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Modulation of Detoxification Gene Expression in Human Colon HT29 Cells by Glutathione-*S*-Transferase Inhibitors

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SUMMARY

We investigated the effects of glutathione-*S*-transferase (GST) inhibitor treatment on human colon HT29 cell mRNA levels of dihydrodiol dehydrogenase (DDH), glyoxalase I, and γ-glutamylcysteine synthetase. Time- and concentration-dependent increases in both DDH and γ-glutamylcysteine synthetase mRNAs resulted from treatment with ethacrynic acid, ethacrynic acid/glutathione conjugate, and T.199 (γ-glutamyl-*S*-(benzyl)-cysteinyl-*R*(−)-phenyl glycine diethyl ester), a selective GSTπ inhibitor. In contrast, glutathione analogue GSTα- and GSTμ-selective inhibitors did not induce expression of these genes. Treatment with ethacrynic acid or T.199 had no effect on the mRNA levels of the glutathione-dependent glyoxalase I gene. Pretreatment of cells with buthionine-DL-sulfoximine, a γ-glutamylcysteine synthetase inhibitor and glutathione depleter, coupled with ethacrynic acid, ethacrynic acid/glutathione conjugate, or T.199 resulted in greater levels of γ-glutamylcysteine synthetase and DDH induction compared with single treatments. Treatment with buthionine-DL-sulfoximine alone resulted in modest increases in both γ-glutamylcysteine synthetase and DDH expression. Analyses of DDH induction by both differential Northern hybridization with specific oligonucleotides as probes and reverse transcriptase-polymerase chain reaction amplification of products, followed by diagnostic restriction digestion with endonucleases, showed that ethacrynic acid induced multiple DDH transcripts in HT29 cells and human HepG2 and SKHep1 hepatoma cells. Possible induction mechanisms include the alteration of sulfhydryl status by the electrophilic properties of EA or by elevations of endogenously generated oxidative stress via transient removal of GSTπ from the cytosolic GST pool.

GST (EC 2.5.1.18) are phase II detoxification enzymes that belong to a multigene family consisting of cytosolic α, π, μ, θ, and microsomal classes. They exhibit overlapping and broad specificities for the ability to conjugate substrates to glutathione (see Ref. 1 for a review). DDH (EC1.3.1.20) and NAD(P)H (quinone acceptor) oxidoreductases (also called DT diaphorase; EC 1.6.99.2) are phase II enzymes that belong to gene families involved in the oxidation and reduction of numerous xenobiotic substrates, including steroids, polycyclic aromatic hydrocarbons, and quinones (see Refs. 2 and 3 for reviews). GST inhibitors are of clinical interest because they have potential for use as modulators of cellular resistance to anticancer drugs. The drug EA inhibits the GST-mediated conjugation of chlorambucil with glutathione (4) and sensitizes human HT29 tumor xenografts in Scid mice to melphalan (5). However, there are limitations to its use: (a) EA both induces and inhibits GSTπ, inhibits NAD(P)H (quinone acceptor) oxidoreductase enzymes, and induces DDH (6–10); (b) neither EA nor EA-SG is a selective inhibitor of either GST α, π, or μ class enzymes (10); (c) EA depletes glutathione and reacts with other cellular sulfhydryls by Michael addition in both normal and tumor tissues (5, 11); and (d) EA causes marked diuresis and concomitant electrolyte imbalance *in vivo* in humans (11).

The Michael addition chemistry of EA may also serve to induce the expression of other proteins such as γ-glutamylcysteine synthetase, which could be critical in the early cellular response to stress. γ-Glutamylcysteine synthetase is the rate-limiting enzyme in glutathione synthesis, and its regulation may be contingent on changes in cellular glutathione levels (6).

Despite these limitations, EA has shown promise as a resistance modulator in combination with thiotepa in a phase

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ABBREVIATIONS: DDH, dihydrodiol dehydrogenase(s); EA-SG, ethacrynic acid/glutathione conjugate; GLXSE, glyoxalase I; GST, glutathione-*S*-transferase(s); hARE, human antioxidant response element; T.183, γ-glutamyl-*S*(octyl)cysteinyl-glycine diethyl ester; T.199, γ-glutamyl-*S*(benzyl)-cysteinyl-*R*(−)-phenyl glycine diethyl ester; T.200, γ-glutamyl-*S*(methylbenzyl)cysteinyl-glycine diethyl ester; T.206, γ-glutamyl-*S*(napthyl)cysteinyl-glycine diethyl ester; T.286, γ-glutamyl-a-amino-B((2-ethyl-*N*, *N,N',N'*-tetrakis(2-chloroethyl)phosphorodiamidate)sulfonyl)-propionyl-(*R*)-(−)phenylglycine; T.317, γ-glutamyl-*S*(benzyl)cysteinyl-*S*(+)-phenylalanine diethyl ester; RT-PCR, reverse transcriptase-polymerase chain reaction; ARE, antioxidant response regulatory element; PBS, phosphate-buffered saline; bp, base pair(s).

I clinical trial (11). More recently, certain glutathione analogue-based GST inhibitors have been found to potentiate chlorambucil cytotoxicity in human HT29 cells (12). Unlike EA, these agents were designed to be selective inhibitors of specific GST isozyme classes (13).

Such cell-permeable glutathione analogues could alter cellular sulfhydryl status. Therefore, we sought to determine whether, like EA, they influenced detoxification gene expression. We report that EA, EA-SG, and the glutathione analogue T.199 increased cellular levels of DDH and γ-glutamylcysteine synthetase mRNAs but not of mRNAs of GLXSE (EC 4.4.1.5) and other housekeeping genes. At least three distinct DDH genes in three human cell lines were induced by EA at concentrations that are known to inhibit GST activity and reduce glutathione levels (14). These evaluations may be useful in understanding how tumor cells regulate a variety of phase II detoxification enzymes and in devising appropriate scheduling for the clinical modulation of alkylating agent drug resistance.

Experimental Procedures

Cell culture and treatment. Cell culture media and other reagents were obtained from Life Technologies (Gaithersburg, MD). Seeding of cells, culturing, and treatment of HT29 subclone HT41 cells (designated wild-type HT29 cells) and of the EA-resistant HT29 subclone HT68 cells (designated EA-resistant HT29 cells) were performed as described previously (6, 8). Human hepatoma HepG2 and SKHep1 cells were cultured in modified essential medium containing 10% fetal bovine serum, 50 units/ml of penicillin G activity, and 50 μg/ml streptomycin activity, supplemented with 2 mM glutamine. Seeding of HepG2 and SKHep1 cells was performed as described for the HT29 cells (8). (±)-Buthionine-DL-sulfoximine was purchased from Aldrich (Milwaukee, WI). Except for (±)-buthionine-DL-sulfoximine, the structures of the drugs used in the present study are shown in Fig. 1. EA was purchased from Sigma Chemical Co. (St. Louis, MO). EA-SG was synthesized according to the methods of Ploemen *et al.* (10). The GSTπ-activatable prodrug T.286 was synthesized as described (15). The glutathione analogues were prepared according to the general methods of Lyttle *et al.* (16). They are esterified to permit cellular uptake. Once inside the cell, they are converted by esterases to the corresponding active-inhibitory moieties. The vehicle used for T.199 was water, except in comparisons of treatments with other glutathione analogues, where DMSO was used (0.1% final vehicle concentration). Concentrations chosen for glutathione analogue treatments were approximately equitoxic with T.199. EA-resistant HT29 cells were maintained continuously in 72 μM EA. They are 2–3-fold resistant to EA (they are also resistant to chlorambucil, melphalan, adriamycin, mitomycin C, prostaglandin D_2, and phenylglyoxal) (6, 8). The EA-resistant cells markedly overexpress DDH (also called H-37 in Ref. 8) compared with wild-type controls (9). Dimethyl maleate, another Michael acceptor, was used as a positive control treatment and is shown in Fig. 2 (A, *lane 6*). After being washed with PBS, the cells were scraped and pelleted in PBS. The pellets were frozen immediately in liquid nitrogen and stored at −80° until further use.

Transcription rate. Transcription rates were measured according to the method of Johnson *et al.* (17) and are reported in Table 1 as the rate in EA-resistant cells relative to wild-type HT29 controls. Cells that were ~60% confluent were placed in prewarmed medium containing 100 μM 4-thiouridine and 5 μCi/ml [^{3}H]uridine. After 1-hr incubation in the presence (EA-resistant cells) or absence (HT29 wild-type cells) of 72 μM EA, the medium was aspirated, and the cells were washed with cold PBS. Total RNA was extracted with the use of guanidine isothiocyanate, and newly synthesized RNA was separated by phenyl-mercury affinity chromatography. After determination of radioactivity, purified newly synthesized RNA was fractionated on a 1% agarose-formaldehyde gel and transferred to a Magna-NT nylon membrane. The membranes were probed with ^{32}P-

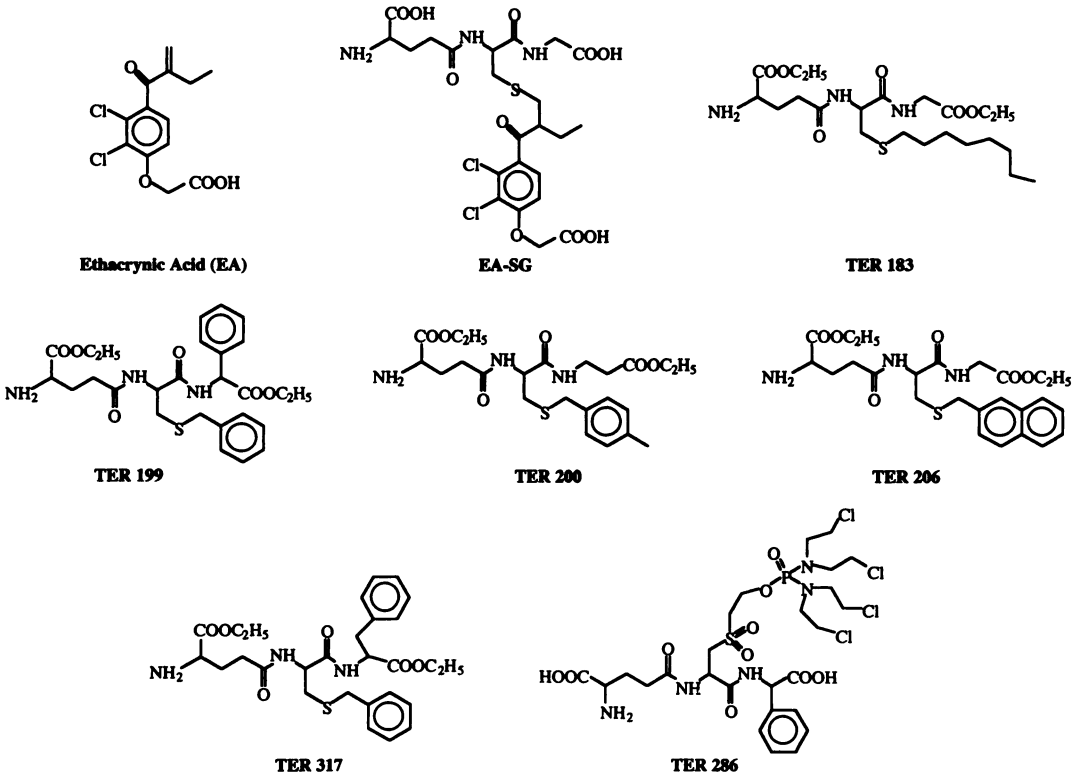

Fig. 1. Chemical structures of EA, EA-SG, a GSTπ activatable prodrug [T.286 (TER 286)], and glutathione analogues T.183 (TER 183), T.199 (TER 199), T.200 (TER 200), T.206 (TER 206), and T.317 (TER 317).

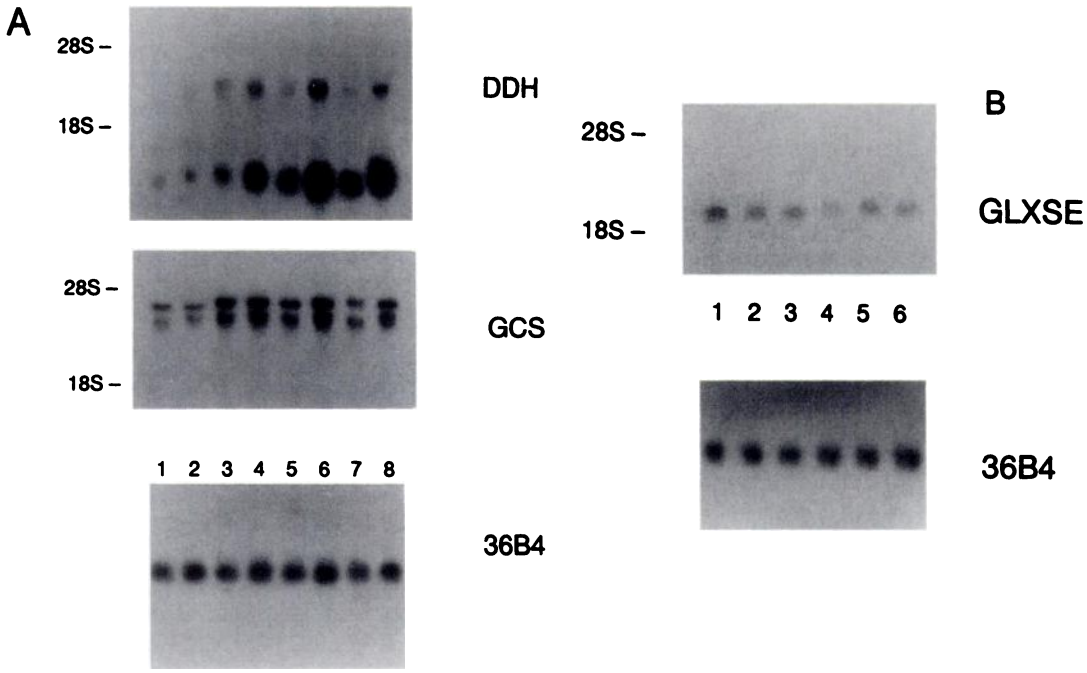

Fig. 2. Effects of EA, dimethyl maleate and T.199 on DDH, γ-glutamylcysteine synthetase (GCS) (A), and GLXSE (B) mRNA levels in HT29 colon cells. There is 20 μg total RNA/lane. Northern blots were performed as described in Experimental Procedures. A, *Lane 1*, wild-type untreated; *lanes 2–5*, wild-type treated with 50 μM T.199 for 1, 6, 10, and 24 hr; *lane 6*, wild-type treated with 50 μM dimethyl maleate for 24 hr; *lane 7*, wild-type treated with 50 μM EA for 24 hr; and *lane 8*, EA-resistant cells maintained in 72 μM EA continuously. B, GLXSE: *lane 1*, wild-type untreated; *lanes 2 and 3*, 6 hr 25 μM and 100 μM T.199; *lanes 4–6*, wild-type treated for 24 hr with 25 μM and 100 μM T.199; and *lane 6*, wild-type treated for 24 hr with 50 μM EA. The 36B4 estradiol-independent human acidic ribosomal phosphoprotein cDNA was used as a probe for control of total RNA loading (23).

TABLE 1

Effect of EA on relative transcriptional rates of GSTπ, DDH, and γ-GCS in human EA-resistant versus wild-type HT29 colon cells (fold increase)

Cell line	GSTπ	DDH	γ-GCS	G3PDH
HT29, wild-type	1	1	1	1
HT29, EA resistant	2.7	21	2.0	0.8

Wild-type cells were untreated. EA-resistant cells were maintained in 72 μM EA continuously. Transcription rates were determined as described in Experimental Procedures.

labeled glyceraldehyde-3-phosphate dehydrogenase, DDH, γ-glutamylcysteine synthetase, or GSTπ cDNAs as described below. Transcription rates were calculated according to Johnson *et al.* (17). This method is suitable for the transcripts with half-lives longer than the labeling period (1 hr). This condition is applicable to these four genes.

Northern blots. Reagents for RNA extraction were purchased from Sigma, and reagents for restriction digestion of plasmids to yield probe fragments were purchased from Life Technologies Inc. Total RNA from untreated, treated, or EA-resistant HT29 cells was extracted from cell pellets by the guanidinium isothiocyanate-phenol-chloroform extraction method (18). Twenty micrograms of total RNA from each sample were denatured, electrophoresed, and transferred onto a nylon membrane (Magna nylon, Micron Separations, Westboro, MA) according to Godwin *et al.* (18). A 5′-end 860-bp fragment of DDH (clone 32) (9) without noncoding 3′-end DNA generated by restriction digestion with *Eco*RI was used as a probe for DDH mRNA. The γ-glutamylcysteine synthetase probe (764-bp *Pst*I fragment) was kindly provided by Dr. T. Mulcahy, (University of Wisconsin Medical School, Madison, WI) (19). The GLXSE probe was a full-length human cDNA from our laboratory (20). The human GSTπ probe (21) was kindly provided by Dr. I. Listowsky (Albert Eistein College of Medicine, New York, NY) (22). The 36B4 estradiol-independent ribosomal phosphoprotein gene (23) was used as a probe to control for RNA loading. DNA probes were labeled with [^{32}P]dCTP by the random primer method with a kit from Boehringer Mannheim (Indianapolis, IN). Membranes were hybridized with probe overnight and washed under stringent conditions as described (18) and exposed to X-ray film (Kodak) for detection of mRNA signal. Messenger RNA signal intensities were measured by densitometry with an LKB Ultrascan XL densitomer.

Differential Northern hybridization. Oligonucleotide probes specific for different DDH gene groups (see Fig. 7) were ^{32}P end-labeled with T_4 polynucleotide kinase and [γ-^{32}P]ATP. Membranes were prehybridized for 4 hr in 6× SSPE (1 × SSPE = 8.8 g/liter NaCl, 1.4 g/liter $NaH_2PO_4 \cdot H_2O$, and 0.37 g/liter EDTA, pH 7.4), 10× Denhardt's solution, 0.1% sodium dodecyl sulfate, and 50 μg/ml denatured salmon sperm DNA. They were then hybridized overnight in 6× SSPE and 1% sodium dodecyl sulfate, containing 2.0 × 10^6 cpm/ml probe at the specific T_H for each probe ($T_H = T_M - 5° = 4(G + C) + 2(A + T) - 5°$). Membranes were then washed at room temperature three times for 15 min each in 6× SSPE and 0.1% sodium dodecyl sulfate, followed by a single wash for 3 min in the same solution at the specific T_H. A final 15-min wash at room temperature contained 6× SSPE. The sequences of the four oligonucleotides used to probe the three groups of DDH genes are shown in Table 2.

RT-PCR. One-and-one-half micrograms total RNA were ethanol salt precipitated, air dried, and resuspended in RNAse-free water. Fifty nanograms of random hexamer primers were added to the RNA, and the samples were heated to 70° for 10 min and placed on ice. DNA was then generated by reverse transcription in 20 μl total volume at 42° for 50 min with the addition of Superscript RT$^-$ and other reaction components provided in a kit from Life Technologies. The samples were heated for 10 min at 70° to inactivate the reverse transcriptase. Two-and-one-half units of RNAse H were added to the samples and incubated at 37° for 20 min. Aliquots (7.5 μl) were then prepared for the PCR amplification of histone controls or 707-bp DDH fragments. Primer sequences were as follows: DDH forward, 5′ CCA TCC GAA GCA AGA TTG C 3′; DDH reverse, 5′ TAG GCC ATC

TABLE 2

Oligonucleotide antisense-probe sequences used in differential Northern hybridization (see Fig. 8)

Probe[a]	T_H	bp	Sequence
A	63°	43–20	5′ AGT GGC CAT CAT TTA GCT TTA CAC 3′
A/M	61°	43–20	5′ AGT GAC CAT AAT TTA GCT TTC CAC 3′
B/C	57°	42–22	5′ GT GAC CAT CAT TCA GCT TCA C 3′
C	63°	118–95	5′ TTT GGT GGC CTC TAA AGC TTT ACT 3′

Probe A, identical to bp 43–20 sequence in genes from group A; nucleotides in bold differ from sequence in genes from both gene groups B and C; probe A/M is a mutated probe A: nucleotides in bold show three mutations; probe B/C, identical to bp 42–22 sequence in genes from group B/C: nucleotides in bold differ from sequence in genes from group A; probe C, identical to bp 118–95 sequence in genes from group C: nucleotides in bold differ from sequences in group A; nucleotides underlined differ from sequences in group.

[a] See Fig. 7 for representative genes detected by these probes.

TAT GGC TTT C 3′; histone 3.3 forward, 5′ CCA CTG AAC TTC TGA TTC GC 3′; and histone 3.3 reverse, 5′ GCG TGC TAG CTG GAT GTC TT3′. In addition to the DNA, PCR reactions contained 20 mM Tris·HCl, pH 8.4, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM each dNTP, 1 μM each primer, and 2.5 units *Taq* DNA polymerase (Life Technologies) in a total volume of 100 μl. After 1 min of denaturation at 94°, DNA was amplified for 28 cycles of 1 min 94° denaturation, 1 min 51° annealing, and 1.5 min 72° extension. Four 20-μl aliquots were removed from each sample for restriction digestion with DNA endonuclease enzymes: no enzyme, *Bam*HI, *Pst*I, or *Hind*III. Diagnostic restriction patterns were obtained by Tris-borate-EDTA Metaphor (2.8%) (FMC Bioproducts, Rockland, ME) agarose electrophoresis of undigested and digested DNA products, followed by ethidium bromide staining. [A restriction map for predicted fragment sizes of the three DDH gene groups (A, B, and C) is shown in Fig. 7.]

Results

Overexpression of detoxification gene mRNA in HT29 cells. EA, EA-SG, and the deesterified metabolite of T.199 are GST inhibitors. On treatment of human colon HT29 cells, EA, EA-SG, and T.199 caused an increased expression of DDH and γ-glutamylcysteine synthetase mRNAs in both a concentration- (data not shown) and time-dependent manner (Fig. 2A, *lanes 1–5*; Fig. 3, *lanes 1–7*). These effects were selective in that GLXSE (Fig. 2B) mRNA was unaffected. Also, GSTπ was unaffected by T.199 treatment (data not shown). Time course treatment of HT29 cells with either EA-SG or EA yielded a similar pattern of response, i.e., DDH mRNA was induced slightly by both agents within 3 hr, and the levels remained elevated at 24 hr (Fig. 3, *lanes 2–7*).

Reductions in glutathione levels will subject the cell to oxidative stress and protein sulfhydryl modification and could serve as an inducing signal for phase II detoxification and glutathione-associated genes. We therefore treated cells with buthionine-DL-sulfoximine, the γ-glutamylcysteine synthetase inhibitor and glutathione depleter. Buthionine-DL-sulfoximine treatment alone resulted in a marked increase in γ-glutamylcysteine synthetase mRNA (Fig. 4, *lane 5*, controlling for RNA loading). This compound also induced DDH, although it did so modestly (Fig. 3, *lane 10*). Compared with induction levels caused by single treatments with EA, EA-SG, or T.199, pretreatment with buthionine-DL-sulfoximine caused greater levels of induction of both DDH and γ-glutamylcysteine synthetase (Fig. 3, *lanes 8 and 9* compared with *lanes 6 and 7*; Fig. 4, *lanes 7–9* compared with *lanes 2–4*).

The metabolite of the diethylester glutathione analogue T.199 is a selective inhibitor of GSTπ class enzyme (13).

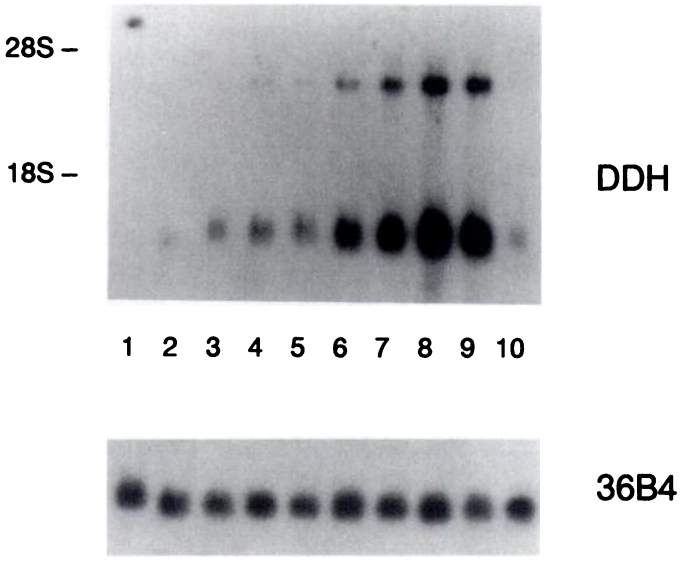

Fig. 3. Time course treatment with EA-SG versus EA; effect of buthionine-DL-sulfoximine on DDH mRNA levels in HT29 colon cells. There was 20 mg total RNA/lane. Northern blots were performed as described in Experimental Procedures. *Lane 1*, wild-type untreated; *lanes 2, 4, and 6*, 50 μM EA for 3, 6, and 24 hr; *lanes 3, 5, and 7*, 100 μM EA-SG for 3, 6, and 24 hr; and *lanes 8–10*, 50 μM pretreatment with buthionine-DL-sulfoximine at 28 hr before isolation followed by 50 μM EA (*lane 8*), 100 mM EA-SG (*lane 9*), and vehicle (*lane 10*) at 24 hr before isolation. The 36B4 cDNA was used as a probe for control of total RNA loading (23).

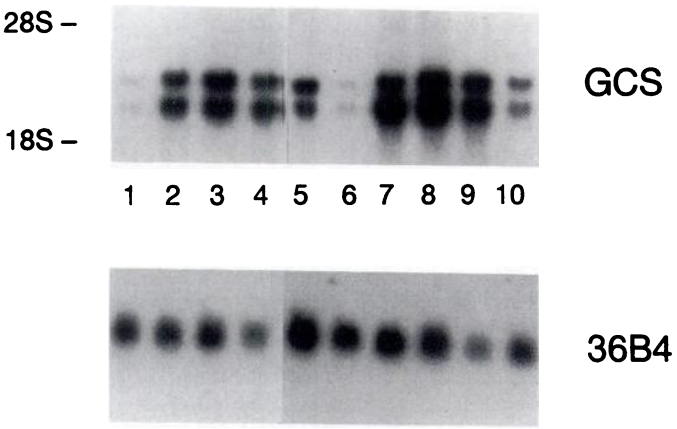

Fig. 4. Effects of buthionine-DL-sulfoximine pretreatment on EA, EA-SG, and T.199 induction of γ-glutamylcysteine synthetase (GCS) mRNA levels in HT29 colon cells. There was 20 μg total RNA/lane. Pretreatment, 28 hr before isolation: *lanes 5 and 7–10*, 50 μM buthionine-DL-sulfoximine; *lane 5*, buthionine-DL-sulfoximine alone). Treatment 24 hr before isolation: *lane 1*, vehicle control; *lanes 2 and 7*, 50 μM EA; *lanes 3 and 8*, 100 μM EA-SG; *lanes 4 and 9*, 50 μM T.199; and *lanes 6 and 10*, 25 μM GSTπ-activatable prodrug. The 36B4 cDNA was used as a probe for control of total RNA loading (23).

Because GSTπ is essentially the only GST class expressed in our HT29 colon cell subclone (6), we sought to determine whether GSTπ enzyme inhibition was required for the observed changes in gene expression. HT29 colon cells were therefore treated with glutathione analogues T.183, T.200, and T.206, which selectively inhibit either GSTα or GSTμ class enzymes, respectively (13). As shown in Fig. 5 at approximately equitoxic concentrations, neither analogue induced DDH or GLXSE (*lanes 2–4*). T.286, a GSTπ activatable prodrug, was also ineffective (Fig, 4, *lane 6*, and data not shown). In addition, neither these agents nor EA induced GSTα or GSTμ mRNAs to detectable levels (data not shown).

To assess which cellular mechanisms may control mRNA levels, determination of relative transcription rates for the effects of EA on γ-glutamylcysteine synthetase, DDH, GSTπ, and glyceraldehyde-3-phosphate dehydrogenase genes was carried out. Fig. 6 shows the corresponding transcript levels

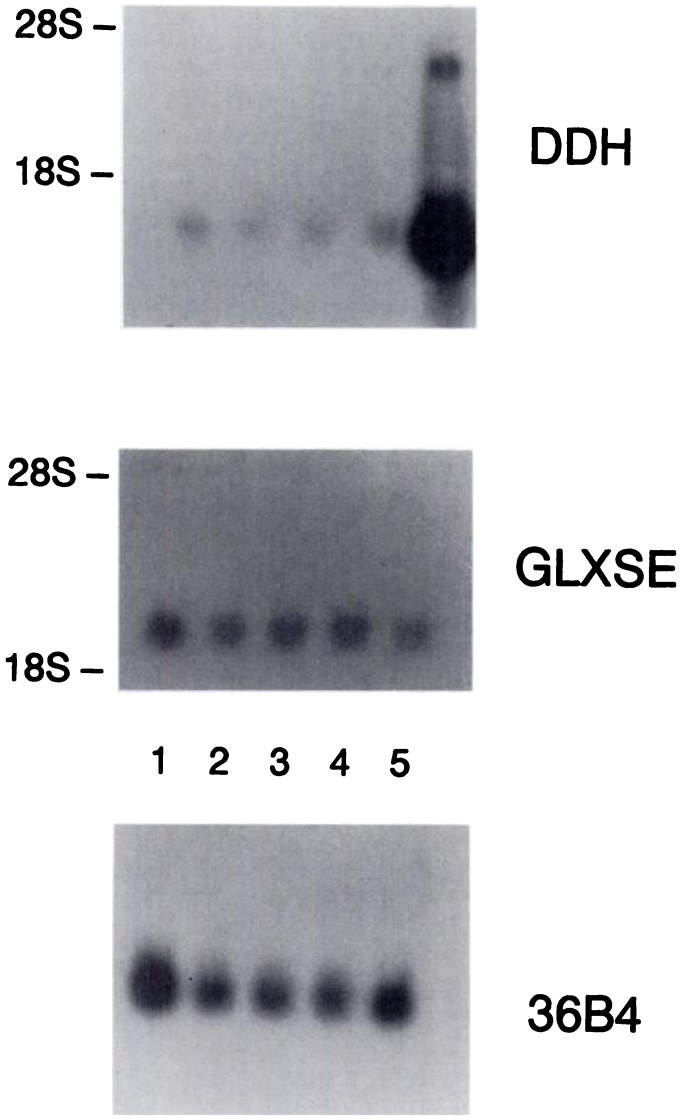

Fig. 5. Effects of glutathione analogues on DDH and GLXSE mRNA levels in HT29 colon cells. There was 20 μg total RNA/lane. Northern blots were performed as described in Experimental Procedures. Treatments were for 24 hr before total RNA isolation. *Lane 1*, wild-type untreated, *lane 2*, 25 μM T.183; *lane 3*, 25 μM T.206; *lane 4*, 25 μM T.317; and *lane 5*, 50 μM T.199. The 36B4 cDNA was used as a probe for control of total RNA loading (23).

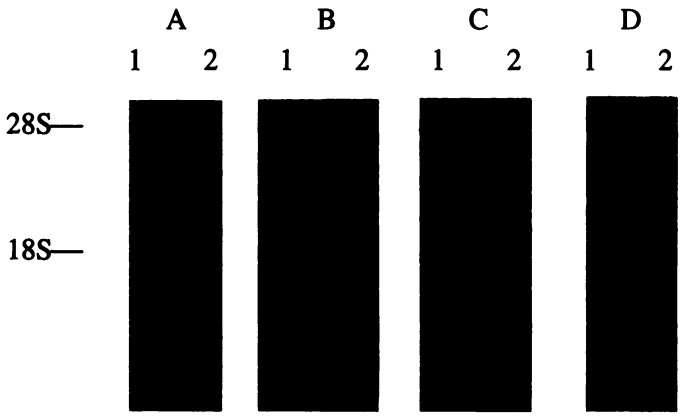

Fig. 6. The effect of EA on relative transcription rates of glyceraldehyde-3-phosphate dehydrogenase, GSTπ, DDH, and γ-glutamylcysteine synthetase by assessment of mRNA levels in newly synthesized RNA. As described by Johnson *et al.* (17) and in Experimental Procedures, total RNA was extracted with guanidine isothiocyanate. Newly synthesized RNA was separated by phenyl-mercury affinity chromatography. Samples were normalized to radiolabel incorporated into newly synthesized RNA, and the RNA was resolved on agarose-formaldehyde gels, as described for other Northern blots. Membranes were then probed with the corresponding specific radiolabeled cDNAs for mRNA level determination. *Lanes 1*, newly synthesized RNA from wild-type untreated cells; *lanes 2*, newly synthesized RNA from EA-resistant cells. A, Glyceraldehyde-3-phosphate dehydrogenase. B, GSTπ. C, DDH(s). D, γ-Glutamylcysteine synthetase.

from affinity purified newly synthesized RNA (1 hr) (*lanes 1 and 2*, wild-type and resistant cells). As shown in Table 1, increases in transcriptional rates with EA exposure were 2.0-fold and 21-fold for γ-glutamylcysteine synthetase and DDH. Consistent with the findings from a previous report from this laboratory, the GSTπ transcription rate increased 2.7-fold in the EA-resistant cells compared with wild-type untreated HT29 cells (6). Also, no effect of EA was observed on the transcription rate of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. For all three enzymes, parallel (but not stoichiometric) increases in protein levels and functional indicators have been reported previously (6, 8, 9): GSTπ protein, 3-fold, 1-chloro-2,4-dinitrobenzene-conjugating activity, 4-fold; DDH protein, 30-fold, 1-acenaphthenol oxidase, 24-fold; γ-glutamylcysteine synthetase (protein was not tested), and reduced glutathione, 4-fold.

Identification of multiple EA-induced DDH transcripts in human cell lines. DDH isozymes are expressed differentially in a number of cell lines and tissues and exhibit varying affinities toward aldehyde, ketone (reductase activity), and polycyclic aromatic hydrocarbon (dihydrodiol dehydrogenase/oxidase activity) substrates (see Ref. 2 for a review). Representative DDH genes are listed in Fig. 7; their trivial names are used. All the genes listed are presumed to belong to the DDH gene family because the nucleotide sequence similarities among them are >85% and because the lowest identity occurs between genes that encode for two functional DDH enzymes (DD2 and DD4). The DDH genes are grouped according to identity in particular restriction sites and short nucleotide sequences corresponding to specific oligonucleotide probes used in differential hybridization Northern blotting (Fig. 8). Thus, for example, the oligonucleotide probe C can be used to recognize more than a single DDH in group C but should not detect genes from group A. Similarly, restriction digestion diagnosis is group specific.

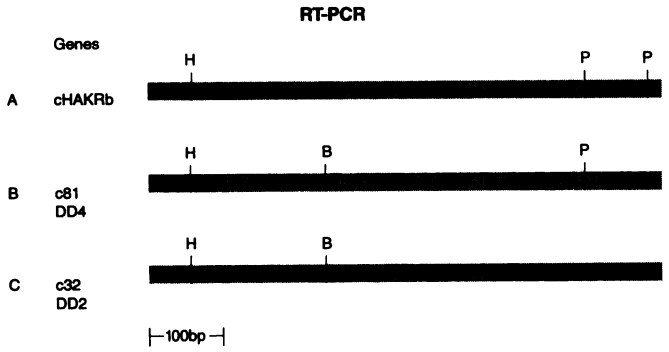

Fig. 7. Restriction map of an RT-PCR-generated 707-bp fragment for three groups of representative DDH genes. DDH trivial nomenclature is used. All of the genes listed are presumed to belong to the DDH gene family because the nucleotide sequence similarities among them are >85% and because the lowest identity occurs between genes that encode for two functional DDH enzymes (DD2 and DD4). The DDH genes are grouped according to identity in particular restriction sites and short nucleotide sequences corresponding to specific oligonucleotide probes used in differential Northern hybridization (see Fig. 8). The identities of three HT29-derived cDNAs from groups A, B, and C were confirmed by sequencing; these include c81, c32, and cHAKRb cDNAs. c81 and c32 were isolated from libraries prepared from EA-resistant HT29 cell mRNA (9, 43). The *Pst*I fragment A (*panel 1*, Fig. 9) was isolated, subcloned, and sequenced as described (43). It corresponds to cHAKRb. cHAKRb, accession number S68288. c81, accession number U05598. DD4 (44), accession number D26125. c32, accession number U05684, also identical to the bile acid binder of Stolz *et al.* (40), accession number U05684. DD2 (44), accession number D26124. *B*, *Bam*HI; *P*, *Pst*I; *H*, *Hind*III.

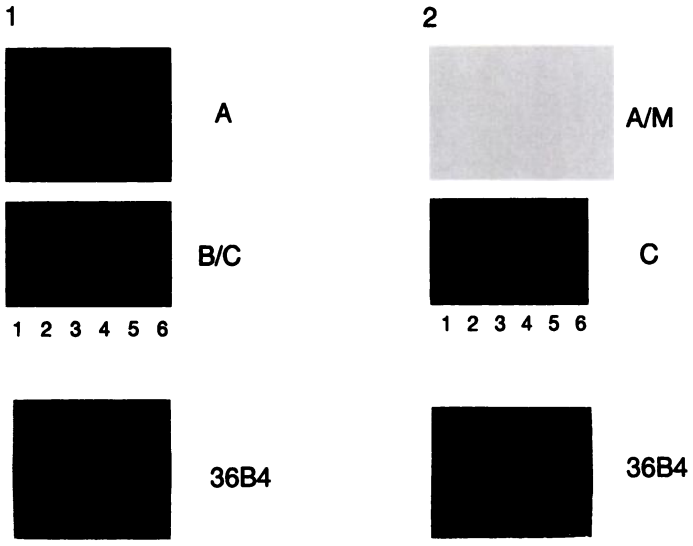

Fig. 8. Differential hybridization detection of several dihydrodiol dehydrogenase mRNAs in human hepatoma HepG2 and SKHep1 cells and HT29 colon cells with specific oligonucleotide probes. DDH mRNA species is 1.4 kb, as shown in Fig. 2, 3, and 5. The larger DDH mRNA species was not detected by this approach. Oligonucleotide probes *A*, *B/C*, and *C* hybridize with representative DDH genes from groups A, B, and C, and C, respectively (see Fig. 6). Three nucleotides have been mutated in oligonucleotide A to yield probe A/M. RNA isolation and Northern blotting methods, oligonucleotide sequences, and T_M are described and listed in the Experimental Procedures section. *Lanes 1*, EA-resistant HT29 cells; *lanes 3 and 5*, EA-treated SkHep1 and HepG2 cells (24 hr); and *lanes 2, 4, and 6*, untreated HT29, SkHep1, and HepG2 cells, respectively. *Panels 1 and 2* represent two different blots containing the same resolved RNA. Each membrane was hybridized, probed, and stripped successively. The 36B4 cDNA was used as a probe for control of total RNA loading (23).

To determine whether the EA-inductive effect was confined to a single DDH gene, we treated three human cell lines with EA and used both Northern hybridization and RT-PCR, followed by restriction digestion, to probe for different DDH mRNAs. As shown in Fig. 8 (*panels 1 and 2*), 50 μM EA treatment for 24 hr induced DDH expression in all three cells lines: HT29 colon cells (*lanes 1*, EA resistant; *lanes 2*, untreated wild-type), SKHep1 (*lanes 3*, treated; *lanes 4*, untreated), and HepG2 (*lanes 5*, treated; *lanes 6*, untreated) hepatoma cells. Group-specific oligonucleotide probes recognized induced DDH mRNAs from gene groups A and C. Mutated oligonucleotide probe A (designated A/M) did not hybridize with mRNA. Oligonucleotide probe B/C hybridizes with DDH mRNA from either group B or C. It also detected induction of DDH mRNAs by EA. As shown in Fig. 9, this profile of induction was confirmed by RT-PCR and restriction digest diagnosis. Compared with samples from untreated cells (Fig. 9, *panels 1–3, lanes 1*), mRNAs from EA-treated or -resistant cells yielded cDNAs in greater abundance (*panel 1, lanes 3*, EA-resistant cells; *panels 2 and 3, lanes 2*, treated). The *Hind*III fragments (*panel 1, lanes 10–12; panels 2 and 3, lanes 7 and 8*) (648 and 59 bp) confirmed the generation of DDH cDNAs because all DDH sequenced to date have contained a *Hind*III site at the border between exon 2 and exon 3. The *Pst*I restriction digest patterns corresponded specifically to groups A (594- and 86-bp *Pst*I fragment; the 27-bp *Pst*I fragment was not detectable) and B (113-bp *Pst*I fragment). A third set of fragments (467- and 240-bp *Bam*HI fragments) were derived from either group B or C. Samples that were resistant to restriction digestion, even after heating to 70° for 10 min, provided indirect, but additional, evidence for the increased expression of specific DDH in treated cells (uncut 707-bp cDNAs in *Bam*HI lanes = group A cDNAs; uncut 707-bp cDNAs in *Pst*I lanes = group C cDNAs). The use of histone 3.3 primers as external controls in the PCR reaction confirmed that reverse transcription of RNA was equal among samples.

Discussion

In the present study, we have shown that the transcription rates of γ-glutamylcysteine synthetase and DDH are increased in EA-resistant HT29 colon cells compared with that of wild-type cells. Based on our work plus previous reports (6, 9, 24), it is clear that with EA exposure increased GSTπ, γ-glutamylcysteine synthetase, and DDH expressions represent composites of increased transcription rates and mRNA stabilization. In the case of GSTπ, protein half-life is also stabilized. Moreover, we have shown that protein levels and corresponding generic substrate activities, or other functional indicators, increase in parallel with increases in mRNA. The complexity of regulation is therefore exemplified by these findings. For DDH, the RT-PCR and differential hybridization studies underscore this observation by revealing that at least three distinct DDH in three different human cell lines are induced by EA.

The electrophilic property of Michael acceptors that contain an α,β-unsaturated carbonyl moiety is believed to provide the inducing signal for some phase II detoxification genes. This signal could be acting on certain genes via alteration of redox (cysteine modification) status of *trans*-activating factors that bind to antioxidant (also called electrophile)

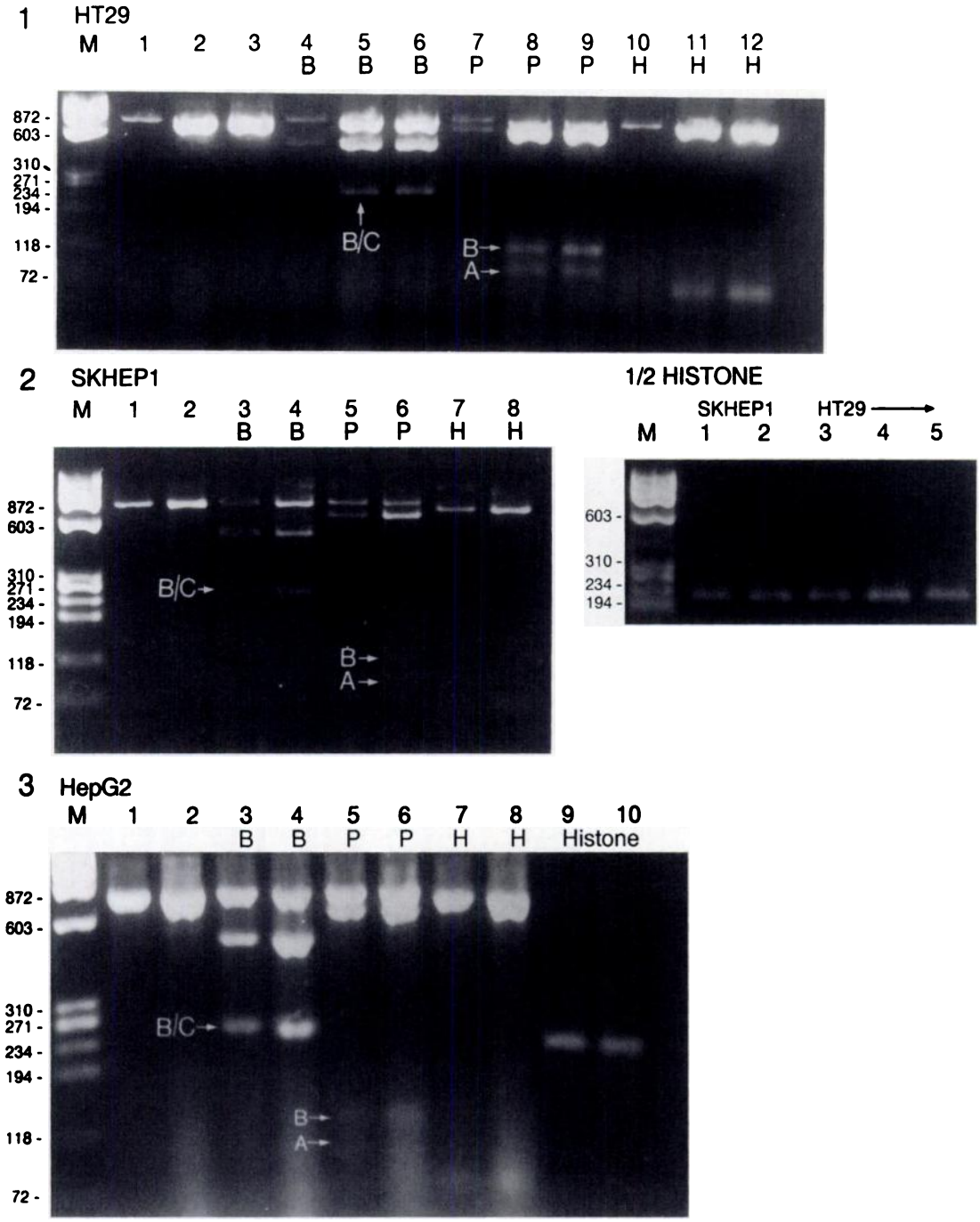

Fig. 9. Restriction digestion diagnosis of RT-PCR-generated DNA fragments. PCR reactions used identical primers for all cDNA samples. Reactions were carried out as described in Experimental Procedures. Undigested (707 bp) and digested samples were resolved on Tris-borate-EDTA Metaphor (2.8%) agarose gels. *Panel 1*, Human HT29 colon carcinoma cells as total RNA source. *Lanes 1, 4, 7, and 10*, wild-type untreated; *lanes 2, 5, 8, and 11*, wild-type treated with 50 μM EA for 24 hr; and *lanes 3, 6, 9, and 12*, EA-resistant cells. *Panels 2 and 3*, Human SKHep1 and HepG2 hepatoma cells as total RNA sources, respectively. *Lanes 1, 3, 5, and 7*, untreated wild-type cells; and *lanes 2, 4, 6, and 8*, wild-type cells treated with 50 μM EA for 24 hr. *Panel 1/2*, Corresponding histone controls for SKHep1 and HT29 cells. *Lanes 1 and 3*, untreated wild-type; *lanes 2 and 4*, EA-treated wild-type; and *lane 5*, EA-resistant cells. *Panel 3, lanes 9 and 10*, Histone controls for HepG2 cells. *M*, molecular weight markers. *B*, *Bam*HI; *P*, *Pst*I; *H*, *Hind*III. Fragments *A*, *B*, or *B/C* correspond to genes from groups A, B, or B and C, respectively (see Fig. 7).

response regulatory elements (25–31). This is supported by the findings that ARE binding activity is composed in part of protein from the redox-sensitive Jun and Fos *trans*-activating families (31) [proteins other than Fos and Jun can bind to an ARE (32, 33)] and that many agents that induce chloramphenicol acetyltransferase in cells transfected with ARE-containing constructs will react with sulfhydryl groups by oxidoreduction or alkylation (29). In light of the recent identification of a putative ARE in the γ-glutamylcysteine synthetase gene (34), the T.199, EA, and EA-SG induction data are consistent with the possible involvement of an ARE in the regulation of γ-glutamylcysteine synthetase. To test the hypothesis that these agents may cause *trans*-activation of an hARE, we treated Hepa1 cells stably transfected with an

NQ01 [NAD(P)H quinone (acceptor) oxidoreductase]) hARE chloramphenicol acetyltransferase reporter construct (30) with these agents for 18 hr. Albeit at modest response levels, 50 μM EA (2-fold response; Ref. 9), 100 μM EA-SG, and 50 μM T.199 increased chloramphenicol acetyltransferase activity 2-fold (data not shown). The data with EA establish a consistent link between ARE activation and the ability of EA to induce expression of murine NAD(P)H (quinone acceptor) oxidoreductase in Hepa1 cells (25) and with data from gel shift assays by the observed increased redox-sensitive NQ01 hARE binding activity in nuclear extracts from EA-resistant HT29 colon cells (9). The data also represent preliminary evidence that these compounds will induce other genes containing an ARE, such as the γ-glutamylcysteine synthetase gene. To date, no ARE have been reported or identified in a DDH gene.

DDH and γ-glutamylcysteine synthetase are also induced in wild-type HT29 colon cells by EA-SG and T.199. The time-dependent, parallel increases of both DDH and γ-glutamylcysteine synthetase after treatment with 50 μM T.199 (Fig. 2) raises the possibility that these two genes are regulated coordinately. Discussion of coordinate regulation is complicated by a number of quantitative issues. It requires careful titration of treatment time course and concentration and assessment of the relative importance of differing basal expression levels and magnitudes of transcript induction. The fact that transcription rates differ between the two genes clearly detracts from a clear-cut case of coordinate regulation. Thus, mechanisms of induction may be different. Nevertheless, that these genes are induced by the same agents and along the same time course suggests that they may share some similar DNA regulatory elements in the promoter regions of these two genes. Transcriptional activating proteins other than ARE binding proteins may mediate induction of phase II detoxification enzymes. Like the ARE, the NFκB motif may be involved in the regulation of numerous proteins that are induced by redox signals (see Ref. 35 for a review). As shown recently (36), the NFκB regulatory element present in the NQ01 gene (31) may mediate the increased expression of NQ01 NAD(P)H (quinone acceptor) oxidoreductase by oltipraz, mitomycin C, hypoxic stress, and heat-shock treatments. The time course of increased NFκB binding activity with treatment in gel shift assays paralleled that of NQ01 mRNA induction. Like the ARE, this element may be involved in a number of stress response situations involving redox processes (35–38). The specificity of gene activation may depend on a number of structural and functional interactions between NFκB and other transcriptional activating proteins. For example, NFκB is known to synergize with Ets, Sp1, and AF-2/c-*jun trans*-activating proteins (35). Assuming a gene has both ARE and NFκB sequences, the relative role these elements may play in regulation could depend on the redox balance of the cell. Additional *cis*-acting elements such as the nuclear redox factor, Ref-1, and the serum response factor element as one of the earlier targets of proxidant stress could also be involved. We have identified a putative NFκB site in the 5′-flanking region of one of the DDH genes that is induced by EA (Figs. 7 and 8, gene from group A, 86-bp *Pst*I fragment, also identified by DNA sequencing as cHAKRb[1]). It is not present in the 5′-flanking region of the DDH gene (39) originally characterized by Stolz *et al.* (40). Functional analyses of these sites are under way. Similarly, putative NFκB sites have been identified in the 5′-flanking region of the γ-glutamylcysteine synthetase gene.[2]

In contrast to induction by EA, gene induction by the glutathione analogue T.199 is not due to any direct property of electrophilicity. T.199, in common with the other inducers reported, is a competitive inhibitor of GSTπ. It is possible that inhibition of GSTπ by T.199 leads to oxidative stress and thus provides the requisite redox-inducing signal. Ploemen *et al.* (41) have demonstrated the *in vitro* reversibility of the conjugation reaction and time-dependent transfer of EA from the glutathione conjugate to other cellular sulfhydryls. That the EA-SG can induce DDH (Fig. 3) approximately in parallel with the time course response to EA treatment suggests that it may be catabolized to an active moiety before or immediately after cellular uptake.

Parallel induction of DDH and γ-glutamylcysteine synthetase by GST inhibitor treatment implies an increased protective role in the metabolism of certain DDH substrates. For example, once the *ortho*-quinone is generated from polycyclic aromatic hydrocarbon dihydrodiols by DDH-mediated oxidation, it can be conjugated to glutathione for elimination (42). Otherwise, *ortho*-quinones are diverted along one-electron reduction pathways to generate toxic oxygen radicals (2). Although EA may not be a direct substrate for DDH, it is possible that its effects on GST and glutathione could produce a cellular environment conducive to the formation of oxidative products.

Like EA (5), T.199 is effective in sensitizing GSTπ-expressing colon cells and tumor xenografts to the cytotoxic effects of nitrogen mustards and supports the concept that glutathione analogues can be used for potentiation of nitrogen mustard effects in tumors overexpressing particular GST classes (12). Results of the present study contribute to our understanding of how GST inhibitors may influence gene regulation when used to modulate the efficacy of other anticancer drugs. We have shown that, like EA, EA-SG and T.199 can cause stress response alterations in expression of enzymes involved in phase II detoxification and glutathione synthesis. Despite marked differences in structure, EA, EA-SG, T.199, and buthionine-DL-sulfoximine probably have a common property of modifying cellular redox balance. In turn, this may lead to downstream enhancer activation involving redox sensitive proteins. Upstream mechanisms may include direct alterations in cellular sulfhydryl status by reaction with glutathione (EA) or indirect elevation of endogenous levels of oxidative stress by transient removal of GSTπ from the cellular cytosolic pool.

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