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THE CHEMOPREVENTIVE AGENT DIALLYL SULFIDE

A STRUCTURALLY ATYPICAL PHENOBARBITAL-TYPE INDUCER

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Abstract—Diallyl sulfide (DAS), a known chemopreventive agent, was administered i.g. (200 or 500 mg/kg body wt/day) to male F344/NCr rats for 4 days. Livers were removed, and hepatic levels of a variety of drug-metabolizing enzymes were determined with either catalytic assays or by quantifying levels of total cellular RNA coding for the individual genes of interest. The high dose of DAS induced the cytochrome P450 (CYP) 2B subfamily to near maximal levels [i.e. similar to those induced by phenobarbital (PB)] and induced the CYP3A subfamily, while having minimal effects on the levels of the CYP1A subfamily. In addition, DAS induced the glutathione *S*-transferase α subfamily, the glutathione *S*-transferase μ subfamily, and epoxide hydrolase. Unlike PB, however, DAS was also able to induce quinone oxidoreductase. In fact, the pleiotropic hepatic response to DAS appeared to be similar to that elicited by PB, with the exception that only DAS induced quinone oxidoreductase. Finally, we determined that DAS induced the levels of a specific nuclear binding protein that appears to be associated with the induction of various genes that are part of the pleiotropic response caused by PB-type inducers.

Key words: diallyl sulfide; phenobarbital; CYP; chemoprevention; liver enzymes; nuclear transcription factors

A number of investigators have hypothesized that many chemopreventive agents, particularly those that inhibit carcinogen initiation, may act by altering the metabolism of procarcinogens [1, 2]. These agents could increase detoxification either by: (1) increasing the levels of phase II conjugating enzymes and thereby increasing conjugation of the carcinogen or its ultimate reactive metabolites; or (2) altering levels of various phase I drug-metabolizing enzymes, such as the cytochromes P450, and thereby altering the toxicokinetics of the procarcinogen. In fact, certain investigators have hypothesized that one can indeed screen for potential chemopreventive agents by measuring the ability of a compound to induce specific non-CYP¶-mediated drug-metabolizing activities, such as quinone oxidoreductase or GSTs [3, 4]. The rationale for such an approach is that compounds that induce these specific enzymes will coordinately induce a wide variety of phase II drug-metabolizing enzymes, which will more readily conjugate, and thereby detoxify, any reactive intermediates produced from procarcinogens.

In the present experiments, we investigated the ability of DAS to induce a variety of drug-metabolizing enzymes in the livers of male F344/NCr rats. DAS, a breakdown product of cysteine, can be found at concentrations up to 100 mg/g in crushed garlic [5]. DAS has been shown previously to be an effective chemopreventive agent when employed during the initiation stage in a variety of rodent tumor model systems [6–9]. Interestingly, both Yang and coworkers [10, 11] as well as ourselves [12] have observed previously that DAS highly induces the CYP2B subfamily, despite the fact that DAS bears little structural resemblance to the prototype CYP2B inducers PB or DDT (Fig. 1). Employing catalytic assays and RNA hybridization techniques, we have examined the induction of a variety of genes that code for specific drug-metabolizing enzymes. DAS caused an induction pattern quite similar to PB, with the exception that DAS induced quinone oxidoreductase, which PB failed to do.

Finally, we examined the ability of DAS to induce a nuclear protein that binds to a specific nucleotide sequence (5′-GAGGAGTGAATAGCCAAAGCAGGAGGCGTG-3′) originally described by Upadhya *et al.* [13]. This nucleotide sequence includes a shorter sequence, designated the BARBIE site, which has been shown to be involved in transcriptional activation of cytochrome $P450_{BM-3}$ by barbiturates in *Bacillus megaterium* [14, 15]. In addition, there is more circumstantial evidence for the involvement of this nuclear binding in PB-mediated transcriptional activation of drug-metabolizing genes in eukaryotes, as well [13, 16].

MATERIALS AND METHODS

Chemicals

DAS, PB and dicumarol were purchased from the Aldrich Chemical Co. (Milwaukee, WI). BZR, ETR, MTR

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¶ Abbreviations: BARBIE site, 5′-ANCNAAAGCNGGNGG3′; BZR, benzyloxyresorufin; CYP, cytochrome P450; DAS, diallyl sulfide; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; ETR, ethoxyresorufin; GST, glutathione *S*-transferase; HCB, 3,3′,4,4′,5,5′-hexachlorobiphenyl; MTR, methoxyresorufin; PB, phenobarbital; PMSF, phenylmethylsulfonyl fluoride; PTR, pentoxyresorufin; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

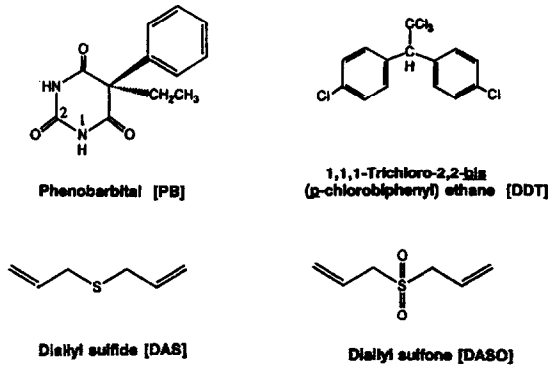

Fig. 1. Chemical structures of PB, DDT, DAS, and DASO.

and PTR were obtained from Molecular Probes, Inc. (Eugene, OR). HCB was purchased from the Accurate Chemical Co. (West Haven, CT).

Animal treatment

DAS was administered to 8-week-old male F344/NCr rats (Animal Production Area, NCI-Frederick Cancer Research and Development Center) via gavage (three successive daily administrations of 200 or 500 mg/kg body wt, in corn oil). The rats were killed by CO_2 asphyxiation 24 hr following the last DAS administration. Additional rats were administered PB (500 ppm in the diet, 7 days feeding), DDT (428 ppm in the diet, 5 days feeding), or HCB (8 ppm in the diet, 5 days feeding).

Catalytic assays for drug-metabolizing enzymes

Alkoxyresorufin *O*-dealkylation assays were performed for 9000 *g* supernatants (S9) as described previously [17, 18]. Quinone oxidoreductase activity was determined by measuring the reduction of resorufin as described previously [18, 19].

Semiquantitative RNA analysis

Isolation of total cellular RNA and hybridization to specific plasmid or oligonucleotide probes was performed as described previously [18, 20]. The plasmids that we employed to determine the levels of the GST α subfamily [21] and microsomal epoxide hydrolase [22] were supplied by Dr. C. Pickett. The specific oligonucleotides that were employed to determine levels of CYP2B1 and CYP3A1 have been described previously [12, 23]. The RNAs examined were all hybridized with a plasmid for β-actin as an internal control, and the loadings were determined to vary by less than 15%.

Nuclear protein purification

Rats were killed by CO_2 asphyxiation. The livers were frozen in liquid N_2 immediately after removal and were stored at −70° until processed. Approximately 3 g of tissue were minced with a scalpel or scissors while still frozen and were put in a 50-mL plastic tube containing 20–25 mL of homogenization buffer on ice [20 mM HEPES (pH 7.9), 20 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 30% sucrose, 100 U/mL aprotinin, 0.5 mM PMSF, 5μg/mL leupeptidin, 5 μg/mL pepstatin, 1 mM 2-mercaptoethanol]. A 1:6 (w/v) ratio (tissue: buffer) gave the highest yield. The samples were homogenized in a Teflon/glass homogenizer by 2–3 slow strokes and filtered through two layers of cheesecloth. The homogenate was spun at 2500 *g* for 15 min in a 50-mL plastic tube. The pellet was resuspended in 25 mL of homogenization buffer on ice while avoiding cell disruption. NP 40 was added to a final concentration of 0.5%. After mixing gently by inverting the tubes, the samples were spun at 1200 *g* for 10 min. The pellet was resuspended in 25 mL of resuspension buffer on ice [20 mM HEPES (pH 7.9), 20 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 100 U/mL aprotinin, 0.5 mM PMSF, 5 μg/mL leupeptidin, 5 μg/mL pepstatin, 1 mM 2-mercaptoethanol] and spun at 1200 *g* for 10 min. The resuspension was repeated twice. A 5-μL drop was put onto a glass slide and stained with crystal violet to assess the quality of the nuclei. After estimating the nuclear pellet volume (npv), 1 npv of 200 mM NaCl was added slowly, followed by 1 npv of 600 mM NaCl, and then 1 npv · 0.85 of 5 M NaCl. The samples were mixed for 30 min at 4°. The contents were transferred to an Oak Ridge tube (cooled on ice) and were spun at 100,000 *g* (33,000 rpm) for 1 hr. The supernatant was transferred to standard dialysis tubing using a Pasteur glass pipette and was dialyzed overnight at 4° against a dialysis buffer [20 mM HEPES (pH 7.9), 100 mM KCl, 20% glycerol, 10 U/mL aprotinin, 0.2 mM PMSF, 0.5 μg/mL leupeptidin, 0.5 μg/mL pepstatin, 1 mM 2-mercaptoethanol]. The dialyzed sample was spun for 5 min in a microcentrifuge, and the resulting pellet was discarded. The protein concentration was determined and the samples divided into 30- to 50-μg aliquots, which were frozen in liquid nitrogen and then stored at −70°. The proteins were stable for up to 3 months at −70°.

DNA gel retardation

The specific double-stranded oligonucleotide that we employed was taken from the published sequence of Upadhya *et al.* [13] and includes the consensus BARBIE sequence of He and Fulco [14]. The sequence of the oligonucleotide is as follows: 5′-GAGGAGTGAATAGCCAAAGCAGGAGGCGTG-3′ and 3′-CTCCTCACTTATCGGTTTCGTCCTCCGCAC-5′. The complementary oligonucleotides were annealed by mixing them in TE (10 nM Tris, 1 mM EDTA) in equimolar concentrations, denaturing by boiling for 5 min, and cooling slowly to room temperature. They were kept at −20° and aliquots were taken for 5′ end-labeling (Lofstrand Laboratories, Gaithersburg, MD). The purified labeled double-stranded probes were diluted in TE to a final activity of 2,500–10,000 cpm/μL. The DNA gel retardation assay was performed as described previously [24]. Briefly, we employed the high ionic strength buffer and the high ionic strength gel mix. The cast gels were always prerun at 100 V for 90 min. The reaction mixture contained 20,000–30,000 cpm double-stranded oligonucleotide, 5–20 μg nuclear protein, 2 μg poly (dI-dC) in a buffer containing 12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 1 mM EDTA, 300 μg/mL bovine serum albumin, 4 mM Tris–HCl (pH 7.9), 1 mM dithiothreitol. The incubation was for 20 min at room temperature. The samples were subjected to electrophoresis at 30–35 mA for 1.5 to 2.0 hr. The gels were dried, and autoradiography was performed without an intensifying screen.

RESULTS

We initially examined the induction of certain of the CYP isozymes. Induction of these CYP proteins was determined either by determining the metabolism of highly specific substrates or by measuring levels of total cellular RNAs coding for the specific CYP genes. Employing a variety of alkoxyresorufin substrates, we examined for the induction of the three major inducible forms of CYP: CYP1A1 (ETR *O*-dealkylation), CYP1A2 (MTR *O*-dealkylation) or CYP2B (BZR and PTR *O*-dealkylation) [17]. Besides determining catalytic levels in rats exposed to DAS, we also determined these levels in rats exposed to PB (500 ppm), DDT (428 ppm) or HCB (8 ppm) in the diet. Phenobarbital and DDT were employed as positive controls for the induction of CYP2B1/2, while HCB was used as a positive control for CYP1A-type induction. DAS and PB both highly induced BZR *O*-dealkylation (35 to 45-fold) while causing only limited induction of the two members of the CYP1A subfamily. In contrast, HCB, a prototype TCDD-type inducer, induced MTR *O*-dealkylation approximately 30-fold and ETR *O*-dealkylation almost 80-fold (Table 1), while having more limited effects on the induction of BZR or PTR *O*-dealkylation activity.

When we examined the induction of these CYPs employing RNA analysis (Fig. 2), we observed similar results. Specifically, strong induction of CYP2B1 by DAS and PB was observed, along with limited induction of the CYP1A subfamily. In addition, DAS and PB strongly induced the levels of the CYP3A gene expression.

We next examined for the induction of the GSTs, employing hybridization techniques. Phenobarbital and DAS both highly induced the expression of the genes coding for the GST α (Fig. 2) and GST μ families (data not shown). Interestingly, HCB also induced these phase II drug-metabolizing genes. The effects of PB and DAS on the induction of epoxide hydrolase were examined next. The present studies demonstrated that both PB and DAS induce this specific gene. Interestingly, HCB treatment similarly induced this gene.

The final non-CYP enzyme we investigated was quinone oxidoreductase (DT-diaphorase), which was determined by a catalytic assay (Table 1). This enzyme was induced markedly (3.7-fold) by DAS, but was not induced by PB or DDT. In addition, the relatively high dose of HCB employed induced this catalytic activity almost 6-fold.

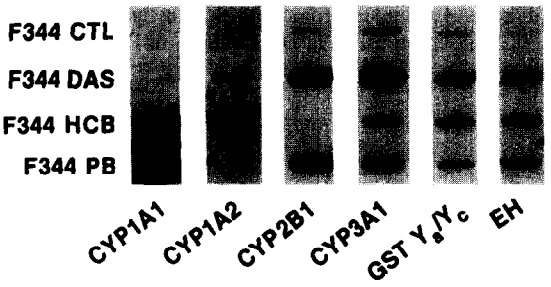

Fig. 2. Levels of the RNAs coding for various drug-metabolizing enzymes in rats exposed to DAS, PB or HCB. Individual slots contained 10 μg of total hepatic RNA and were hybridized to the appropriate oligonucleotide (CYP1A1, 1A2, 2B1 or 3A1) or plasmids GST Y_a/Y_c (GST subfamily α) or EH (epoxide hydrolase) as described in Materials and Methods. Treatments were as follows: PB, 500 ppm in the diet for 7 days; HCB, (8 ppm in the diet for 5 days); DAS (500 mg/kg body wt, i.g., for 4 days; killed on day 5); and control (corn oil, i.g.).

Finally, we determined the ability of DAS and PB to alter the levels of a specific nuclear binding protein that is increased in the nuclei of animals treated with PB. This binding protein is defined by its ability to bind to the specific oligonucleotide that we synthesized. This oligonucleotide includes the sequence for the BARBIE site, which appears to be involved in PB-mediated transcriptional activation of certain CYP proteins in both bacteria and mammals [13–16]. Extracts from DAS-, DDT- and PB-treated rats revealed a lower molecular weight nuclear binding protein that was not observed in control rats. Although extracts from rats exposed to all three inducers exhibited this low molecular weight binding protein, levels of this protein were apparently highest in the PB-treated rats. In contrast, there is a second binding protein (of higher molecular weight) that was readily

Table 1. Induction of alkoxyresorufin *O*-dealkylation activities and quinone oxidoreductase by various xenobiotics

Treatment*	*O*-Dealkylase activity†				Quinone oxidoreductase‡
	MTR	ETR	PTR	BZR	
Control	16 ± 6	32 ± 2	18 ± 2	30 ± 7	1.9 ± 0.30
DAS (low dose)	68 ± 9§	116 ± 17§	360 ± 23§	735 ± 46§	ND‖
DAS (high dose)	92 ± 8§	152 ± 11§	472 ± 34§	912 ± 64§	5.3 ± 0.64§
PB	59 ± 6§	125 ± 10§	507 ± 42§	1042 ± 127§	2.4 ± 0.43§
DDT	52 ± 6§	97 ± 7§	453 ± 27§	884 ± 51§	2.2 ± 0.35§
HCB	550 ± 43§	2430 ± 143§	84 ± 9§	137 ± 13§	10.6 ± 1.1§

* Animal treatment: control rats were administered corn oil, i.g., for 4 days. DAS was administered i.g. at 200 mg/kg body wt/day (low dose) or 500 mg/kg body wt/day (high dose) for 4 days. PB was administered in the diet (500 ppm) for 7 days. DDT was administered in the diet (428 ppm) for 5 days. HCB was administered in the diet (8 ppm) for 5 days.

† Specificity of alkoxyresorufin substrates: MTR, methoxyresorufin (CYP1A2); ETR, ethoxyresorufin (CYP1A1); PTR, pentoxyresorufin (CYP2B); BZR, benzyloxyresorufin (CYP2B). Activities are in pmol resorufin produced/min/mg S9 protein.

‡ Quinone oxidoreductase activity was determined with the reduction of resorufin [19]. Activity is expressed as nmol resorufin reduced/min/mg S9 protein at 28°.

§ Significantly different from controls, $P < 0.05$ (Student's *t*-test).

‖ ND, Not determined.

observed in extracts from control animals but that was apparently diminished in DAS-, DDT- or PB-treated animals (Fig. 3).

DISCUSSION

In the present studies, we determined the levels of a variety of drug-metabolizing enzymes in the livers of male F344/NCr rats administered DAS by gavage. We initially attempted to administer DAS in the diet; however, the rats were adverse to consuming the feed. In addition, the compound is volatile and apparently not stable in the diet (data not shown). We therefore administered this compound via gavage. Perhaps the most striking aspect of the response to DAS is the fact that this compound, despite minimal structural similarity to PB, is a profound inducer of the CYP2B family. However, prior studies both by Yang and coworkers [10, 11] and Lubet *et al.* [12] had shown that DAS markedly induces the CYP2B family. Recent work by Pan *et al.* [25] implies that it is the sulfone derivative of DAS rather than DAS itself which is the primary inducer of CYP2B. However, this intermediate, like DAS itself, is structurally quite distinct from the prototype CYP2B inducers PB and DDT (Fig. 1). Interestingly, when DAS was compared with PB for the induction of various other drug-metabolizing genes, a similar pattern emerged. Specifically, both DAS and PB induced the GST α subfamily, epoxide hydrolase, and CYP3A1 (Fig. 2) and simultaneously induced the GST μ subfamily (data not shown). In contrast, neither compound markedly induced CYP1A1 or CYP1A2 or the class III (TCDD-inducible) aldehyde dehydrogenase (data not shown), a set of genes reproducibly induced by compounds that interact with the *Ah* receptor. In fact, HCB induces all three of these genes to a high degree [26]. The only striking difference between the two compounds is the fact that DAS induced quinone oxidoreductase, whereas PB failed to do so. Thus, DAS appears to be a member of the PB-type inducers, which include such structurally diverse chemicals as PB, DDT, α-hexachlorocyclohexane and 2,2′,4,4′,5,5′-hexachlorobiphenyl [12]. Prior studies by this laboratory have shown that all of these chemicals appear to induce the same combination of genes [12] in the rat. The induction of quinone oxidoreductase, however, seems to set DAS apart from the typical PB-type inducers, since the prototype inducers such as PB and DDT (Table 1) failed to induce this enzyme. These results imply that DAS or its metabolites may function both as typical PB-type inducers as well as inducers via the electrophilic mechanisms described by Tallalay and coworkers [3, 4]. Direct confirmation of the ability of DAS to functionally interact with the ARE element might most readily be accomplished in cell culture with cell lines such as those described by Tallalay *et al.* [4] that contain the ARE response element.

In screening for potential chemopreventive agents, the tendency has been to avoid those chemicals that induce the "phase I" (activating) activities, most of which are mediated by various isozymes of the cytochromes P450. The tendency to avoid these phase I inducers is due primarily to the finding that certain of the CYP enzymes are involved in the activation of a variety of procarcinogens. However, the preponderance of this specific activation (e.g. polycyclic hydrocarbons [2] and aromatic amines [27, 28]) has been associated with induction of the CYP1A family by TCDD-type inducers. In fact, PB and lindane, two strong CYP2B inducers, have proven to be effective chemopreventive agents during the initiation phase in inhibiting aflatoxin B_1 or acetylaminofluorene-induced liver tumorigenesis in rats [29, 30]. Interestingly, recent data have shown that limonene, indole-3-carbinol, and phenethyl isothiocyanate, three other compounds with chemopreventive activity, induce CYP2B in rat liver [31–33]. Potentially most intriguing is epidemiologic evidence that PB treatment decreases the incidence of cigarette smoke-induced bladder cancer in epileptics [34]. This result is presumably due to altered metabolism, rapid conjugation of the presumed carcinogenic metabolites of 4-aminobiphenyl, a bladder carcinogen that is found in cigarette smoke [34].

Given the limited structural similarities between PB and DAS or its sulfone metabolite, it is difficult to imagine how these compounds might act similarly to induce a variety of genes. This potential problem of coordinate induction of various genes is further compounded by the fact that a specific receptor has not been isolated even for the prototype inducer PB. Recent data of He and Fulco have shown that there is a consensus DNA sequence [14] found in the 5′-flanking regions of barbiturate-inducible CYP genes in both bacteria and rats (ANCNAAAGCNGGNGG). This site has been designated the BARBIE site. Interestingly, various genes coding for PB-inducible activities, including most of the genes that we examined in the present studies (e.g. CYP2B1, CYP3A2, epoxide hydrolase and the GST α family) all possess homologs of this same consensus sequence 100–200 bp 5′ from the start site [see Ref. 12]. This DNA sequence can be bound by a PB-mediated nuclear binding protein that can apparently serve as a transcriptional activator for CYP2B [13, 16]. Fournier *et al.* [35] have observed recently that a modified BARBIE site (5′-GCCCAAAGCTGGCTT-3′) found approximately 130 bp upstream from the start site of the PB-inducible gene, α_1-acid glycoprotein, readily binds a PB-inducible nuclear protein, presumably the same nuclear protein that we have observed. In the present experiments, we employed an oligonucleotide described initially by Upadhya *et al.* [13], which includes not only the BARBIE site but an additional 15 nucleotides 5′ to the BARBIE site [16]. As can be seen in Fig. 3, treatment of rats with PB, DDT or a high dose of DAS increased the levels of a nuclear protein that can bind to this consensus sequence. In contrast, there was a second higher molecular weight nuclear protein that bound particularly in control nuclear extracts but was also seen at significant levels in nuclear extracts derived from the DAS-treated rats. This second protein was expressed in control rats, and its level of binding was decreased in nuclear extracts of rats exposed to DAS and DDT, and most strikingly with PB. The binding of the control protein was apparently competed off by the PB-inducible protein. The reason we feel that this reflects competition off rather than a complex of the two is that one would expect a complex to run at a higher molecular weight than either of the two proteins alone. If indeed a constitutive protein is competed off by a barbiturate-inducible protein, this would be strikingly similar to the derepression mechanisms involved in transcriptional activation of the CYP proteins by barbiturates in *B. megaterium* [15]. The presence of such a DNA binding protein may ultimately help to explain the ability of these structurally diverse compounds

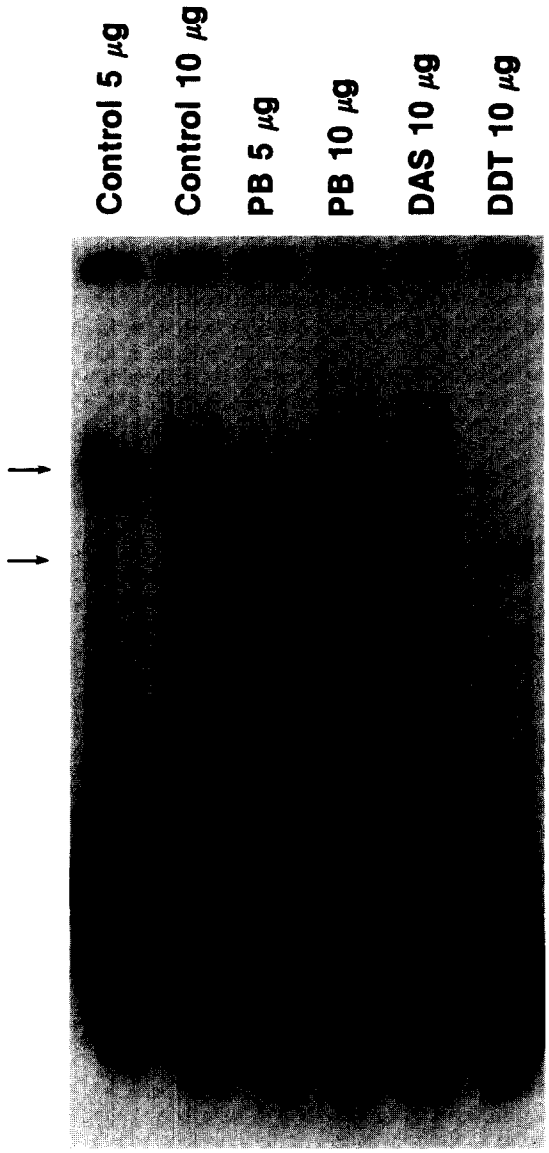

Fig. 3. Induction of a DNA-binding protein by PB, diallylsulfide DAS, and DDT. Gel mobility shift analysis with a 30-mer double-stranded oligonucleotide 5′ GAGGAGTGAATAGCCAAAGCAGGAGGCGTG-3′ [13]. Lane 1, control liver extract 5 μg; Lane 2, control liver extract 10 μg; Lane 3, PB nuclear extract 5 μg; Lane 4, PB nuclear extract 10 μg; Lane 5, high-dose DAS extract 10 μg; Lane 6, DDT nuclear extract 10 μg. The top arrow indicates a nuclear binding protein(s) that was observed most strongly in the control extract. The lower arrow indicates the appearance of a new nuclear binding protein band following treatment with PB, DAS, or DDT.

(barbiturates, DDT, α-hexachlorocyclohexane and DAS) to rapidly induce a wide variety of genes [12, 36], as well as their ability to induce a wide variety of other biological phenomena (e.g. liver hypertrophy, hepatocytomegaly and liver tumor promotion in carcinogen-initiated rats [37–40]). Interestingly, recent work in the rat by Takahashi *et al.* [41] has shown that DAS, similar to most of the strong inducers of CYP2B, including PB and 5-ethyl-5-phenylhydantoin, can promote both liver and thyroid tumorigenesis in carcinogen-initiated rats.

Although we have briefly discussed the induction of this nucleotide-specific binding factor and its potential role in transcriptional activation by PB-type inducers, this specific area is somewhat controversial. Thus, recent work by Omiecinski and coworkers [42] employing a rat CYP2B2 construct placed in transgenic mice has shown that although the 5′ region including the known BARBIE sequence is apparently necessary for PB-mediated induction, it is not sufficient for this induction to occur.

The present studies would appear to be compatible with a role for induction of various drug-metabolizing enzymes (phase I and II) as a significant contributor to the chemopreventive activities of DAS in a variety of tumor models. Interestingly, DAS has shown activity against both nitrosamines as well as polycyclic hydrocarbons [6–9], two classes of carcinogens that are preferentially metabolized by different forms of cytochrome P450 [2, 27, 28]. However, direct effects of increased detoxification of proximate carcinogens or altered paths of metabolism by the CYP proteins may be only a portion of the chemopreventive activity of DAS. Thus, DAS or certain of its metabolites have been shown by Yang and coworkers [43] to directly inhibit the activities of certain CYP proteins, both *in vivo* and *in vitro*. Therefore, the total chemopreventive activity of this compound is likely to reflect a variety of mechanisms.

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