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Isolation of cDNAs representing dithiolethione-responsive genes

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Dithiolethiones inhibit tumorigenicity elicited by many structurally diverse carcinogens in numerous target tissues. These protective actions are associated with the induction of several carcinogen detoxification enzymes, some of which have only recently been discovered. In order to identify additional novel inducible detoxification response genes, a cDNA library was prepared from liver of rats treated with 1,2-dithiole-3-thione (D3T) and was screened by a differential hybridization method. Complementary DNA clones for several known D3T-inducible genes were isolated, such as epoxide hydrolase, aflatoxin B_1-aldehyde reductase, quinone reductase and multiple subunits of glutathione S-transferase. Clones representing genes not previously associated with detoxification were isolated, including those for ferritin heavy and light subunits, ribosomal proteins L18a and S16 and two novel genes, termed dithiolethione-inducible genes (or DIG-1 and DIG-2). Levels of mRNA recognized by each clone were increased from 2- to 31-fold, with maximum induction between 6 and 30 h after treatment with D3T. Except for epoxide hydrolase, the kinetics of induction of each mRNA was coordinate with increased rates of gene transcription. However, based on the time of response to D3T, at least two sets of responsive genes were identified. One set of genes, including glutathione S-transferase Yp, aflatoxin B_1 aldehyde reductase, quinone reductase and DIG-1, had low constitutive and highly inducible expression (~20-fold) and the other, including glutathione S-transferase Ya and Yb, epoxide hydrolase, ferritin heavy and light subunits, ribosomal proteins L18a and S16 and DIG-2, had relatively high constitutive and modestly inducible expression (~5-fold). The simplest explanation for this differential expression of D3T-inducible genes is that multiple regulatory mechanisms govern their response. The transcriptional activation of ferritin, ribosomal protein, DIG-1 and DIG-2 genes in conjunction with those of carcinogen detoxification enzymes suggests that they participate in the pleiotropic cellular defense response to dithiolethiones that inhibits chemically produced tumorigenesis.

Introduction

Chemoprevention of cancer is a means of cancer control in which the carcinogenic process is blocked, retarded or reversed through the administration of one or several compounds (1). A particularly active class of chemopreventive agents is the dithiolethiones, which are constituents of cruciferous vegetables (2). Dithiolethiones inhibit the tumorigenicity of many structurally diverse carcinogens, for example forestomach and/or pulmonary tumors induced by benzo[*a*]pyrene, diethylnitrosamine and uracil mustard in mice and liver tumors induced by aflatoxin B_1 in rats (3,4). As a consequence of this broad-based anticarcinogenic activity, its low toxicity and prior clinical use as an antischistosomal drug, the substituted dithiolethione 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (oltipraz*) is currently undergoing a phase II clinical trial in humans as a chemopreventive agent in a high risk population that is seasonally exposed to high levels of aflatoxin B_1-contaminated grain (5).

The chemopreventive actions of dithiolethiones have been largely associated with the induction of carcinogen detoxification enzymes (6–8). These detoxification enzymes are widely distributed in mammalian cells and tissues and include NAD(P)H:quinone reductase (QR), epoxide hydrolase (EH), UDP-glucuronosyltransferase (UGT) and glutathione S-transferase (GST). The induction of these enzymes facilitates the metabolic clearance of chemical carcinogens and results in markedly reduced levels of carcinogen–DNA adducts in target tissues (6).

The increased expression of QR and GST Ya subunit genes in response to dithiolethiones is associated with enhanced rates of transcription in both mice (9) and rats (10). Molecular analyses of these genes indicate that their activation requires the presence of a *cis*-acting DNA response element known as the antioxidant response element (ARE) or electrophile response element (reviewed in 11), i.e. an 'AP-1-like' binding site. Similar, but not identical, sequences are found in the genes of UGT (12) and EH (13). While the critical transduction pathway for the activation of this element remains unknown, studies measuring GST Ya ARE enhancer–reporter gene activity demonstrate the ability of multiple, structurally distinct chemicals to activate ARE-mediated transcription (14,15). The only chemical property shared by these different compounds is that they bind to sulfhydryl groups, suggesting that a critical, as yet unknown, regulatory protein is modified during the initial cellular recognition of these chemical signals.

Despite our knowledge of the broad-based activity of the dithiolethiones, much remains to be learned about the regulation and function of this set of genes that, through their concerted expression, afford protection against carcinogens. There is emerging evidence that the dithiolethiones, like many chemopreventive agents, may act through multiple mechanisms. In a systematic screen for efficacy of 90 compounds for chemopreventive activity, oltipraz was shown to inhibit carcinogen–DNA adduct formation, as well as 12-*O*-tetradecanoylphorbol-13-acetate-induced tyrosine kinase activity and free radical formation (16). Recent studies indicate that additional carcinogen metabolizing enzymes are relevant to

*Abbreviations: oltipraz, 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione; QR, NAD(P)H:quinone reductase; EH, epoxide hydrolase; UGT, UDP-glucuronosyltransferase; GST, glutathione S-transferase; ARE, antioxidant response element; AFAR, aflatoxin B_1 aldehyde reductase; D3T, 1,2-dithiole-3-thione; FH, ferritin heavy chain; FL, ferritin light chain.

chemoprevention. Both aflatoxin B_1 aldehyde reductase (AFAR), an enzyme which hydrolyzes the cytotoxic dialdehydic phenolate metabolite of aflatoxin B_1 (17,18), and GST Yc_2 (19), an enzyme with high activity towards the DNA-reactive aflatoxin B_1 8,9-epoxide, were found to be induced by oltipraz (18,19). Identification of additional gene products not previously characterized as responding to cancer chemopreventive agents may provide clues as to the components of the inducible cellular defense against chemically produced tumors.

To expand the understanding of the chemopreventive actions of the dithiolethiones, a differential hybridization method was used to isolate cDNA clones representing several known and novel dithiolethione-responsive genes. Their isolation and results suggesting that they are regulated by multiple pathways is presented. The consequences of identifying additional gene products not previously characterized and their participation in cancer chemoprevention is discussed.

Materials and methods

Chemicals and materials

1,2-Dithiole-3-thione (D3T) was provided by Dr T.J.Curphey (Dartmouth Medical School, Hanover, NH). Probes for hybridization were the GST Ya clone GTB38 and QR clone pDTD55 (20; provided by Dr C.Pickett, Schering-Plough Inc., Kenilworth, NJ). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from ICN (Costa Mesa, CA). [α-^{32}P]UTP (3000 Ci/mmol) and [α-^{35}S]dATP (1000 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). The restriction endonuclease *Eag*I and accompanying reagents were obtained from New England Biolabs (Beverly, MA). Bacterial growth medium was obtained from Difco (Detroit, MI). Reverse transcription and random primed labeling kits and all RNA and Southern blot reagents were purchased from Boehringer-Mannheim (Indianapolis, IN). Nitrocellulose filters and NytranPlus™ membranes were purchased from Schleicher & Schuell (Keene, NH). All other reagents were of the highest quality purchased from Sigma Chemical Co. (St Louis, MO).

Animals and treatments

Male F344 rats (100 g) were obtained from Harlan Sprague–Dawley (Indianapolis, IN) and housed under controlled conditions of temperature, humidity and lighting. Food and water were available *ad libitum*. Purified diet of the AIN-76A formulation without the recommended addition of 0.02% ethoxyquin was used. Rats were acclimated to this diet for 1 week before beginning the experiments. Rats were treated by gavage with 0.5 mmol/kg D3T given in 200 µl suspensions consisting of 1% Cremaphor (BASF Wyandotte Inc., Parsippany, NJ) and 25% glycerol in distilled water.

RNA blot analysis

Total and poly(A)$^+$ RNA isolation and RNA blot analysis were performed according to established procedures (21,22). Total RNA was reacted with glyoxal, electrophoretically separated and capillary transferred to NytranPlus™ membrane according to the manufacturer's instructions. Hybridizations were performed according to standard procedures using 100 ng cDNA probes which were random primed labeled with [α-^{32}P]dCTP to a sp. ac. of 1×10^8 c.p.m./µg. The labeled denatured probe (1×10^6 c.p.m./ml) was added to begin hybridization and incubated for 18 h. Blots were washed twice with 1× SSC (0.15 M NaCl, 0.025 M sodium citrate), 1.0% SDS at 50°C. Signals were visualized by autoradiography using Kodak X-Omat AR film with a Cronex LightningPlus™ intensifying screen (DuPont, Wilmington DE) for 1 or 2 days at –80°C. Levels of RNA were quantitated using a Fuji BAS1000 phosphorimaging system with correction for local background and normalized for RNA loading by stripping and reprobing the blots with a cDNA probe for albumin.

Isolation of poly(A)$^+$ RNA and cDNA library construction

A cDNA library was constructed from poly(A)$^+$ RNA isolated from liver of rats 24 h after treatment with D3T. The D3T-induced cDNA library was prepared as described (23) by Invitrogen (San Diego, CA). A bidirectional library consisting of 1×10^7 primary clones in *Escherichia coli* DH5α cells with an average insert size of 1.3 kb was prepared in the 3 kb pcDNAII expression vector, which contains a multiple cloning site flanked by T7 and Sp6 promoters.

Differential hybridization screening

Subtracted single-stranded cDNA probes enriched for D3T-induced sequences were prepared according to Gastel and Sutter (24). Briefly, a 10-fold excess of the biotinylated control poly(A)$^+$ RNA was hybridized at a total nucleic acid concentration to single-stranded cDNA prepared from reverse transcribed poly(A)$^+$ RNA from vehicle- or D3T-treated rats. The hybridization reaction proceeded for 24 h at 42°C in 50 mM HEPES, pH 7.6, 50 mM NaCl, 2 mM EDTA, 50% formamide, 0.05% SDS. Double-stranded cDNA:RNA hybrids were washed with a 25-fold excess of buffer (50 mM HEPES, pH 7.6, 50 mM NaCl) and subsequently subtracted by adding 10 µg streptavidin and extracting 10 times with phenol:chloroform. The subtracted probes were random primed labeled and used to differentially screen 30 000 replica plated clones of the D3T-induced cDNA library (23). A differential color coding aid for colony screening analysis (25) was used to analyze the autoradiographs from the first round of screening. Bacterial colonies that contained plasmid DNA which hybridized to subtracted probe from D3T-treated and not to vehicle-treated rats were chosen as potential positive clones. A subsequent second screen of the 670 potential positive clones from the primary screen was performed to remove false positive clones, resulting in 280 chosen positive clones. These 280 clones were subjected to cross-hybridization and RNA blot analysis to group related clones.

Southern blot and cross-hybridization analyses

Southern blot analysis was performed as described (22). Each clone (1 µg) was digested with the restriction endonuclease *Eag*I overnight at 37°C. The digested products were separated by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide. The DNA contained within the gels was denatured, then capillary transferred to NytranPlus™ nylon membrane using 10× SSC buffer. Blots were hybridized for 18 h at 65°C and washed according to standard methods. Signals were visualized by autoradiography for 6–24 h at –80°C using an intensifying screen.

Determination of DNA sequence

Double-stranded DNA sequencing by the dideoxy chain termination method was performed according to the instructions of the Sequenase version 2.0 kit (US Biochemicals, Cleveland, OH) with [α-^{35}S]dATP. The specific primers (Sp6 and T7; Invitrogen) to the multiple cloning site of the vector pcDNAII have been previously used for the purpose of DNA sequence determination (23). DNA sequences from both the plus and minus strands were compared with those in Genbank, EMBL and other databases available from the National Library of Medicine. DNA identity scores were generated by matching the query sequence to a subject found in the database using the BLAST algorithm (26). Comparisons resulting in 95% nucleotide sequence identities or greater were considered definitive matches.

Nuclear run-on analysis

Nuclear run-on assays were performed as described (27). Livers from rats treated with D3T or vehicle were obtained 6 and 18 h after treatment. Nuclei were isolated from 5 g tissue. The tissues were lysed in hypotonic buffer (10 mM HEPES, pH 7.4, 0.25 M sucrose) and disrupted with a Dounce homogenizer. Lysis was completed by the addition of Triton X-100 (0.15% final concentration) and the homogenate centrifuged through a 2 M sucrose gradient for 60 min at 60 000 *g*. Nuclei were resuspended in homogenization buffer containing 30% glycerol and stored at –80°C. The transcription reaction mix contained 20 mM HEPES, pH 7.5, 100 mM potassium glutamate, 5 mM sodium fluoride, 10 mM magnesium chloride, 1 mM EGTA, 1 mM ATP, 1 mM CTP, 1 mM GTP, 10 mM creatine phosphate, 100 µg/ml creatine kinase, 20% glycerol and 1 mM dithiothreitol. A 25 µl aliquot containing 250 µCi [α-^{32}P]UTP was added to give a final reaction volume of 200 µl. The nuclear RNA was isolated as described (28). *Eco*RI endonuclease-digested DNA samples (2 µg) were immobilized onto NytranPlus™ membrane and hybridized at 42°C for 48 h. The membrane was washed twice in 2× SSC, 0.1% SDS for 15 min at 65°C, then once at 37°C with 10 µg/ml RNase A for 1 h, then twice more with 2× SSC, 0.1% SDS for 15 min at 65°C. Membranes were autoradiographed for 4 weeks at –80°C using an intensifying screen or were exposed to a phosphorimaging plate for 48 h and quantitated using a Fuji phosphorimaging system as described for the RNA blot analysis.

Results

Isolation of cDNA clones representing D3T-inducible genes

The strategy to isolate new D3T-inducible genes was based on determining the time of maximal increases in the levels of mRNA for GST Ya and QR. Hepatic RNA levels were measured by RNA blot analysis of total RNA isolated at 0, 6, 12, 24, 30, 36, 48 and 72 h after treatment of rats with 0.5 mmol/kg D3T. Levels of both GST Ya and QR transcripts were elevated to a maximum at 24 h post-treatment. Based on these results, a cDNA library was constructed from mRNA

Table I. Assignment of cDNA clone identities based on database search results

Assignment	DNA size (kb)	Query strand	Nucleotide length query/ subject (% identity)	Subject region	Subject accession no.[a]	Reference
Glutathione S-transferase Yb1	0.9	+	119/119 (100)	27–145	gbM11719	29
Glutathione S-transferase Yb1		–	208/208 (100)	782–989		
Glutathione S-transferase Yp	0.8	+	123/130 (95)	605–734	emX02904	30
Glutathione S-transferase Yp		–	131/132 (99)	13–144		
Epoxide hydrolase	1.3	+	82/84 (97)	183–266	gbM26125	31
Epoxide hydrolase		–	94/96 (98)	1518–1650		
Aflatoxin B_1 aldehyde reductase	1.1	+	118/120 (98)	219–338	gbX74673	32
Aflatoxin B_1 aldehyde reductase		–	98/98 (100)	1000–1097		
Ferritin L chain	0.8	+	301/301 (100)	87–387	gbL01122	33
Ferritin L chain		–	205/205 (100)	623–827		
Ferritin H chain	0.8	+	266/266 (100)	1–265	gbM18053	34
Ferritin H chain		–	342/342 (100)	478–820		
Ribosomal protein L18a	0.6	+	154/158 (97)	88–245	emX02904	35
Ribosomal protein L18a		–	113/114 (99)	446–559		
Ribosomal protein S16	0.6	+	83/84 (98)	38–134	gbX17665	36
Ribosomal protein S16		–	121/121 (100)	343–503		

Partial sequences of both strands of the isolated cDNA clones were compared against entries in the DNA sequence databases available from the National Library of Medicine using the BLAST algorithm.
[a]gb, Genbank; em, EMBL.

isolated from liver of rats 24 h after treatment with D3T. Subtracted single-stranded cDNA probes from either D3T- or vehicle-treated rat liver were used to screen 30 000 cDNA clones of this library by differential hybridization techniques. Following the initial screening of the library, 670 potential positive clones representing genes induced by D3T were isolated. After a second round of screening the 670 potential positive clones from the primary screen, the number of potential positive clones was narrowed to 280 clones. Of these 280 potential positive clones, 28 GST Ya and eight QR clones were found by cross-hybridization analyses to a Southern blot of all 280 of the remaining clones. In order to isolate induced cDNA clones, RNA blot analyses were performed on a randomly chosen set of 20 clones. Those cDNA clones not found to represent induced genes were radiolabeled and were found to cross-hybridize to 135 additional redundant clones that were thereby removed from further analysis. By continuing this cross-hybridization method, 27 clones representing 12 different genes were isolated. These cDNA clones recognized D3T-induced RNA transcripts, as evidenced by RNA blot analysis, and were subsequently sequenced.

Partial sequences were obtained for each of these cDNA clones for both the plus and minus strands (Table I). As expected, of the isolated cDNA clones, several known D3T-inducible genes, including clones for GST Ya, GST Yb1, GST Yp, EH, QR and AFAR, were identified. For each isolated cDNA clone, the location and size of the sequenced regions were consistent with those of published sequences. Each of the isolated cDNA clones was nearly full length and had sequence identities of >95% for both DNA strands with those found in the database search.

Four isolated cDNA clones with sequences for genes not previously associated with carcinogen detoxification pathways were identified. These were the ferritin heavy (FH) and light (FL) chain subunits and the ribosomal proteins L18a and S16 (Table I). In addition, two isolated cDNA clones representing novel genes were found and designated as the D3T-inducible genes DIG-1 and DIG-2 (Genbank accession nos U66322 and U66323).

The complete primary structure of the DIG-1 clone was determined and its sequence was found to not be highly related (>70% identity) to any characterized gene in the Genbank or EMBL DNA databases. An open reading frame of 1041 nucleotides starting with a Kozak consensus site was found. The deduced amino acid sequence had a length of 347 amino acids, yielding a calculated isoelectric point of 8.5 and molecular mass of ~38 kDa. Although searching the protein sequence databases identified no characterized protein highly related to DIG-1, the region of DIG-1 spanning amino acids 134–190 was found to have identities of 57% with rabbit intestinal protein AdRab-F (37), 36% with yeast alcohol dehydrogenase 3 (38), 26% with a putative quinone reductase from *Leishmania amazonensis* (39), 22% with human ζ-crystallin/quinone reductase (40) and 17% with fission yeast alcohol dehydrogenase (41), as shown in Figure 1. Interestingly, these enzymes each possess carbonyl metabolizing functions. Moreover, the region spanning amino acids 134–190 of DIG-1 represents a highly conserved NADP(H) binding site for several alcohol dehydrogenases and quinone reductases (42,43).

Approximately 50% of the DIG-2 clone has been sequenced and it possesses a 250 bp region at its 5′-end with homology to a 250 bp DNA sequence distal to the promoter region of *c-raf-1* found in certain human tumor cells (44). However, sequencing from either end of the DIG-2 cDNA clone did not reveal any regions with identity to the coding region of human *c-raf-1*, suggesting that this 5′-portion of the DIG-2 cDNA encodes a different gene product than the activated *c-raf-1* found in human tumors.

Inducible expression of isolated cDNA clones by D3T

To confirm that these cDNA clones represent D3T-inducible genes, RNA blot analyses were performed. The level of induction of mRNA transcripts measured 24 h after treatment with D3T is presented in Table II and their corresponding RNA blots are shown in Figure 2A. Relative to control, mRNA levels of EH, AFAR, QR and the GST Ya, Yb and Yp subunits were increased from 3- to 30-fold by D3T 24 h following treatment (Table II and Figure 2A). The mRNA levels for FL and FH subunits were increased 5.1- and 2.1-fold respectively

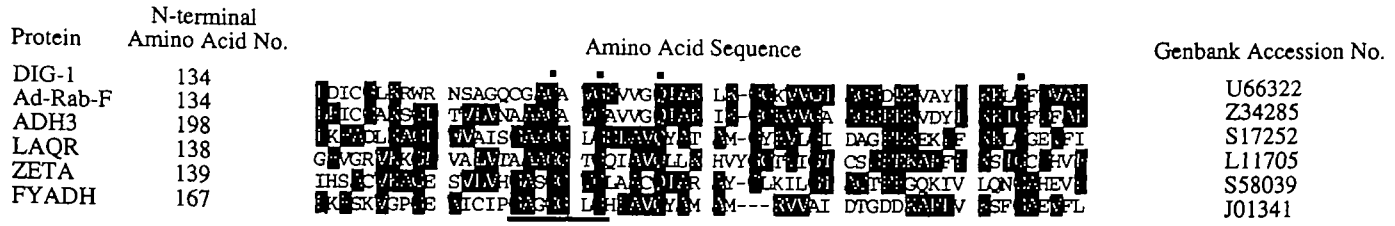

Fig. 1. Comparison of putative NADP(H) binding regions of DIG-1 (134–190) and carbonyl metabolizing enzymes. Shown are the sequences for DIG-1, AdRab-F, *L.amazonensis* quinone reductase (LAQR), human liver ζ-crystallin (ZETA), yeast alcohol dehydrogenase (ADH3) and fission yeast alcohol dehydrogenase (FYADH). N-Terminal positions for each amino acid sequence and Genbank accession numbers are shown. Positions with exact identities for all sequences are identified by closed squares (top) and other positions with identity between multiple sequences are shaded. The glycine-rich region corresponding to the Rossmann fold motif (43) of NADP(H) binding proteins ADH3 (38), ZETA (40) and FYADH (41) is underlined.

24 h after administration of D3T (Table II and Figure 2A). In every case, the size of the mRNA transcript identified by each cDNA clone was consistent with respective, previously published sizes, if available, providing further confirmation of the identity of the cDNA clones that were isolated in this study.

The highest levels of induction, ~20- to 25-fold, were found for GST Yp, AFAR and DIG-1. The magnitude of the increase in the level of mRNA is attributable to the low constitutive level of expression of each of these genes, as shown in lanes 1–3 in Figure 2A. In contrast, the inductions of L18a, S16 and DIG-2 were ~5-fold and were similar to that observed for GST Ya, Yb and EH. Thus, several of these genes are normally expressed in liver and are moderately induced, while others have low constitutive expression and are highly induced in response to D3T.

Time course of D3T induction of mRNA

Evidence suggesting that multiple gene regulatory mechanisms govern this concerted gene response to administration of D3T was first obtained from studies of the time course of mRNA induction. Total RNA was isolated at 0, 6, 12, 24, 30, 36, 48 and 72 h after treatment with D3T. RNA blot analyses revealed that the level of each mRNA transcript increased in a time-dependent manner, with maximal induction between 24 and 30 h after treatment (Figure 2B). Since the data presented in Figure 2B is the ratio of the level of treated to control, it is noteworthy that the RNA levels from untreated rats did not vary significantly over time (T.Primiano, T.W.Kensler and T.R.Sutter, unpublished results). Furthermore, the fold increase in induction was consistent between the time course (Figure 2B) and 24 h RNA blots (Figure 2A).

Differences in the time course of induction following treatment with D3T were found. The mRNA levels of the ferritin subunits, especially FL, appeared to increase in a biphasic manner, with a rapid increase by 6 h after treatment, followed by a slower increase to maximum levels by 24–30 h. The level of AFAR mRNA was elevated ~10-fold by 6 h and, like GST Yb1, showed an apparent linear increase to a maximum at 24 h. Increased levels of GST Ya, EH, QR, L18a, S16 and DIG-2 mRNA appeared slightly delayed, while increased levels of GST Yp and DIG-1 were clearly delayed, with significant increases occurring subsequent to 12 h after treatment with D3T (Figure 2B).

Nuclear run-on analyses

In order to determine whether increased mRNA levels resulted from transcriptional activation of these genes, nuclear run-on analyses were performed at 6 and 18 h following D3T treatment. Corresponding increases in the rates of transcription of each gene were demonstrated (Table III), with the exceptions

Table II. Summary of RNA blot quantitation for phase 2 and novel cDNA clones

cDNA clone	Message size (kb)	Treated/control
Glutathione S-transferase Ya	1.2	8.5 ± 1.0[a,b]
Glutathione S-transferase Yb1	1.2	3.4 ± 0.6[b]
Glutathione S-transferase Yp	1.0	31.3 ± 3.3[b]
Epoxide hydrolase	1.6	14.6 ± 1.2[b]
Quinone reductase	1.1	6.2 ± 1.1[b]
Aflatoxin aldehyde reductase	1.3	24.4 ± 6.3[b]
Ferritin L chain	0.9	5.1 ± 0.4[b]
Ferritin H chain	0.9	2.1 ± 0.2[b]
Ribosomal protein L18a	0.8	4.8 ± 0.1[b]
Ribosomal protein S16	0.8	5.1 ± 0.3[b]
DIG-1	1.3	22.3 ± 2.0[b]
DIG-2	1.4	5.3 ± 0.1[b]

Listed are ratios of signals measured from hybridizations of total RNA isolated from liver using GST Ya, GST Yb1, GST Yp, EH, QR, AFAR, FL, FH, L18a, S16, DIG-1, DIG-2 or albumin cDNA probes 24 h after treatment with D3T compared with vehicle alone. Quantitative signals generated using a Fuji phosphorimaging system were corrected for local background and normalized to values for albumin.

[a]Results are expressed as mean ± SE of determinations from nuclei isolated from three separate rats at each time point.

[b]Indicates significant difference from the mean ($P < 0.05$) based on ANOVA followed by Tukey's test.

of EH (no increase) and S16 (not determined). The time corresponding to the highest rate of transcription was gene dependent. The rates of transcription of GST Yb1, AFAR, FL and FH and DIG-2 were greater at 6 than at 18 h after D3T treatment. In contrast, the rates of transcription of GST Ya, GST Yp, QR, L18a and DIG-1 were greater at 18 than at 6 h (Table III). Except for EH, increases in levels of mRNA paralleled enhanced rates of transcription in both time and magnitude of response to D3T. For EH, additional post-transcriptional mechanisms may contribute to the observed mRNA increase (Table III). Despite the >10-fold increase in steady-state mRNA levels of EH 24 h after administration of D3T (Table II), no significant increase in the rate of EH gene transcription was detected at either the 6 or 18 h times of analysis.

Discussion

In order to further understand the molecular mechanisms underlying the cancer chemopreventive properties of dithiolethiones, a differential hybridization method was used to isolate cDNA clones for D3T-inducible genes. In addition to cDNA clones for genes of known carcinogen detoxification

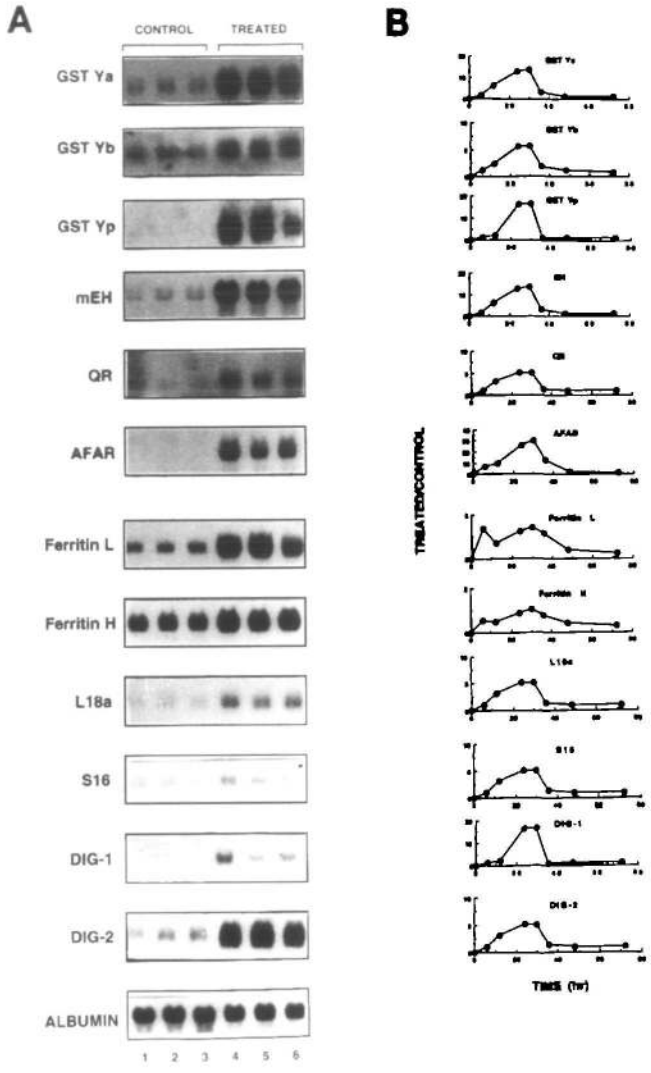

Fig. 2. Analysis of induction of RNA levels by RNA blot hybridization following D3T treatment. (**A**) Total RNA isolated from liver of vehicle-treated rats (**lanes 1–3**) and total RNA from 0.5 mmol/kg D3T-treated rats (**lanes 4–6**) at 24 h post-treatment were hybridized using GST Ya, GST Yb1, GST Yp, EH, QR, AFAR, FL, FH, L18a, S16, DIG-1, DIG-2 and albumin standard cDNA probes respectively. (**B**) Quantitative analysis of time-dependent induction of RNA levels by RNA blot hybridization following treatment with D3T. Results are expressed as the ratios of treated to control values at the time points 0, 6, 12, 24, 30, 36, 48 and 72 h post-treatment. Quantitation was performed using a Fuji BAS1000 phosphorimaging system and was normalized for loading using signals from albumin probed blots.

enzymes, clones corresponding to genes encoding FH and FL subunits, the ribosomal protein L18a and S16 and two novel gene products were identified. The roles of these newly identified gene products in mediating protection from carcinogenesis are uncertain, but their isolation and future characterization will likely provide new insights into the cellular responses governing the anticarcinogenic effects of dithiolethiones. The results of this screen provide new insights into the multiplicity of cellular gene regulatory and functional components that contribute to the preventive actions of this class of compounds.

In this study, cDNA clones representing 12 distinct D3T-inducible genes were identified and analyzed. For the known carcinogen detoxification enzymes, except for EH, the kinetics of induction of each mRNA was coordinate with increased rates of gene transcription (Figure 2B and Table III). The

Table III. Quantitation of the rate of transcription by nuclear run-on analysis

cDNA clone	Treated/control	
	6 h	18 h
Glutathione S-transferase Ya	1.7 ± 1.2[a]	3.2 ± 0.8[a,b]
Glutathione S-transferase Yb1	4.3 ± 0.8[b]	2.1 ± 0.9[b]
Glutathione S-transferase Yp	3.5 ± 0.6[b]	13.8 ± 0.8[b]
Quinone reductase	0.7 ± 0.6	3.8 ± 0.8[b]
Aflatoxin aldehyde reductase	50.2 ± 0.7[b]	17.2 ± 0.2[b]
Epoxide hydrolase	1.2 ± 1.1	1.8 ± 0.9
Ferritin L chain	9.8 ± 0.7[b]	4.7 ± 0.5[b]
Ferritin H chain	4.8 ± 1.0[b]	1.7 ± 0.3
Ribosomal protein L18a	1.0 ± 0.9	3.3 ± 0.4[b]
Ribosomal protein S16	ND[c]	ND
DIG-1	1.0 ± 0.9	3.7 ± 0.7[b]
DIG-2	3.1 ± 1.0[b]	1.5 ± 0.6

Listed are ratios of signals measured from hybridizations of radiolabeled mRNA isolated from rat liver nuclei 6 or 18 h after treatment with D3T compared with vehicle alone. Quantitative signals generated using a Fuji phosphorimaging system were corrected for local background and normalized to values for albumin.

[a]Results are expressed as mean ± SE of determinations from nuclei isolated from three separate rats.

[b]Indicates significant difference from the mean ($P < 0.05$) based on ANOVA followed by Tukey's test.

[c]ND, not determined.

results indicating different temporal responses for the members of the dithiolethione-inducible gene battery, such as the relatively early response of AFAR compared with GST Yp, suggest that alternatives to a solitary signal transduction pathway through the ARE exist. Further evidence supporting the involvement of multiple regulatory mechanisms in response to chemopreventive dithiolethiones is the apparent lack of transcriptional activation of the EH gene; a result suggesting that increased EH mRNA levels occur by post-transcriptional mechanisms. Thus, it appears that the 'ARE-like' sequence in the promoter region of EH (13) is not sufficient for transcriptional activation of this gene by D3T (Table III).

In addition to the known carcinogen detoxification enzymes, the mRNA levels for two ribosomal proteins were both elevated ~5-fold following treatment with D3T (Table II). The genes for these ribosomal proteins were transcriptionally activated as early as 6 h post-treatment, resulting in increased steady-state levels of mRNA, which reached maximum levels between 24 and 30 h after treatment with D3T. This time course was similar in magnitude and temporal response as those of the known carcinogen detoxification enzymes, suggesting that coordinate regulation of these genes may occur in response to D3T. Little information exists concerning xenobiotic induction of ribosomal proteins and the consequences of elevating levels of ribosomal components in response to dithiolethiones can only be speculated upon. In this regard, enhanced translation of detoxification enzymes may be occurring. For example, Davidson *et al.* (45) found that continued administration of oltipraz to rats resulted in a persistent elevation of GST activity long after the GST Ya mRNA levels had decreased from a maximum level. This maintenance of GST activity may be due to increased stability of GST protein or an enhanced translational efficiency of the GST Ya mRNA. Further investigations into the potential relationship of increased ribosomal and GST Ya levels is warranted. Additionally, since the full 5′-flanking regions of these genes have not been published,

the existence of an ARE-like regulatory sequence which mediates induction by dithiolethiones remains to be discovered.

The expression of ferritin protein is tightly regulated at the levels of transcription and translation for both the FH and FL subunits (reviewed in 40,41). It is interesting to note that increases in FL and FH RNA parallel the increases in transcription rates at 6 h, but do not coincide at later time points. These results suggest that by 18 h after administration of D3T, the post-transcriptional mechanism of ferritin regulation may contribute to the total increase in steady-state levels of RNA. Enhanced ferritin gene transcription occurs in response to treatments with iron and cytokines and during certain cell differentiation programs (46,47). The preferential stimulation of the FL subunit observed in our study (Figure 2 and Tables II and III) is similar to the pattern of ferritin gene response to iron and is clearly distinct from the pattern of FH activation that occurs following cytokine treatment or during cell differentiation. Initially, these results suggest that D3T may induce ferritin expression, in part, through an iron-dependent mechanism. However, recent experiments utilizing pretreatment with the iron chelator desferrioxamine indicated that intracellular iron levels did not initially affect enhancement of the levels of RNA or protein of either the FL or FH genes by D3T (48). Induction of ferritin by D3T appears to occur through iron-independent transcriptional mechanisms. Such an iron-independent mechanism would differ from the proposed regulation of ferritin and heme oxygenase in the liver of rats subjected to oxidative stress by a chemical-mediated glutathione depletion response (49).

Two novel clones were also identified whose mRNA levels were induced significantly by D3T. Comparisons of these sequences with those found in the database searches showed that DIG-1 did not significantly match any previously characterized genes. DIG-1 expression parallels that of GST Yp and AFAR, in that its response to D3T is similar in time course and magnitude and is transcriptionally activated. The similarity of a region of DIG-1 to the reported NADP(H) binding regions for adult rabbit AdRab-F, *L.amazonensis* quinone reductase, human liver ζ-crystallin, yeast alcohol dehydrogenase and fission yeast alcohol dehydrogenase suggests the presence of a putative NADP/NADPH binding site in DIG-1 (42). The potential for NADP/NADPH binding by DIG-1 indicates that it may utilize the cofactor to function as a dehydrogenase or reductase. Enhancement of DIG-1 activity by D3T, in conjunction with increased QR and AFAR activities, may serve to increase carbonyl metabolism within cells and tissues, thereby protecting them from aldehyde, ketone and quinone carcinogens and their metabolites. The expression of DIG-1 and characterization of its specific cellular function may elucidate its involvement as an additional component of the chemopreventive actions of dithiolethiones.

DIG-2 expression, in contrast, follows a pattern similar to GST Ya and Yb, QR and the ribosomal protein L18a and S16 genes. A region of 250 bp of DIG-2 has an 84% identity to a DNA sequence that associates with a distal upstream portion of c-*raf-1* by DNA recombination in human tumors (44). This recombinant sequence is not found in normal tissue. The recombinant product was therefore reported to function as an activated form of c-Raf-1, leading to an enabling of the c-*raf-1*/MAP kinase signal transduction pathway, resulting in enhanced growth of transformed cells. The forthcoming sequencing and expression of full-length DIG-2 cDNA will help to clarify whether any relationship exists between activation of the c-*raf-1* gene and the role of DIG-2 in cancer chemoprevention.

In summary, the identification of the FH and FL, ribosomal proteins L18a and S16 and DIG-1 and DIG-2 as part of the set of genes that are activated in response to D3T suggests important additional mechanisms for cancer chemoprevention. Also, differences in their time-dependent expression suggest multiple levels of gene regulation in response to D3T. While much work will be required to understand the functional significance of the elevation of these gene products to cancer chemoprevention, the observation of increased synthesis of ferritin may likely result in an increased sequestration of free intracellular iron. The subsequent decreased levels of intracellular iron may then reduce production of reactive oxygen through Fenton-type reactions (50). Agents like the dithiolethiones, with the capacity to activate multiple protective pathways, may prove to be especially potent and effective cancer chemopreventive compounds.

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