig. 5F). Therefore, the specific binding complex in rat liver and in the four cell lines, Hep G2, H4IIEC3, Hepa 1c1c7, and F9, appears to be mediated only by the proximal half-site.

Interestingly, the human collagenase TRE was also found to compete (partially or completely) with the ARE in all instances including the F9 mouse embryonal carcinoma cell nuclear extracts (Figs. 4A and 5, B, C, D, and F). Since this cell line has been shown to contain low levels of AP-1 (18, 19), nuclear extracts from F9 cells were tested in the electrophoretic mobi-

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**Fig. 3.** Electrophoretic mobility shift analysis using mutant ARE oligonucleotides as competitors. A 150-fold molar excess of synthetic double stranded oligonucleotides containing the point mutations described in Fig. 2 were incubated with nuclear extract from untreated Hep G2 cells as described under “Experimental Procedures.” The arrow points to the specific ARE-nucleoprotein complex. Lanes 1–15 contained 15 \(\mu\)g of Hep G2 nuclear extract. Lanes 2–15 contained the following double-stranded oligonucleotides: lane 2, wild type ARE; lane 3, M\textsuperscript{1}; lane 4, M\textsuperscript{2}; lane 5, M\textsuperscript{3}; lane 6, M\textsuperscript{4}; lane 7, M\textsuperscript{5}; lane 8, M\textsuperscript{6}; lane 9, M\textsuperscript{7}; lane 10, M\textsuperscript{8}; lane 11, M\textsuperscript{9}; lane 12, M\textsuperscript{10}; lane 13, M\textsuperscript{11}; lane 14, M\textsuperscript{12}; lane 15, M\textsuperscript{13}.

**Fig. 4.** The GST-P enhancer GPEI recognizes the same nucleoprotein complex in Hep G2 cells and is functionally an ARE. A, electrophoretic mobility shift analysis of the ARE and competition with GPEI was performed as described in Fig. 3. Lanes 3–7 contained a 150-fold molar excess of the following double-stranded oligonucleotides: lane 3, quinone reductase ARE; lane 4, glutathione S-transferase Ya ARE; lane 5, human collagenase TRE; lane 6, glutathione transferase GPEI; lane 7, random oligonucleotide. B, CAT assays of lysates from Hep G2 cells transfected with constructs containing the ARE from the rat quinone reductase gene, the ARE from the rat glutathione S-transferase Ya gene, and the GPEI from the glutathione transferase P gene. See Fig. 1 for details. Cells were treated for 20 h with either Me\textsubscript{2}SO (solid bars), 60 \(\mu\)M T\textsuperscript{BHQ} (striped bars) or 100 nM TPA (stippled bars). CAT activity is reported relative to \(\beta\)-galactosidase activity.
Characterization of the Antioxidant Response Element

**Fig. 5. Electrophoretic mobility shift analysis comparing the ARE and TRE.** A, nuclear extracts from each cell line were incubated with either the ARE or TRE as described under “Experimental Procedures.” Lanes 1–6, ARE probe; lanes 7–12, TRE probe. Lanes 1 and 7, no extract; lanes 2 and 8, Hep G2 nuclear extract (30 μg); lanes 3 and 9, rat hepatoma H4IIEC3 nuclear extract (20 μg); lanes 4 and 10, mouse hepatoma 1c1c7 nuclear extract (40 μg); lanes 5 and 11, mouse embryonal carcinoma F9 nuclear extract (30 μg); lanes 6 and 12, human epithelial carcinoma HeLa nuclear extract (10 μg). B, competition studies were performed using nuclear extracts from H4IIEC3 cells and a 150-fold molar excess of the following double stranded oligonucleotides: lane 1, nuclear extract; lane 2, rat quinone reductase ARE; lane 3, rat glutathione S-transferase Ya subunit ARE; lane 4, glutathione transferase GPEI; lane 5, random oligonucleotide; lane 6, TRE; lane 7, ARE-M1; lane 8, ARE-M2; lane 9, human quinone reductase ARE. C, as in B except the nuclear extract was from 1c1c7 cells. D, as in B except the nuclear extract was from Hep G2 cells. E, as in B except the nuclear extract was from Hep G2 and HeLa cells. F, as in B except the nuclear extract (10 μg) was from rat liver.

**Fig. 6. Electrophoretic mobility shift analysis of the ARE/TRE probe.** Hep G2 nuclear extract from untreated cells was incubated with the radiolabeled ARE/TRE probe (lanes 2–8). Lane 1 contained only the probe. 5'-TCTAGAGTCACAGTGACTCAGCAAAATCTGA-3'. The following oligonucleotides, at a 150-fold molar excess, were added prior to addition of the probe: lane 3, quinone reductase ARE; lane 4, human collagenase TRE; lane 5, mutant ARE/TRE; lane 6, rat Ya ARE; lane 7, GPEI; lane 8, random oligonucleotide.

The antioxidant response element found in the rat quinone reductase gene has been shown to be responsible for both basal level and inducible transcription in hepatoma cells (4). It is composed of a palindromic sequence in which the two half-sites are separated from each other by a single nucleotide. Footprinting assays indicate that both the proximal and distal half-sites...
are protected from DNase I digestion by crude nuclear extracts from Hep G2 cells (7). However, we previously showed by electrophoretic mobility shift competition assays and by methylation protection analysis that only the proximal half-site is required for high affinity binding of the ARE to a protein found in crude Hep G2 nuclear extracts (7).

In this communication we have extended our analysis of this enhancer by mutagenesis of nucleotides in the “core sequence” previously defined for the ARE found in the rat glutathione S-transferase Ya subunit gene (11). Mutational analysis of the ARE indicates that neither the proximal nor the distal half-site alone is sufficient for high basal level gene expression. However, together in the intact enhancer they function synergistically. Furthermore, additional adenine residues are required in the 3′-flanking region for high level basal expression in Hep G2 cells. Mutations in the distal half-site decrease basal activity, although some level of inducible activity is conserved as long as the proximal half-site remains intact. In the absence of the distal half-site, however, the proximal half-site alone does not function as an enhancer in Hep G2 cells. In contrast, mutations in the proximal half-site result in a complete loss of basal and inducible activity. The organization of the rat quinone reductase ARE is similar to the mouse glutathione S-transferase Ya subunit EpRE in that both half-sites are required for constitutive and inducible expression (20). Results presented here indicate that mutations in 3 residues found in the proximal half-site, 5′-GAGTggtgca-3′, completely eliminate basal and inducible activity. However, a number of other residues in both the distal and proximal half-sites are important for maximum basal and inducible activity. Synthetic oligonucleotides containing mutations of these three residues are no longer able to compete with the wild type ARE sequence for the binding protein. Additionally, mutating C+10 which decreases basal expression to background also eliminates competition with the wild type ARE. Taken together, these results indicate that while the proximal half-site is essential for induction by β-naphthoflavone, the intact enhancer is required for maximum basal and inducible activity.

These results are similar to results previously obtained for the ARE found in the glutathione S-transferase Ya subunit gene (11). The slight functional differences between the quinone reductase and glutathione S-transferase Ya subunit gene ARE mutants may arise from the differences in the surrounding sequences both proximal and distal to the core sequences. The rat quinone reductase ARE is a more potent enhancer than the glutathione S-transferase Ya ARE (Fig. 4B), although the fold induction is the same (4, 11). Regardless, both enhancers appear to bind to a similar nuclear factor(s) and both enhancers are responsive to the same stimuli, i.e. phenolic antioxidants, metabolizable planar aromatic compounds, and hydrogen peroxide. Similarly, the human quinone reductase ARE has also been shown to consist of two half-sites, both of which are required for functional activity (21).

Based on results from in vitro assays such as DNase I and methylation protection and interference footprinting assays as well as functional assays deploying mutations of the wild type ARE, several observations about the structure and function of the enhancer may be made. Although only the proximal half-site is required for binding, at least part of the distal half-site is required for maximal functional activity. Further, point mutations of residues in the same relative position in each half-site affect basal and inducible activities in different ways, suggesting that the two half-sites are not equivalent. Results presented here indicate that both basal level expression and β-NF responsiveness are greater when the two half-sites are arranged in a palindromic orientation. Interestingly, using two copies of a consensus TRE sequence, Okuda et al. (22) showed that the palindromic orientation was 3–7 times more active in F9 and HeLa cells than the tandem repeat orientation.

It is interesting to note that in F9 cells, which lack AP-1 activity and c-j un, an ARE-nucleoprotein complex with similar properties to the complex found in Hep G2 cells can be detected. Furthermore, in F9 cells the mouse glutathione S-transferase Ya EpRE (ARE) and the human quinone reductase ARE are responsive to t-BHQ or β-NF, respectively (20, 21, 23). Thus, the ARE is responsive to inducers in the absence of AP-1. Electrophoretic mobility shift assays conducted with nuclear extracts from treated Hep G2 cells revealed no complexes that would be consistent with j un/Fos binding to the ARE (7). Based on these results, we have concluded that induction is mediated by a nuclear protein(s) expressed constitutively which is distinct from j un/Fos (7).

In undifferentiated F9 cells, the only jun family member expressed is j un-D (24, 25). j un-D and j un-B have been shown, along with c-Fos, to be part of the human quinone reductase ARE-nucleoprotein complex in mouse Hep 1 cells (9, 26). All three j un proteins contain a conserved cysteine residue in the DNA-binding domain which has been implicated in the redox regulation of j un and Fos binding activity (25, 27). The human ARE contains a perfect TRE motif and has been shown to bind j un/Fos proteins in vitro and in nuclear extracts (7, 9, 26). We and others have shown that the rat ARE does not contain a high affinity site for j un/Fos proteins nor does it compete with the human ARE for j un/Fos proteins (7, 28). Therefore, the 2-nucleotide difference in the core sequence of the rat ARE is sufficient to abolish high affinity binding to j un/Fos.

Recently, a number of transcription factors have been identified which recognize sequences similar to the ARE (29–33). One of these, Maf, has been reported to bind to the AREs found in the rat and human quinone reductase, the rat glutathione S-transferase Ya, and the rat glutathione transrepressor P genes (34). However, it is not known if any of these factors are present in rat liver or the cell lines which show nucleoprotein binding activity to the ARE.

A number of structural and functional similarities exist between the rat quinone reductase ARE and the glutathione transrepressor P GPEI enhancers (7, 15). Both enhancers contain palindromic sequences which are required for full basal level gene expression. Electrophoretic mobility shift analyses indicate that along with the ARE from the rat glutathione S-transferase Ya gene, the GPEI also competes the nucleoprotein complex formed with the quinone reductase ARE. Constructs containing the GPEI enhancer inserted upstream from the minimal promoter CAT fusion gene are responsive to t-BHQ and TPA when they are transfected into Hep G2 cells. Recent evidence indicates that although j un and Fos proteins bind GPEI, another binding factor also exists which represents the main mechanism for transactivation of the glutathione transrepressor P gene (35). These results may provide a common link between these three rat genes which are elevated in persistent hepatocyte nodules (13, 36). A transgenic rat model containing the upstream regulatory sequence of the GST-P gene fused to the CAT reporter gene has recently been described (37). Liver foci and nodules produced by chemical carcinogens were found to express high CAT enzyme levels indicating that during hepatocarcinogenesis a trans-acting mechanism activates the expression of the GST-P gene. Preliminary results indicate that it is the GPEI motif which is required for tumor-specific expression of the GST-P gene (38).

In summary, we have characterized further the quinone reductase ARE by identifying nucleotides that are required for basal and inducible activity. These nucleotides are similar to
those previously identified for the rat glutathione S-transferase Ya subunit ARE. We have also demonstrated that nuclear extracts from human and rat hepatoma cell lines as well as from rat liver contain a similar nucleoprotein(s) which interact(s) with the rat ARE. The ARE-binding protein can be distinguished from AP-1 by its different mobility in gel shift assays, its presence in F-9 cells which lack AP-1 activity and by its unique nucleotide requirements for binding and functional activity. Finally, competition assays indicate that the enhancer from three rat genes (i.e. quinone reductase and the glutathione S-transferase P and Ya subunits) which are elevated in liver preneoplastic nodules recognize a similar nucleoprotein complex in rat liver, human Hep G2, and rat H4IEC3 nuclear extracts. The purification, identification, and cloning of this factor will lead to an understanding of how this factor regulates gene expression as a response to the oxidative stress produced by a large number of chemical inducers.

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REFERENCES
1. Ernster, L., Ljunggren, M., and Danielson, L. (1960) Biochem. Biophys. Res. Commun. 2, 88–92
2. Prochaska, H. J., and Talalay, P. (1988) Cancer Res. 48, 4776–4782
3. Prestera, T., Hatclaw, W. D., Zhang, Y., and Talalay, P. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2965–2969
4. Favreau, L. V., and Pickett, C. B. (1991) J. Biol. Chem. 266, 4556–4561
5. Denison, M., Fisher, J. M., and Whitlock, J. P., Jr. (1988) J. Biol. Chem. 263, 17221–17224
6. Paulson, K. E., Darnell, J. E., Jr., Rushmore, T. H., and Pickett, C. B. (1990) Mol. Cell. Biol. 10, 1841–1852
7. Favreau, L. V., and Pickett, C. B. (1993) J. Biol. Chem. 268, 19875–19881
8. Jaiswal, A. K. (1991) Biochemistry 30, 10647–10653
9. Li, Y., and Jaiswal, A. K. (1992) J. Biol. Chem. 267, 15097–15104
10. Rushmore, T. H., King, R. G., Paulson, K. E., and Pickett, C. B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3826–3830
11. Rushmore, T. H., Morton, M. R., and Pickett, C. B. (1991) J. Biol. Chem. 266, 11632–11639
12. Friling, R. S., Bensimon, A., Tichauer, Y., and Daniel, V. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6258–6262
13. Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I., and Sato, K. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3964–3968
14. Sakai, M., Okuda, A., and Muramatsu, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4956–4960
15. Okuda, A., Imagawa, M., Maeda, Y., Sakai, M., and Muramatsu, M. (1989) J. Biol. Chem. 264, 16919–16926
16. Dignat, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1476–1489
17. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051
18. Kryszke, M. H., Piette, J., and Yaniv, M. (1987) Nature 328, 254–256
19. Yang-Yen, H.-F., Chiu, R., and Karin, M. (1990) New Biol. 2, 351–361
20. Friling, R. S., Bergelson, S., and Daniel, V. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 668–672
21. Xie, T., Belinsky, M., Xu, Y., and Jaiswal, A. K. (1995) J. Biol. Chem. 270, 6894–6900
22. Okuda, A., Imagawa, M., Sakai, M., and Muramatsu, M. (1990) EMBO J. 9, 1131–1135
23. Yoshiba, K., Deng, T., Cavaglioni, M., and Karin, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4972–4976
24. Chiu, R., Angel, P., and Karin, M. (1989) Cell 59, 979–986
25. Ryder, K., Lanahan, A., Perez-Albuerne, E., and Nathans, D. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1500–1503
26. Li, Y., and Jaiswal, A. K. (1992) Biochem. Biophys. Res. Commun. 188, 992–996
27. Albone, C., Patel, L., Rauscher, F., III, and Curran, T. (1990) Science 249, 1157–1161
28. Yao, K.-S., Xanthoudakis, S., Curran, T., and O’Dwyer, P. J. (1994) Mol. Cell. Biol. 14, 5997–6003
29. Andrews, N. C., Erdjument-Bromage, H., Davidson, M. B., Tempst, P., and Orkin, S. H. (1993) Nature 362, 722–728
30. Mi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9926–9930
31. Kataoka, K., Noda, M., and Nishizawa, M. (1994) Mol. Cell. Biol. 14, 700–712
32. Kataoka, K., Fujiwara, K. T., Noda, M., and Nishizawa, M. (1994) Mol. Cell. Biol. 14, 7581–7591
33. Fujiwara, K. T., Kataoka, K., and Nishizawa, M. (1993) Oncogene 8, 2371–2380
34. Kerppola, T. K., and Curran, T. (1994) Oncogene 9, 3149–3158
35. Diccianni, M. B., Imagawa, M., and Muramatsu, M. (1992) Nucleic Acids Res. 20, 5153–5158
36. Pickett, C. B., Williams, J. B., Lu, A. Y. H., and Cameron, R. G. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 5091–5095
37. Marimura, S., Suzuka, T., Mochi, S.-I., Yuki, A., Nomura, K., Kitagawa, T., Nagatsu, I., Imagawa, M., and Muramatsu, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2065–2068
38. Suzuki, T., Okuda, A., Fukushima, A., Hiraibayashi, M., Moriya, T., Yuki, A., Nomura, K., Kitagawa, T., Imagawa, M., and Muramatsu, M. (1994) Proc. Am. Assoc. Cancer Res. 35, 611