Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6258–6262, August 1990
Biochemistry

Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element

(planar aromatic compounds/gene regulation/DNA sequence/DNA-binding proteins)

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Communicated by Paul Talalay, May 30, 1990 (received for review April 26, 1990)

ABSTRACT **Glutathione S-transferase (GST) Ya subunit gene expression is induced in mammalian tissues by two types of chemical agents: (*i*) planar aromatic compounds (e.g., 3-methylcholanthrene, β-naphthoflavone, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and (*ii*) electrophiles (e.g., *trans*-4-phenyl-3-buten-2-one and dimethyl fumarate) or compounds easily oxidized to electrophiles (e.g., *tert*-butylhydroquinone). To study the mechanism of this induction, we have introduced deletions in the 5′ flanking region of a mouse GST Ya subunit gene, fused it to the coding sequence for chloramphenicol acetyltransferase (CAT) activity, and transfected the Ya-CAT genes for expression into hepatoma cells. We show that a single cis-regulatory element, between nucleotides −754 and −713 from the start of transcription, is responsible for the induction by both planar aromatic and electrophilic compounds. Using murine hepatoma cell mutants defective in either the *Ah*-encoded aryl hydrocarbon receptor (BP^{r}c1 mutant) or in cytochrome P_1-450 gene (c1 mutant), we show that induction by planar aromatic but not by electrophilic inducers requires a functional Ah receptor and cytochrome P_1-450 activity. From this it is concluded that Ya gene activation by planar aromatic compounds involves metabolism of these inducers by the phase I xenobiotic-metabolizing cytochrome P_1-450 system into electrophilic compounds, which is consistent with a recently proposed model [Prochaska, H. J. & Talalay, P. (1988) *Cancer Res.* 48, 4776–4782]. Therefore, the regulatory sequence of the Ya gene should be considered an electrophile-responsive element (EpRE) activated exclusively by inducers containing an electrophilic center. An EpRE-containing 41-bp oligonucleotide ligated at the −187 site of the Ya gene promoter confers upon it an increase in basal activity and xenobiotic inducibility. The basal activity augments with the number of EpRE copies. DNase I protection patterns show the protection of the EpRE domain by a nuclear factor(s) that becomes more abundant upon exposure of Hepa 1c1c7 cells to *tert*-butylhydroquinone.**

The glutathione S-transferases (GST) are a family of enzymes that catalyze the conjugation of electrophilic compounds with glutathione (for a review, see ref. 1). They belong, together with UDP-glucuronosyltransferases and NAD(P)H:quinone reductase, to a group of xenobiotic metabolizing phase II enzymes (2) that function as intracellular detoxication systems of mutagens, carcinogens, and other toxic compounds. The induction of phase II enzymes is considered an important mechanism of protection against chemical stress and carcinogenesis (3–5). The variety of chemical compounds that induce these enzymes are of two types: (*i*) planar aromatic compounds (polycyclic, azo, halogenated), which activate also phase I enzymes such as cytochrome P-450 (cytochrome P_1-450)* and hence are termed bifunctional inducers (3); and (*ii*) selective inducers for phase II enzymes, monofunctional inducers (3), including a variety of compounds such as diphenols, isothiocyanates, thiocarbamates, unsaturated dicarboxylic acid esters, etc., which contain or acquire by metabolism electrophilic centers. Many monofunctional inducers contain olefinic or acetylenic bonds rendered electrophilic by conjugation with electron-withdrawing substituents and, in fact, are Michael reaction acceptors (6).

To elucidate the mechanisms of regulation of GST Ya subunit gene expression by the two types of inducers, we have previously isolated a mouse GST Ya gene (7, 8) and have shown the presence of xenobiotic responsive elements in the 5′ flanking region (9). In the present study, we demonstrate that this region contains between nucleotides −754 and −713 an inducible element that activates Ya gene transcription in cis in response to a variety of inducers such as 3-methylcholanthrene, β-naphthoflavone, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), *tert*-butylhydroquinone, and *trans*-4-phenyl-3-buten-2-one. These results raise the question of the mechanisms proposed for the regulation of GST gene expression by planar aromatic and electrophilic inducers (4, 5). Our results show that, in order to function as Ya gene inducers, the planar aromatics have to be metabolized by the cytochrome P_1-450 system into electrophilic compounds. In this paper we bring evidence that the inducible expression of GST Ya subunit gene is controlled by a single electrophile-responsive element (EpRE), which is activated exclusively by an electrophilic signal. Protein–DNA binding experiments indicate the presence of an EpRE binding factor(s) that is abundant in nuclei of Hepa 1c1c7 cells after treatment with *tert*-butylhydroquinone.

MATERIALS AND METHODS

Cell Lines and Extracts. Mouse hepatoma cell line Hepa 1c1c7 and the aromatic hydrocarbon induction-defective BP^{r}c1 variant were provided by J. Whitlock, Jr. (Stanford University), and the c1 variant, defective in the production of an active cytochrome P_1-450, was obtained from O. Hankinson (University of California, Los Angeles). The human hepatoma line Hep G2 was provided by Y. Shaul (Weizmann Institute). The cell lines were propagated as described (9). Nuclear and cytosolic extracts were prepared from the cell lines as described by Dignam *et al.* (10), and DNase I protection patterns (footprint assays) have been described (9).

Transfections. The plasmid constructions containing fragments of the 5′ flanking region of the GST Ya subunit gene

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Abbreviations: GST, glutathione S-transferase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CAT, chloramphenicol acetyltransferase; EpRE, electrophile-responsive element.

*Phase I xenobiotic metabolizing enzymes (e.g., cytochrome P_1-450) introduce by oxidation or reduction functional groups into chemical compounds.

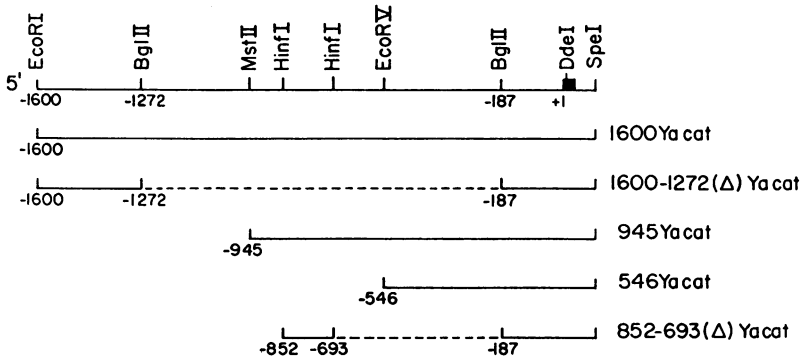

FIG. 1. The 5′ flanking region of the mouse GST Ya subunit gene and the deletions used to construct the fused Ya-CAT genes.

fused to the chloramphenicol acetyltransferase (CAT) gene sequence, transfections, and measurements of CAT activities were carried out as described (9).

Probes and Competitor DNA. The 159-base-pair (bp) fragment (−852 to −693) of the GST Ya subunit gene 5′ flanking region, isolated by *Hin*fI digestion and subcloned into the *Sma* I site of pGEM-1 (Promega) plasmid was alternatively labeled at the 3′ end of the coding or the noncoding strand by [α-^{32}P]dATP and Klenow DNA polymerase and was used as a probe for electrophoretic mobility-shift and DNase footprint assays. A synthetic 41-bp oligonucleotide containing the sequences between −754 to −713 of the Ya gene 5′ flanking region,

5′-*GATC*TAGCTTGGAAATGACATTGCTAATGGTGACAAAGCAACTTT

ATCGAACCTTTACTGTAACGATTACCACTGTTTCGTTGAAA*CTAG*-5′,

was used as a specific competitor for protein–DNA interactions along the 159-bp DNA fragment and ligated into the −187 site of the Ya gene promoter (italicized residues indicate linker regions).

RESULTS

Functional Analysis of Xenobiotic-Inducible Elements in the GST Ya Subunit Gene. The 5′ flanking region of a mouse GST Ya subunit gene was shown to contain elements responsive to induction by planar aromatics such as 3-methylcholanthrene and β-naphthoflavone between nucleotides −1600 and −187 from the start of transcription (9). To characterize the regulatory sequences, we have deleted DNA fragments in this region and made the Ya-CAT fusion gene constructs described in Fig. 1. The recombinant plasmids were transfected for expression into Hep G2 cells. The inducibility of Ya-CAT gene expression was tested by treatment of the transfected Hep G2 cells for 24 hr with planar aromatic or electrophilic inducers and assays of CAT activity in the cell extracts. The relative CAT activities presented in Fig. 2*A*

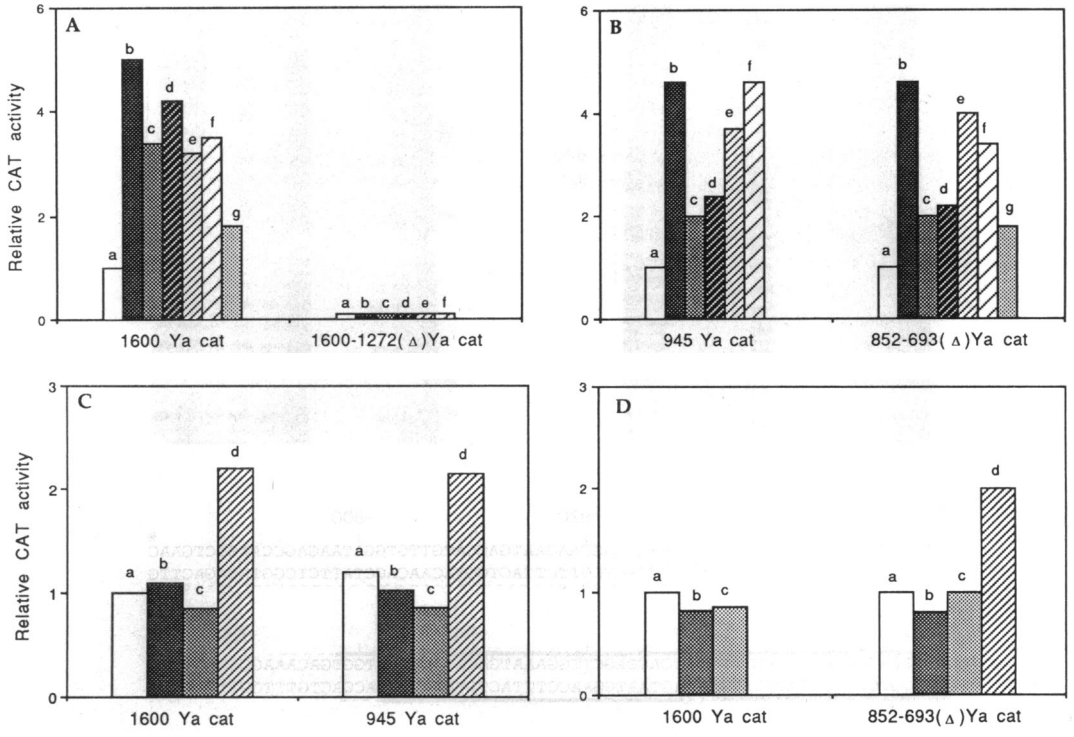

FIG. 2. Histograms of relative CAT activities expressed from the Ya-CAT gene constructs transfected into hepatoma cells. (*A* and *B*) Hep G2 cells. After transfection the cells were untreated (a) or were exposed for 24 hr to the following inducers: 50 μM β-naphthoflavone (b), 50 μM 3-methylcholanthrene (c), 30 μM *trans*-4-phenyl-3-buten-2-one (d), 30 μM *tert*-butylhydroquinone (e), 30 μM dimethyl fumarate (f), or 5 nM TCDD (g). (*C*) BP^{r}c1 cells defective in Ah receptor translocation were untreated (a) or were induced for 24 hr by β-naphthoflavone (b), 3-methylcholanthrene (c), or *tert*-butylhydroquinone (d). (*D*) c1 cells defective in the cytochrome P_1-450 gene were untreated (a) or were induced for 24 hr by 3-methylcholanthrene (b), TCDD (c), or *tert*-butylhydroquinone (d).

show that removal of the region between −1272 and −187 causes a complete loss of basal as well as inducible activity of the Ya gene promoter. A similar loss of activities is produced by deletion to −546 (data not shown). Further deletions in the region between −945 and −546 locate the inducible element in the 159-bp DNA fragment between −852 and −693 (Fig. 2*B*). It should be observed that this Ya gene inducible element is responsive to both types of inducers: planar aromatics (β-naphthoflavone, 3-methylcholanthrene, and TCDD) and electrophiles or compounds easily converted to electrophiles (*trans*-4-phenyl-3-buten-2-one, dimethyl fumarate, and *tert*-butylhydroquinone).

The Ya-CAT gene constructs were transfected for expression also into two mouse hepatoma Hepa 1c1c7 cell variants: BP^{r}c1 defective in Ah (aryl hydrocarbon) receptor translocation into the nucleus (11, 12) and c1, which, because of a mutation in the structural gene for cytochrome P_1-450, produces a truncated, inactive enzyme (13). Fig. 2 *C* and *D* show that the Ya-CAT gene constructs, transfected into BP^{r}c1 and c1 cells, respectively, express only their basal CAT activity and are not induced by planar aromatics such as β-naphthoflavone, 3-methylcholanthrene, or TCDD. On the other hand *tert*-butylhydroquinone functions as an inducer in these cells to increase by 2- to 2.5-fold the CAT activity.

Interaction of the Inducer-Responsive Region of GST Ya Subunit Gene with Nuclear Extracts. The 159-bp (−852 to −693) region of the GST Ya subunit gene, which appears to contain elements responsive to planar aromatic and electrophilic inducers, was analyzed by interaction with nuclear extracts in footprinting assays for the binding of proteins capable of protecting specific sequences from digestion by DNase I. In these experiments we have compared the binding activities of nuclear extracts from Hepa 1c1c7 cells untreated or incubated for 24 hr with 10 μM 3-methylcholanthrene or with 35 μM *tert*-butylhydroquinone. Fig. 3 *A* and *B* show that the nuclear extract of *tert*-butylhydroquinone-treated cells is abundant in the DNA-binding protein(s) that protects sequences between −820 and −720 on both coding and noncoding strands of the 159-bp DNA fragment from DNase I digestion. DNase I-hypersensitive sites are observed at nucleotides −760 and −759 on the noncoding strand and at nucleotide −782 of the coding strand. A summary of the

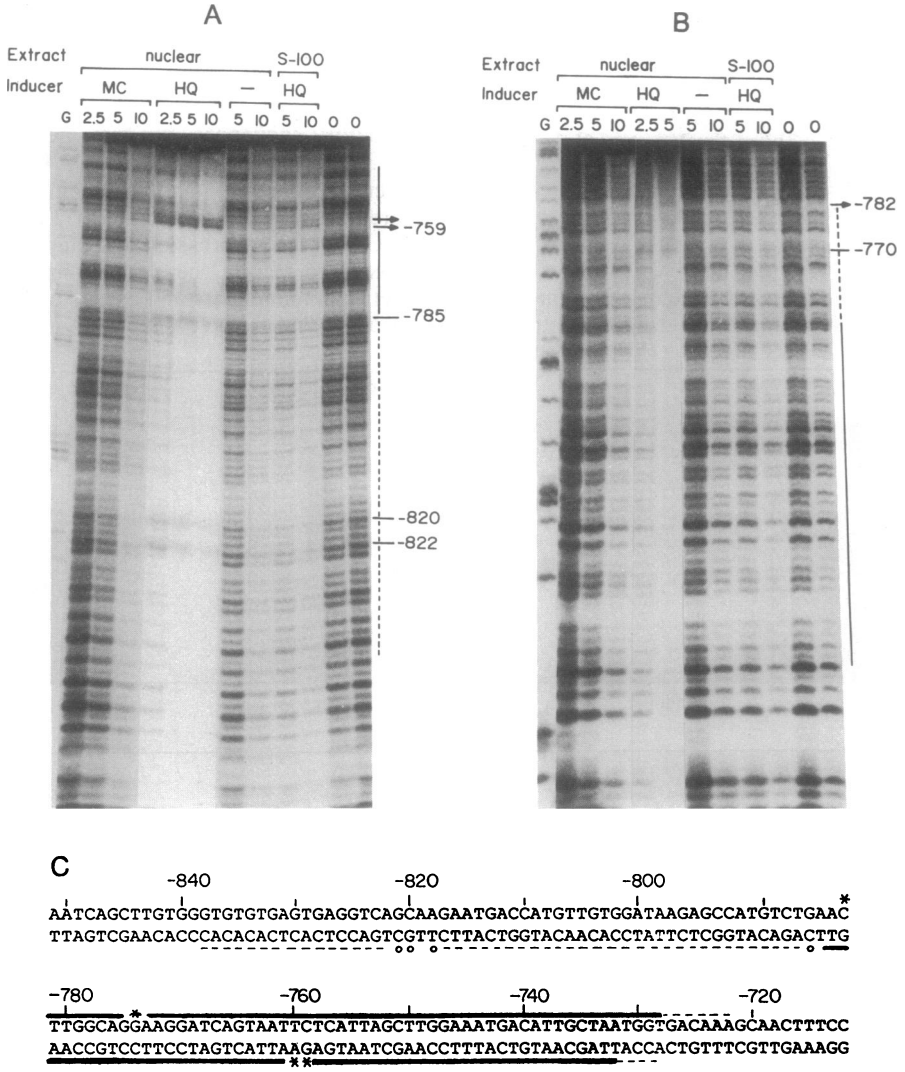

FIG. 3. Footprint analysis of −852 to −693 sequences of the GST Ya subunit gene 5′ flanking region. The 159-bp DNA fragment, 3′-end-labeled at the noncoding strand (*A*) or the coding strand (*B*), was assayed for protection against DNase I digestion by incubation with nuclear or S-100 extracts from Hepa 1c1c7 cells untreated (−) or exposed for 24 hr to 10 μM 3-methylcholanthrene (MC) or 35 μM *tert*-butylhydroquinone (HQ). The amounts of protein extract are indicated in micrograms at the tops of lanes. Lanes 0 indicate free DNA or no protein addition. Lane G indicates chemical cleavages at purine guanosine residues. (*C*) Summary of the footprinting results. Sequences protected from DNase I cleavage are marked by lines alongside the respective strand. Asterisks indicate positions of enhanced DNase I cleavage, and small circles indicate DNase-sensitive nucleotides in a protected domain.

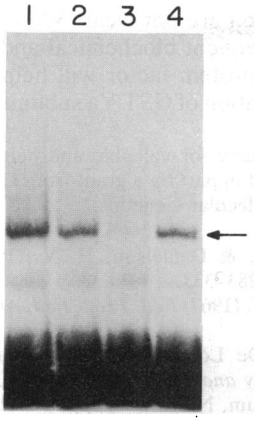

FIG. 4. Electrophoretic mobility shift of the 159-bp (−852 to −693) DNA fragment by interaction with nuclear extract from 35 μM *tert*-butylhydroquinone-treated Hepa 1c1c7 cells. Reaction mixtures (20 μl) containing 5 μg of nuclear extract, 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 10% glycerol, and 4 μg of poly(dI-dC) were incubated for 15 min at 20°C with the ^{32}P-labeled 159-bp probe (2×10^4 cpm; 1–2 ng) in the absence (lane 1) or presence of 10 or 100 ng of the 41-bp oligonucleotide specific competitor (lanes 2 and 3, respectively) or 500 ng of the nonspecific competitor 200-bp DNA fragment (lane 4) and electrophoresed in a 4% acrylamide gel. An arrow indicates the retarded protein–DNA complex.

footprinting results is shown in Fig. 3*C*. The DNA-binding protein(s) that recognizes sequences in the −820 to −720 region of the Ya gene seems to be abundant only in the nuclear extract and not in the S-100 fraction of *tert*-butylhydroquinone-treated Hepa 1c1c7 cells. However, this protein also is observed in amounts smaller by a factor of about 10 in nuclear extracts from uninduced or 3-methylcholanthrene-treated cells (Fig. 3). Electrophoretic mobility-shift assays using nuclear extracts from *tert*-butylhydroquinone-treated Hepa 1c1c7 cells and the ^{32}P-labeled 159-bp DNA fragment revealed the formation of a protein–DNA complex that could be competitively blocked by a 41-bp synthetic oligonucleotide containing the sequences −754 to −713 and not by an unrelated DNA fragment (Fig. 4).

Activation of the GST Ya Subunit Gene Promoter by the Inducer-Responsive Element. Assuming that the 41-bp sequence contains an element responsible for the xenobiotic inducible expression of the GST Ya subunit gene, which is specifically recognized by a nuclear trans-acting factor, we have ligated this oligonucleotide upstream of the −187 site of the Ya gene promoter fused to the CAT coding sequence. Constructs containing one or four tandem copies of the 41 bp in a 3′-to-5′ and 5′-to-3′ orientation, respectively, were transfected into Hep G2 cells and tested for basal and inducible CAT activity. Fig. 5 shows that sequences present in the 41-bp (−754 to −713) oligonucleotide, independent of orientation, confer inducibility on the Ya gene promoter by 3-methylcholanthrene, β-naphthoflavone, TCDD, *trans*-4-phenyl-3-buten-2-one, and *tert*-butylhydroquinone. A quantitative comparison between Ya-CAT gene constructs containing a single copy of the 41-bp oligonucleotide [852–693(Δ) Ya-CAT and 754–713 Ya-CAT] or four 41-bp copies [$(754–713)_4$ Ya-CAT] indicates that multiple copies of this sequence cause about a 20-fold increase in the basal activity of the Ya gene promoter (Fig. 5).

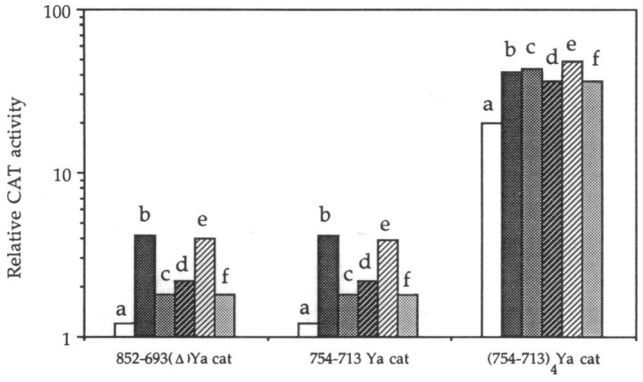

FIG. 5. Histogram of relative CAT activities expressed in Hep G2 cells from constructs 852–693(Δ) Ya-CAT and 754-713 Ya-CAT or from construct $(754–713)_4$ Ya-CAT containing respectively only one copy or four copies of the 41-bp oligonucleotide. The cells were untreated (a) or were exposed for 24 hr to 50 μM β-naphthoflavone (b), 50 μM 3-methylcholanthrene (c), 30 μM *trans*-4-phenyl-3-buten-2-one (d), 30 μM *tert*-butylhydroquinone (e), or 5 nM TCDD (f). CAT activities are presented on a logarithmic scale.

DISCUSSION

This study shows that the 5′ flanking region of the mouse GST Ya subunit gene contains a single positive regulatory element responsible for the inducible expression of this gene by xenobiotic compounds. The regulatory element, located between nucleotides −754 and −713 upstream of the transcription start site, was found to be responsive to both planar aromatic inducers (β-naphthoflavone, 3-methylcholanthrene, and TCDD) and to electrophilic compounds (*tert*-butylhydroquinone, dimethyl fumarate, and *trans*-4-phenyl-3-buten-2-one) (Figs. 2 and 5). In view of these surprising findings, we have reconsidered the different mechanisms proposed for GST gene activation by the two classes of chemical inducers (4, 5). Transfection experiments of Ya-CAT gene constructs into mouse hepatoma Hepa 1c1c7 cell mutants—BP^rc1, defective in nuclear translocation of a liganded Ah receptor, and c1, defective in the production of an active cytochrome P_1-450—show that in these cells the planar aromatic compounds do not induce CAT activity, while the electrophilic compounds are effective inducers (Fig. 2 *C* and *D*). It may be concluded that Ah receptor and cytochrome P_1-450 activity are involved in the induction of Ya gene expression by planar aromatics but not by the electrophilic compounds. The absence of a xenobiotic-responsive-element (XRE) sequence from the 41-bp Ya gene regulatory element argues against a mechanism of induction involving direct binding of an Ah receptor–aromatic compound complex similar to the transcriptional activation of cytochrome P_1-450 genes (14–16). The lack of inducibility of Ya-CAT genes by planar aromatics in the c1 mutant cells, which were shown to contain intact Ah receptors (12), also excludes a role for the liganded Ah receptor in the control of Ya gene expression either by direct interaction or indirectly via activation of transcription of a putative regulatory gene producing a trans-acting factor (17). These observations, together with the finding of a common responsive element in the Ya gene for both planar aromatic and electrophilic inducers, lead us to assume that the activation of Ya gene expression actually involves only an electrophilic chemical signal. The presence of an electrophilic (e.g., Michael reaction acceptor) center is a characteristic of monofunctional inducers of phase II enzymes (6). The planar aromatic compounds probably have to be metabolized by the cytochrome P_1-450 system to acquire the electrophilic properties responsible for Ya gene induction. This metabolism would require functional Ah receptors and the activation of cytochrome P_1-450 gene expression. Therefore, our results support the metabolic cascade model proposed by Prochaska and Talalay (5) for phase II enzyme induction by planar aromatic compounds. The case of TCDD, which although considered a slowly metabolizable compound (18) neverthe-

less induces CAT activity via the same Ya gene regulatory element as the other planar aromatics, is puzzling (Fig. 5). In view of the present findings, the −754 to −713 regulatory sequence of the Ya gene appears to contain an EpRE. This element confers an increase in basal activity as well as xenobiotic inducibility on the Ya gene promoter. The basal activity increases with the number of the EpRE copies (Fig. 5). Although a consensus sequence for EpRE has not yet been determined, we observe a DNA motif containing the inverse repeat TGGAAAT(GACATTGC)TAATGGT.

DNase I footprinting experiments indicate extensive regions of protection by Hepa 1c1c7 cell nuclear proteins on both DNA strands between nucleotides −820 and −720 (Fig. 3). The protected DNA domains include, in addition to the EpRE element, a half-recognition site for TGGCA-binding nuclear factor I (19) at nucleotides −780 to −776. While the pattern of DNase I protection suggests that the region from −820 to −720 of the GST Ya subunit gene binds a number of different proteins, the protein factor(s) recognizing the EpRE sequence may play a key role in the formation of these complex interactions. In support of this assumption, gel mobility shift assays show that the formation of a protein–DNA complex between crude nuclear extracts and the −852 to −693 fragment is specifically blocked by the EpRE-containing 41-bp oligonucleotide (Fig. 4). From DNase I protection assays, it is evident that the DNA-binding protein factor(s) is abundant only in the nuclear extracts of Hepa 1c1c7 cells exposed to *tert*-butylhydroquinone (or *trans*-4-phenyl-3-buten-2-one; data not shown) but not to 3-methylcholanthrene (Fig. 3). We also observe that nuclear extracts from 3-methylcholanthrene-treated cells do not produce a different pattern of DNase I protection, a finding that argues against the presence of specific aromatic planar compound-induced proteins. However, exposure of Hepa 1c1c7 cells to electrophilic inducers seems to cause a quantitative increase within the nuclear fraction (caused by stimulation of synthesis or modification) of DNA-binding protein(s) already present in the uninduced cells. From DNase I footprints (Fig. 3) and gel mobility-shift assays with the 41-bp oligonucleotide probe (data not shown), we observe that, in addition to its presence in the nuclear extracts, an EpRE-binding protein is present also in the cytosolic fraction. The molecular mechanisms by which the electrophilic inducers interact with protein factor(s) to recognize EpRE sequences and activate Ya gene expression are not clear. We expect that the purification and subsequent biochemical and molecular characterization of the protein factor will help to understand the electrophile regulation of GST Ya subunit gene transcription.

We thank P. Talalay for valuable and helpful discussions. This work was supported in part by a grant from Leo and Julia Forscheimer Center for Molecular Genetics.

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