A human oxidoreductase (H-37) that is overexpressed in ethacrynic acid-resistant HT29 colon cells (Ciaccio, P. J., Stuart, J. E., and Tew, K. D. (1993) Mol. Pharmacol. 43, 845-853) has been identified as a dihydrodiol dehydrogenase. Translated protein from a dihydrodiol dehydrogenase cDNA isolated from a library prepared from ethacrynic acid-resistant HT29 cell poly(A) RNA was recognized by anti-H-37 IgG and was identical in molecular weight with H-37. The isolated cDNA was identical in both nucleotide and amino acid sequences with the recently cloned liver dihydrodiol dehydrogenase (Stolz, A., Hammond, L., Lou, H., Takikawa, H., Ronk, M., and Shively, J. E. (1993) J. Biol. Chem. 268, 10448-10457). Using this cDNA as probe, we have examined its induction by Michael acceptors. The steady state dihydrodiol dehydrogenase mRNA level in the ethacrynic acid-resistant line was increased 30-fold relative to that of wild-type cells. Twenty-four hour treatment of wild-type cells with ethacrynic acid or dimethyl maleate increased mRNA 10-fold and 5-fold, respectively. These changes are accompanied by both increased protein expression and increased NADP-dependent 1-acenaphthenol oxidative activity in cell cytosol. In gel shift assays, compared with wild type controls, increased binding of NAD(P)H quinone oxidoreductase human antioxidant response element (hARE) DNA to redox labile protein complexes present in treated and resistant cell nuclear extract was observed. Ethacrynic acid induced CAT activity 2-fold in Hepal cells stably transfected with NAD(P)H quinone oxidoreductase hARE-tk-CAT chimeric gene construct.

Experimental Procedures

Materials—Ethacrynic acid, dimethyl maleate, all buffer components, β-NADP, and poly(dI-dC) were purchased from Sigma. 1-Acenaphthenol was purchased from Aldrich. Restriction enzymes for Taq polymerase, cell culture media, and other reagents were obtained from Life Technologies, Inc. Uni-Zap cDNA synthesis and cloning kits were purchased from Stratagene. Magna nylon transfer membranes for Northern blots were obtained from Micron Separations, Inc. (Westboro, MA).

1 The abbreviations used are: NQO1, NAD(P)H quinone oxidoreductase; hARE, human antioxidant response element(s); CRE, cyclic AMP response element; DDH, dihydrodiol dehydrogenase(s); DTT, dithiothreitol; TRE, TPA responsive elements; XRE, xenobiotic responsive elements; CAT, chloramphenical acetyltransferase; PAGE, polyacrylamide gel electrophoresis.
Cell Culture—Seeding of cells, culturing, and treatment of HT29 cells were performed as described previously (10). Ethacrynic acid-resistant cells were maintained continuously in 72 μM ethacrynic acid. They are 2- to 5-fold resistant to ethacrynic acid (also to chlorambucil, melphalan, adriamycin, mitomycin C, progestagen D₃, and phenylglyoxyl). After washing with phosphate-buffered saline, cells were scraped and pelleted in phosphate-buffered saline. The pellets were frozen immediately in liquid nitrogen and stored at −80°C until further use.

E3 Acenaphthenol Oxidation—Cytosol was isolated as described previously (10). Cytosol was resolved by two-dimensional isoelectric focusing gels as described by Schisselbauer et al. (17). Cytochrome c from pig heart was resolved on a 2-dimensional isoelectric focusing gel stained with silver (Fig. 1). The gel shown in panel A was developed for a longer period of time than gel in panel B.

Northern Blots—Total RNA from untreated or treated HT29 cells was extracted from cell pellets by the guanidinium isothiocyanate-phenol-chloroform extraction method (22). Twenty μg of total RNA from each sample was denatured, electrophoresed, and transferred onto a nylon membrane as described by Godwin et al. (22). A 5'-end 860-base pair fragment of DDH (c32) without noncoding 3'-end DNA generated by double-stranded oligonucleotide probe in a total volume of 25 μl of buffer containing 25 μM Hepes (K⁺), pH 7.8, 12.5 mM MgCl₂, 1 mM Dm, 250 μg β-NADP, and 250 μg of cytosol. NADPH production was linear with time and protein concentration. Boiled protein was used as a blank.

Library Preparation and Screening—Poly(A) RNA from ethacrynic acid-resistant HT29 colon cells was isolated by affinity chromatography using oligo(dT)-cellulose supplied in a kit from Collaborative Biomedical Products (Bedford, MA). An amplified Uni-Zap cDNA library (cloned directionally with EcoRI and XhoI sites) and subsequent in vivo excision to form Bluescript phagemid were prepared according to the manufacturer (Strategene) instructions. Three rounds of screening to isolate plaque-pure bacteriophage clones were performed as described by Graves et al. (19) using as probe a 505-base pair fragment of the aldo-keto reductase bovine lung prostaglandin F synthase (20). Cloned male bovine lung DNA was generated by reverse transcription of mRNA purchased from Clontech Laboratories. This step was followed by the polymerase chain reaction. The 100-μl polymerase chain reaction mixture contained 20 μM Tris-HCl, pH 8.4, 50 mM KCl, 100 pmol of each primer, 1 μg of bovine lung cDNA, 200 μM dNTP mixture, and 1.5 mM MgCl₂. The reaction was extended for 40 cycles for 2 min at 94°C, 1.5 min at 65°C, and 2 min at 72°C. Primers containing EcoRI (forward) and XhoI (reverse) restriction sites for subcloning into Bluescript were prepared at the Fox Chase Cancer Center DNA synthesis laboratory. 5'-GGG GAA TAC AAT GGA TCC AAG CAA AAG TCA GAC-3' (forward); GGC CTC GAG GCA CCC AAT GAT CTT GGT CAG-3' (reverse). Probing with the bovine lung prostaglandin F synthase fragment, a positive clone designated c32, was isolated and sequenced from both directions using double-stranded DNA in Bluescript phagemid by the Sanger's dideoxy chain termination method with the Sequenase kit (US Biochemical Corp.). The DNA data bank (GenBank/EMBL) was searched for homology with other sequences by the FASTA method. The deduced amino acid and nucleotide sequences of the independently isolated c32 clone (accession number U05684) were found during this search for homology with other sequences by the FASTA method. The deduced amino acid and nucleotide sequences of the independently isolated c32 clone (accession number U05684) were found during this search for homology with the independently isolated c32 clone (accession number U05684) were found during this search for homology with other sequences by the FASTA method.

RESULTS

H-37 Identity—Overexpression of H-37 protein in ethacrynic acid-resistant cells is illustrated by a two-dimensional isoelectric focusing gel stained with silver (Fig. 1). The gel shown in panel A (wild-type cytosol) was developed for a greater length of time to demonstrate that H-37 is present constitutively. Panel B shows that this protein is overexpressed markedly and has an approximate M₉ of 37,500. The approximate pl value determined from isoelectric focusing was 7.6. This value is equivalent to that of a human liver 36-kDa DDH characterized by Harra et al. (26), called DD-2. Other human liver DDHs exhibit pl values of 5.2, 5.4, 5.9, 9.1, and 9.7 (26). Sequence analysis of an H-37 peptide fragment revealed that it is identical with the bile acid binder (DDH) in 21/21 amino acids (10). To confirm the identity between H-37 and the bile acid binder, a DDH cDNA (c32) that is identical with the bile acid binder in both nucleotide and protein sequence was isolated from a library prepared from ethacrynic acid-resistant HT29 cell poly(A) RNA. It was then transcribed and translated. The translation product (37.5 kDa) was immunoprecipitated with antibodies raised against H-37 (Fig. 2, lane 4). This protein is identical in molecular weight with H-37 overexpressed in cytosol from ethacrynic acid-resistant cells was isolated by affinity chromatography using oligo(dT)-cellulose supplied in a kit from Collaborative Biomedical Products (Bedford, MA). An amplified Uni-Zap cDNA library (cloned directionally with EcoRI and XhoI sites) and subsequent in vivo excision to form Bluescript phagemid were prepared according to the manufacturer (Strategene) instructions. Three rounds of screening to isolate plaque-pure bacteriophage clones were performed as described by Graves et al. (19) using as probe a 505-base pair fragment of the aldo-keto reductase bovine lung prostaglandin F synthase (20). Cloned male bovine lung DNA was generated by reverse transcription of mRNA purchased from Clontech Laboratories. This step was followed by the polymerase chain reaction. The 100-μl polymerase chain reaction mixture contained 20 μM Tris-HCl, pH 8.4, 50 mM KCl, 100 pmol of each primer, 1 μg of bovine lung cDNA, 200 μM dNTP mixture, and 1.5 mM MgCl₂. The reaction was extended for 40 cycles for 2 min at 94°C, 1.5 min at 65°C, and 2 min at 72°C. Primers containing EcoRI (forward) and XhoI (reverse) restriction sites for subcloning into Bluescript were prepared at the Fox Chase Cancer Center DNA synthesis laboratory. 5'-GGG GAA TAC AAT GGA TCC AAG CAA AAG TCA GAC-3' (forward); GGC CTC GAG GCA CCC AAT GAT CTT GGT CAG-3' (reverse). Probing with the bovine lung prostaglandin F synthase fragment, a positive clone designated c32, was isolated and sequenced from both directions using double-stranded DNA in Bluescript phagemid by the Sanger's dideoxy chain termination method with the Sequenase kit (U.S. Biochemical Corp.). The DNA data bank (GenBank/EMBL) was searched for homology with other sequences by the FASTA method. The deduced amino acid and nucleotide sequences of the independently isolated c32 clone (accession number U05684) were found during this investigation to be identical with the recently published sequence of human bile acid binder protein or DDH (11). The nucleotide sequence of this form is 70% identical with rat 3α-hydroxysteroid dehydrogenase (a DDH) (21), and 80% identical with bovine lung prostaglandin F synthase (20).

Northern Blots—Total RNA from untreated or treated HT29 cells was extracted from cell pellets by the guanidinium isothiocyanate-phenol-chloroform extraction method (22). Twenty μg of total RNA from each sample was denatured, electrophoresed, and transferred onto a nylon membrane as described by Godwin et al. (22). A 5'-end 860-base pair fragment of DDH (c32) without noncoding 3'-end DNA generated by restriction digestion with EcoRI was employed as a probe. Probe DNA was labeled with [³²P]dCTP by the random primer method. Membranes were washed under stringent conditions and exposed to x-ray film (Kodak) for detection of mRNA signal. Messenger RNA signal intensities were measured by densitometry using an LKB Ultrascan XL densitometer.
Induction of Dihydriodiol Dehydrogenase by Xenobiotics

FIG. 2. SDS-PAGE and immunoblotting illustrating identity between H-37 and DDH protein translated from c32. c32 DDH was transcribed and translated using kits purchased from Ambion and Stratagene, respectively. SDS-PAGE and blotting were performed as described previously for protein in lanes 1 and 2 (10). Proteins in lane 1 were detected with antibodies raised against H-37. Protein in lanes 3 and 4 were resolved on a 10% SDS-polyacrylamide gel, air-dried, and exposed to x-ray film. Lane 1, 100 μg of cytosol from ethacrinic acid-resistant HT29 cells (H-37 is lower band); lane 2, c32-translated product using 0.1 μm NaN50 M methionine supplement (1200 Ci/mmol) (DuPont NEN); lane 3, c32 translation product and incubation with preimmune sera; lane 4, c32-translated product immunoprecipitated by incubation with anti-H-37 IgG. For immunoprecipitation, lysate was denatured by boiling for 1 min in 100 μl of NET buffer without detergent and azide (NET: 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). Lanes 2 through 4 were run on a 10% SDS-polyacrylamide gel. Preimmune sera did not precipitate c32-translated product (lane 3).

Acenaphthenol Oxidation—In our previous report (10), it was thought that H-37 might be homologous to an NADPH-dependent prostaglandin F synthase which primarily exhibits NADPH-dependent prostaglandin F synthase activity (27). DDHs such as polycyclic aromatic hydrocarbon trans-dihydrodiols, 9,10-phentarathrenequinone (reduce), and secondary alcohols such as acenaphthenol were recognized by anti-H-37 IgG when using 0.1 mM ~[35S]methionine (DuPont NEN). Preimmune sera did not precipitate two bands in lane 4, a higher molecular weight band (lane 1) and a lower molecular weight protein that was recognized by anti-H-37 IgG when using 0.1 mM ~[35S]methionine. SDS-PAGE and blotting were performed as described previously for protein in lanes 1 and 2 (10). Proteins in lane 1 were detected with antibodies raised against H-37. Protein in lanes 3 and 4 were resolved on a 10% SDS-polyacrylamide gel, air-dried, and exposed to x-ray film. Lane 1, 100 μg of cytosol from ethacrinic acid-resistant HT29 cells (H-37 is lower band); lane 2, c32-translated product using 0.1 μm ~[35S]methionine (DuPont NEN); lane 3, c32 translation product and incubation with preimmune sera; lane 4, c32-translated product immunoprecipitated by incubation with anti-H-37 IgG. For immunoprecipitation, lysate was denatured by boiling for 1 min in 100 μl of NET buffer without detergent and azide (NET: 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide). Purified anti-H-37 IgG was added, and samples were incubated for 3 h at room temperature, followed by incubation for 1 h with 10 μg of protein A-Sepharose (Sigma). Samples were washed three times with NET buffer. Protein was solubilized by adding SDS-PAGE gel buffer and boiled for 4 min. Protein A-Sepharose was precipitated by centrifugation, and the supernatant was run on a 10% SDS-polyacrylamide gel. Preimmune sera did not precipitate c32-translated product (lane 3).

FIG. 3. Northern blot analysis of human DDH in HT29 cells. Analysis was performed using DDH (c32 5′-end-EcoRI restriction fragment) as probe with 20 μg/lane total RNA. Lane 1, wild-type, 0 h; lanes 2 and 3, wild-type treated with 50 μM ethacrinic acid for 6 h and 24 h; lane 4, wild-type treated with 50 μM dimethyl maleate for 24 h; lane 5, RNA from ethacrinic acid-resistant cells. Lower panel is β-actin as control.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td><strong>NADP-dependent oxidation of 1-acenaphthenol by cytosol from HT29 cells and association with H-37 levels and phenylglyoxal reductase activity</strong></td>
</tr>
<tr>
<td><strong>Cell type</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>50 μM EA</td>
</tr>
<tr>
<td>50 μM DMM</td>
</tr>
<tr>
<td>80 μM tBHQ</td>
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<td>R</td>
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* Except for resistant cells which were maintained with ethacrinic acid continuously, cells were treated with drugs 48 h before cytosol isolation.

- Fold increases are in parentheses.
- Data taken from Ref. 10. NADPH-dependent, dicumarol-insensitive phenylglyoxal reductase. H-37 protein levels were determined by densitometry on immunoblot x-rays.
- WT, wild-type; R, resistant; EA, ethacrinic acid; DMM, dimethyl maleate; tBHQ, tert-butylhydroquinone.
- Statistically significant difference between untreated wild-type control and sample listed by Student's t-test, p < 0.05.
- Treatment concentrations used were 80 μM DMM.
- In vitro pretreatment of cytosol sample with 100 μM indomethacin for 2 min at room temperature caused 52% inhibition of this activity.

Induction of DDH in HT29 Cells—To determine possible mechanisms of induction by Michael acceptor treatment, we probed DDH mRNA in Northern blots using a 5′-end EcoRI restriction fragment of c32 DDH. DDH mRNA (1.3 kilobases) was overexpressed 30-fold in ethacrinic acid-resistant cells and 10-fold and 5-fold in cells treated with 50 μM ethacrinic acid or 50 μM dimethyl maleate for 24 h (Fig. 3). The increase in mRNA by ethacrinic acid was time-dependent and could be seen as early as 6 h (2-fold). The time-dependent induction of mRNA paralleled the 2-fold induction of H-37 protein by 6 h and the 11-fold induction of H-37 protein at 24 h (10). Also, as was the case with protein, the constitutive mRNA levels in wild-type cells were very low. An additional unknown larger mRNA was detected and was also induced by ethacrinic acid and dimethyl maleate. HT29 Nuclear Protein Binding to the Human NQO1 ARE and CAT Assay with NQO1 hARE-th-CAT Construct—Michael acceptors induce expression of certain Phase II enzymes via activation of an ARE (7-9). Since we have shown that DDH is induced by the same agents (10), we sought to determine whether potential binding to the NQO1 hARE (9) would be modified in treated HT29 colon cells or HT29 colon cells resistant to ethacrinic acid. As shown in a gel shift assay (Fig. 4), specific binding of nuclear protein to the radiolabeled double-stranded oligonucleotide NQO1 hARE sequence was exhibited by all preparations. Three bands of nuclear protein complexes (a, b, and c) were observed. Band b appeared to consist of a doublet since much but not all of this band is competed away by excess unlabeled NQO1 hARE and since it appeared as such in lanes 4 and 6 (resistant cell extract). Overall binding in bands a and b was lower in extracts from untreated wild-type cells. Binding in band c was approximately equal among treatments. Specificity of binding was illustrated by the ability of 100-fold molar excess unlabeled hARE to compete with binding in complexes a (completely), b (partially), and c (most) where 100-fold molar excess unlabeled CRE did not. Since it is known that...
**Induction of Dihydrodiol Dehydrogenase by Xenobiotics**

**Fig. 4.** Gel shift assay of hARE binding to nuclear protein from wild-type and ethacrylic acid-resistant HT29 cells. Isolation of nuclear protein and gel shift assay was performed as described under "Experimental Procedures." Lane 1, extract from untreated wild-type cells; lanes 2 and 3, extract from wild-type cells treated with 50 μM dimethyl maleate and 50 μM ethacrylic acid for 48 h; lane 4, ethacrylic acid-resistant cells; lane 5, resistant cell extract incubated with 100 μM excess unlabeled hARE as positive control competitor; lane 6, resistant cell extract incubated with 100 μM excess cold CRE as negative control competitor; lanes 7–9, prior to incubation with [35S]hARE: lane 7, resistant cell extract treated with 2 μM diamide at 37 °C for 1 h; lane 8, resistant cell extract treated with 5 μM excess DTT for 1 h at 37 °C; lane 9, wild-type extract treated with 5 μM excess DTT for 1 h at 37 °C; lane 10, 5 μg of bovine serum albumin only. Protein complexes in a and c are specifically competed by unlabeled hARE. Protein complex b is a doublet, competed in part by unlabeled hARE. Protein(s) in complex c appear to represent oxidized protein.

**TABLE II**

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>αC acetylation of chloramphenicolb</th>
<th>pmol/min/mg</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>3.17 (1.0)c</td>
<td></td>
</tr>
<tr>
<td>1 μM EA</td>
<td>3.40 (1.1)</td>
<td></td>
</tr>
<tr>
<td>10 μM EA</td>
<td>3.56 (1.1)</td>
<td></td>
</tr>
<tr>
<td>50 μM EA</td>
<td>5.29 (1.7)</td>
<td></td>
</tr>
<tr>
<td>100 μM EA</td>
<td>6.24 (2.0)</td>
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a EA, ethacrylic acid.
b Constructs and CAT assays were performed as described previously (23) using 420 μg reaction.
c Fold change.

Because of the marked differences in constitutive expression observed in vivo (28) (and in cell lines), induction of DDH by xenobiotics may have important toxicological implications for organs exposed to polycyclic aromatic hydrocarbon carcinogens. Penning (14) has argued that DDH may not always be protective in polycyclic aromatic hydrocarbon metabolism. Indeed, in some cases, it may represent a pathway of activation to carcinogenic metabolites. Products of rat liver DDH reactions can auto-oxidize to quinones which may react with DNA, RNA, and protein or with glutathione to water soluble conjugates. Hydroquinone metabolites may also enter redox cycles to generate superoxide anion radicals (14, 32, 33). The precise role that human DDH may play in these pathways has yet to be determined.

Talalay and co-workers (5, 35, 36) have made the compelling case that most inducers of Phase II protective enzymes contain or acquire distinctive electrophilic centers by metabolism. Many such compounds are classical Michael reaction acceptors that are susceptible to attack by nucleophiles and are electrophilic by conjugation with electron-withdrawing groups (5). Their structures include CH₂=CH–Z, Z=CH=CH–Z (also quinones), and R=C=C–Z, where Z is an electron-withdrawing group (5). Ethacrylic acid, dimethyl maleate, and tert-butyldihydroquinone contain or are converted to compounds that contain α, β-unsaturated carbonyl groups. The inducing signal may be due either to the parent drug or metabolite, depending on whether it requires reversible oxidation to the cognate quinone.

The DDH described here (H-37), γ-glutamyl cysteine synthase, and glutathione S-transferase-α (38) are induced by ethacrylic acid and thus may be coordinately regulated in HT29 cells. Several important regulatory DNA sequences have been identified in the 5'-flanking regions of glutathione S-transferase-α (TRE) (39, 40), glutathione S-transferase-α (7, 8, 41, 42) (XRE and ARE), and the NAD(P)H:quinone oxidoreductase genes (XRE and ARE) (8, 9). The ARE is required for transcriptional activation by phenolic antioxidants, redox-labile quinones, hydrogen peroxide, and, as suggested here,

\[ \text{Induction occurs in the human colon HT29 cell line by de novo synthesis of protein from mRNA. In our previous work, when tested in cultured cells, the effect of ethacrylic acid on levels of DDH appeared to be cell type-specific in that induction was marked in two types of human colon carcinoma cells, did not occur in human prostate cells, and was not apparent in rat breast carcinoma Walker cells (10). Constitutive levels in these cells appeared low. We have since probed cell lines for their steady state level of DDH mRNA. In contrast to the HT29 colon cell line subclone from our laboratory (designated HT-418), message was expressed constitutively to widely varying degrees in a number of lines derived from human tumors, including colon (data not shown).} 

\[ \text{Because of the marked differences in constitutive expression observed in vivo (28) (and in cell lines), induction of DDH by xenobiotics may have important toxicological implications for organs exposed to polycyclic aromatic hydrocarbon carcinogens. Penning (14) has argued that DDH may not always be protective in polycyclic aromatic hydrocarbon metabolism. Indeed, in some cases, it may represent a pathway of activation to carcinogenic metabolites. Products of rat liver DDH reactions can auto-oxidize to quinones which may react with DNA, RNA, and protein or with glutathione to water soluble conjugates. Hydroquinone metabolites may also enter redox cycles to generate superoxide anion radicals (14, 32, 33). The precise role that human DDH may play in these pathways has yet to be determined.} 

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ethacrynic acid. A binding site for AP-1 is contained within the NQO1 ARE, and three proteins in an hARE binding complex have been identified (43, 44). These are Jun-D, c-Fos, and Jun-B. We have identified Jun-related proteins (91 kDa) in HT29 nuclear extract by Western blotting and a protein of the same molecular weight by Southwestern blotting using the NQO1 hARE (data not shown), suggesting that Jun-related proteins may be present in the complexes detected by the gel shift assay with HT29 cell extract. It is understood that not all proteins involved in hARE binding and regulation have been identified. Moreover, there may be several hARE-like sequences that differ in structure and function throughout the human genome (44).

Further characterization of the regulatory proteins that bind to hAREs will help identify additional molecules that are involved in receiving redox signals from Michael acceptors. We have found that relative to control samples nuclear extract from treated or ethacrynic acid HT29 cells exhibited increased binding to the NQO1 hARE. The trans-activating factor(s) involved in binding were not induced by new protein synthesis but rather by altered redox state, as indicated by the ability of DTT to increase the binding of the NQO1 hARE to wild-type redox active proteins. The trans-activating factor(s) involved in binding were not induced by new protein synthesis but rather by altered redox state, as indicated by the ability of DTT to increase the binding of the NQO1 hARE to wild-type redox active proteins.

Evidence for the involvement of an element similar to hARE may be related to induction of glutathione S-transferase-rr, involving redox state, as indicated by the ability of DTT to increase the binding of the NQO1 hARE to wild-type redox active proteins. The trans-activating factor(s) involved in binding were not induced by new protein synthesis but rather by altered redox state, as indicated by the ability of DTT to increase the binding of the NQO1 hARE to wild-type redox active proteins.

Acknowledgments—We thank Jeanette E. Stuart and Tao Xie for technical assistance, Donna Bunch for typing the manuscript, and Dr. Gary Kruh of FCCC for his helpful advice on utilization of the polymerase chain reaction.

REFERENCES


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