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Glutathione *S*-transferase Ya subunit gene: Identification of regulatory elements required for basal level and inducible expression

(cis-acting regulatory element/β-naphthoflavone induction/DNA transfection/drug metabolism)

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ABSTRACT **The function of the 5′-flanking region of a rat glutathione *S*-transferase Ya subunit structural gene has been examined in homologous and heterologous cells. By using the 5′-flanking region of the Ya subunit gene fused to the structural gene encoding chloramphenicol acetyltransferase, we have identified two cis-acting regulatory elements in the upstream region of the Ya gene. One element is required for maximum basal level expression in homologous cells, whereas the second element is required for inducible expression of the Ya gene by planar aromatic compounds such as β-naphthoflavone. The cis-acting element required for inducible expression of the Ya gene by β-naphthoflavone is functional only in cells with normal dioxin receptors.**

The rat liver glutathione *S*-transferases (RX:glutathione R-transferase, EC 2.5.1.18) are a family of proteins that catalyze the conjugation of glutathione to various electrophilic ligands (1–8). In addition, they bind heme, bilirubin, steroids, and polycyclic aromatic hydrocarbons with high affinity (1–8). Nucleotide and amino acid sequence analysis of cDNA clones and purified proteins indicate a minimum of four glutathione *S*-transferase gene families in the rat (9–17). From Southern blot analysis of genomic DNA, we have estimated that five to seven genes constitute the glutathione *S*-transferase gene family encoding the basic subunits Ya and Yc* (18).

Work from our laboratory has demonstrated (21) that members of the Ya gene family are transcriptionally activated by phenobarbital and 3-methylcholanthrene. The transcriptional activation of the Ya subunit genes leads to an elevation in the steady-state level of the Ya mRNA in rat liver (10). We have isolated and characterized a rat glutathione *S*-transferase structural gene. The gene encodes a Ya subunit, spans ≈11 kilobases (kb), and is separated into seven exons by six introns (22).

In this study, we have begun to analyze the role of the 5′-flanking region of a glutathione *S*-transferase Ya subunit gene and have identified two cis-acting regulatory elements. One regulatory element confers responsiveness to β-naphthoflavone, a flavonoid that like 2,3,7,8-tetrachlorodibenzodioxin (TCDD) and 3-methylcholanthrene binds to the dioxin receptor (23). The second regulatory element is necessary for maximum basal level expression. By using variant mouse cell lines defective in the dioxin receptor we have demonstrated that the responsiveness of the regulatory element to β-naphthoflavone requires the presence of functional receptors.

MATERIALS AND METHODS

Cell Lines. The rat cell lines H5-6 (H4IIEC3) and 2M0 (FA0-H4IIEC3) and the mouse cell line Hepa 1c1c7 were obtained from Oliver Hankinson (University of California, Los Angeles). The mouse class I and class II variant cells were obtained from James P. Whitlock, Jr. (Stanford University, Palo Alto, CA). These cell lines were grown on minimal essential medium (α-MEM), L-glutamine, 10% (vol/vol) fetal bovine serum, penicillin at 10 units/ml, and streptomycin at 10 units/ml at 37°C in humidified 7% CO_2/93% air. In the induction studies the mouse hepatoma cell lines were treated with 15 μM β-naphthoflavone, whereas the rat cell lines were treated with 50 μM β-naphthoflavone. These concentrations were chosen from dose–response curves and gave maximum induction with maximum cell viability.

Human hepatoma cells, HepG2, were grown in Eagle's minimum essential medium, nonessential amino acids, sodium pyruvate, 10% (vol/vol) fetal bovine serum, penicillin at 10 units/ml, and streptomycin at 10 units/ml at 37°C in humidified 7% CO_2/93% air. In the induction studies the human hepatoma cell line was treated with 50 μM β-naphthoflavone.

RNA Isolations and Slot Blot Analysis. Total RNA was isolated from cells by the method of Chirgwin *et al.* (24). Various concentrations of RNA were applied to nitrocellulose filters with the aid of a slot blot apparatus (Schleicher & Schuell), the filter was baked at 80°C for 2 hr in a vacuum oven, and the RNA was hybridized to nick-translated DNA probes (25).

Construction of Glutathione *S*-Transferase Promoter-Chloramphenicol Acetyltransferase (CAT) Fusion Genes. *Construction of pGTB.7cat.* A genomic subclone, pGTB45-15-5.5, which has intron 1, exon 1, and the 5′-flanking region of the Ya gene, was digested with *Sma* I and *Pst* I. The 0.7-kb *Sma* I–*Pst* I fragment was blunt-ended with T4 DNA polymerase. *Hin*dIII linkers were added, and the fragment was ligated into the *Hin*dIII site of pSV0cat using T_4 ligase.

Construction of pGTB1.6cat. The genomic subclone pλGTB45-15-5.5 was digested with *Xma* I and *Nco* I. The 1.6-kb *Xma* I–*Nco* I fragment was isolated, the ends were filled in using the Klenow fragment of DNA polymerase I and blunt-end ligated into the *Hin*dIII site of pSV0cat. The *Hin*dIII site of pSV0cat had been filled in using the Klenow fragment of DNA polymerase I.

Construction of pGTB4.0cat. The genomic subclone pλGTB45-15-5.5 was digested with *Sma* I and *Eco*RI. The 4.0-kb *Sma* I–*Eco*RI fragment was isolated, the ends filled in with the Klenow fragment of DNA polymerase I, *Hin*dIII

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Abbreviations: CAT, chloramphenicol acetyltransferase; TCDD, 2,3,7,8-tetrachlorodibenzodioxin.

*A new nomenclature has been proposed for the rat liver glutathione *S*-transferases by Jakoby *et al.* (19). In this system the Ya subunit is subunit 1, and the Yc is subunit 2. We have retained the nomenclature originally proposed by Bass *et al.* (20).

linkers added, and the fragment was ligated into the *Hin*dIII site of pSV0cat.

Transfection Assays. Transfections were done by a modified calcium phosphate method (26). Cells were trypsinized and plated at a density of 1–2 × 10^5 cells per 25-cm^2 flask. The cells were allowed to recover overnight at 37°C, medium was changed, and 10 μg of calcium phosphate-precipitated DNA was added. The cells were incubated at 37°C for 4–5 hr. Following incubation, medium was removed, and the cells were washed with isotonic phosphate-buffered saline followed by a 2-min glycerol shock. The cells were then rinsed, allowed to recover overnight at 37°C, and treated for 24 hr with the appropriate xenobiotic.

Measurement of CAT Activity. Approximately 2 × 10^5 cells were washed with isotonic phosphate-buffered saline and rinsed with trypsin/EDTA. The cells were washed, resuspended in 100 μl of 0.25 M Tris·HCl (pH 7.8), and lysed by three cycles of freezing and thawing. The cell debris was removed by centrifugation, and the supernatant was assayed for protein (27) and for CAT activity (26). The assay contained in a final volume of 180 μl, 5–20 μl of cell extract, 20 μl of 4 mM acetyl coenzyme A, 0.5 μCi of [^{14}C]chloramphenicol (50–60 mCi/mmol; 1 Ci = 37 GBq), and 0.25 M Tris·HCl (pH 7.5). All assays were linear with regard to time of incubation and protein concentration. After a 60-min incubation at 37°C, the reaction mixture was extracted with ethyl acetate and analyzed by TLC in a chloroform/methanol, 95:5 (vol/vol), system and subsequent autoradiography. The acetylated products of the reaction (1-acetylchloramphenicol, 3-acetylchloramphenicol, and the 1,3-diacetylchloramphenicol) were scraped from the TLC plate and quantitated by liquid scintillation counting.

Construction of a Stable HepG2 Cell Line with pGTB1.6-CAT. The HepG2 cell line was cotransfected with the pGTB1.6CAT and pTK-neo plasmids at a DNA weight ratio of 10:1, respectively. Cells were grown on Eagle's minimum essential medium plus G418 at 400 μg/ml for 48 hr, on medium with G418 at 500 μg/ml for 7 days, and finally on medium with G418 at 1 mg/ml for 2 weeks. Viable cells were trypsinized and plated into multiwell dishes in the presence of G418 at 500 μg/ml. Cells exhibiting CAT activity were isolated and expanded.

RESULTS

Construction of Glutathione *S*-Transferase-CAT Fusion Genes and Transient Transfection into Rat Hepatoma Cells. To examine the function of the 5′-flanking region of the glutathione *S*-transferase Ya subunit structural gene, we isolated fragments from a genomic subclone corresponding to the 5′ end of the Ya structural gene (22) and ligated these fragments into the *Hin*dIII site of pSV0cat. All the fragments contain 23 base pairs of intron 1, all of exon 1 (43 base pairs), and various lengths of the 5′-flanking region of the Ya gene (Fig. 1). The pGTB.7cat construct contained nucleotides −1 to approximately −650, pGTB1.6cat contained nucleotides −1 to approximately −1550, and pGTB4.0cat contained nucleotides −1 to approximately −3550 relative to the transcription start site. The transferase-CAT fusion genes were used in all subsequent transfection studies along with pSV2cat as a control. The plasmid, pSV2cat, contains the CAT structural gene under the control of the simian virus 40 enhancer and promoter (26).

When pGTB.7cat was transfected into rat hepatoma cells, H5-6, CAT activity was significantly lower than observed with pSV2cat (Fig. 2). When the larger construct, pGTB1.6cat, was used in the transfection assay, the absolute CAT activity was routinely 7- to 10-fold higher than observed for the pGTB.7cat construct and was similar to that seen with pSV2cat (Fig. 2). Transfection of the longest construct into the rat hepatoma cell line resulted in CAT activity similar to the pGTB1.6cat construct (Fig. 2). In a typical transient transfection assay, if we assign the relative CAT activity directed by pSV2cat as 100, the relative CAT activities of pGTB.7cat, pGTB1.6cat, and pGTB4.0cat were 13, 96, and 100, respectively. When the rat H5-6 cell line was treated with 50 μM β-naphthoflavone, a 3-fold elevation in CAT activity was observed in transient assays with the pGTB1.6cat and pGTB4.0cat constructs; however, no elevation in CAT activity was seen with the pGTB.7cat construct or with the control plasmid pSV2cat (Fig. 2).

These data suggest that between nucleotides −650 and −1550 there are two regulatory elements: One that allows for maximum basal activity of the promoter and a second that is responsive to β-naphthoflavone. When total RNA was isolated from this cell line and hybridized to the rat Ya cDNA probe pGTB38 (10), a 3- to 4-fold elevation in Ya mRNA was observed 16–24 hr after induction (Fig. 3). Therefore, the extent of induction of the Ya mRNA correlates very well with the elevation in CAT activity seen after β-naphthoflavone administration. In the rat 2M0 cell line, which is not responsive to planar aromatic compounds, β-naphthoflavone administration had no effect on CAT activity (Fig. 4). However, like the H5-6 cell line, maximum promoter activity was observed with the pGTB1.6cat construct (Fig. 4). Phenobarbital (2 mM) and dexamethasone (1 μM) produced no elevation in CAT activity in the H5-6 cell line, whereas the polycyclic aromatic hydrocarbon 3-methylcholanthrene produced a 3-fold elevation in CAT activity (data not shown).

Expression of Glutathione *S*-Transferase-CAT Fusion Genes in Heterologous Cells. We have transfected the transferase promoter-CAT constructs into human HepG2 and mouse Hepa 1c1c7 hepatoma cells. By using transient assays, we have found that β-naphthoflavone induces CAT activity ≈4-fold in the mouse cell line (Fig. 5) and ≈7-fold in the human cell line (Fig. 6*A*). These data indicate that the β-naphthoflavone responsive element is functional in heterologous cells. Interestingly, in the mouse cell line the pGTB.7cat, pGTB1.6cat, and pGTB4.0cat constructs pro-

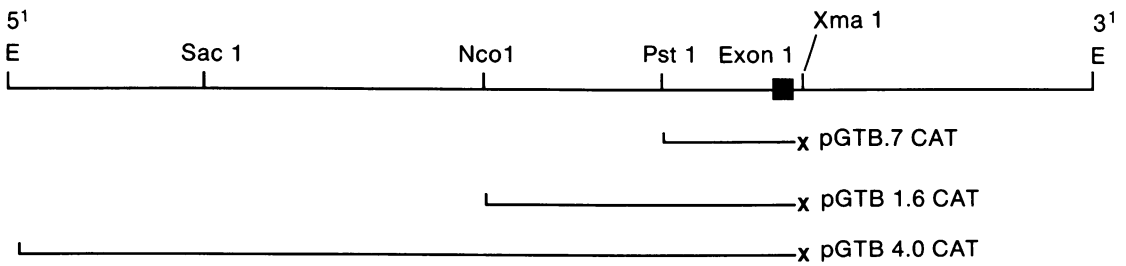

FIG. 1. Construction of 5′-flanking regions of the glutathione *S*-transferase Ya subunit gene to the structural gene encoding CAT. Restriction fragments containing various lengths of the 5′-flanking region of the Ya gene were ligated into the *Hin*dIII site of pSV0cat. The three constructs are indicated below the restriction map of the 5.5-kb subclone of pGTB45-15-5.5.

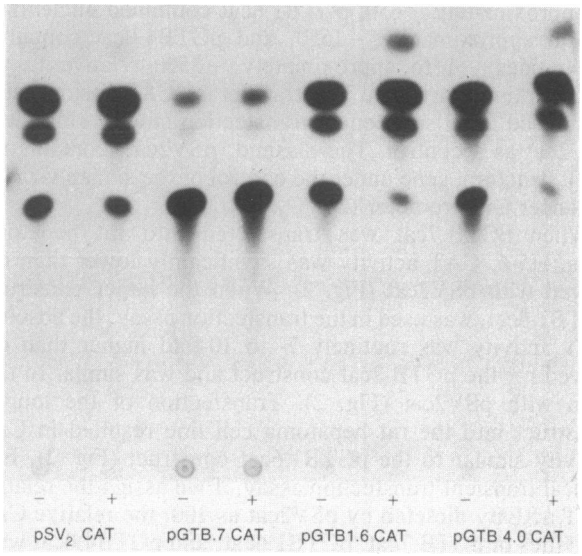

FIG. 2. CAT assay with lysed rat H5-6 cells transfected with glutathione *S*-transferase-CAT chimeric plasmids. Transient assays in the presence (+) and absence (−) of 50 μM β-naphthoflavone were performed in rat H5-6 cells by using pSV2cat, pGTB.7cat, pGTB1.6cat, and pGTB4.0cat. The extent of induction of CAT activity by β-naphthoflavone in cells transfected with pGTB1.6cat was 2.95 ± 1.0-fold. This value is the mean ± SD of three separate experiments. All assays were done in duplicate. Similarly, transient assays with the pGTB4.0cat construct gave similar levels of induction by β-naphthoflavone, ≈3-fold. The substrate of the reaction ([^{14}C]chloramphenicol) migrates closest to the origin on the TLC plate, whereas the acetylated products have a faster mobility in the solvent system.

duced equivalent CAT activity in the absence of β-naphthoflavone. However, the absolute CAT activity directed by all three CAT constructs was equivalent to the CAT activity directed from pGTB.7cat in rat and human cells. These findings are in contrast to the marked elevation in CAT activity seen with the pGTB1.6cat and pGTB4.0cat constructs in human and rat cells. Therefore, the basal activity of the transferase promoter is significantly less in mouse cells compared to rat or human cells and suggests the absence of a trans-acting protein(s) in mouse cells that can regulate basal level expression. Furthermore, these data suggest that the basal regulatory element and the β-naphthoflavone responsive element can function independently of each other.

We have also constructed a stable cell line by cotransfecting HepG2 cells with the pGTB1.6cat and pTK-neo plasmids. The pTK-neo plasmid contains the gene (*neo*) that confers resistance to neomycin-kanamycin antibiotics under control of the thymidine kinase promoter. The stable cell line

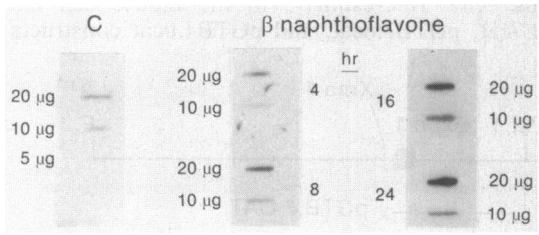

FIG. 3. Slot blot analysis of total RNA isolated from rat H5-6 cells treated with β-naphthoflavone. Various concentrations of total RNA were pipetted onto nitrocellulose filters and probed with a nick-translated probe of the Ya clone pGTB38 (10). The extent of induction at 16 hr was 3.2-fold, and at 24 hr it was 4.0-fold. These values were obtained from densitometric analysis of the x-ray film. C, untreated cells.

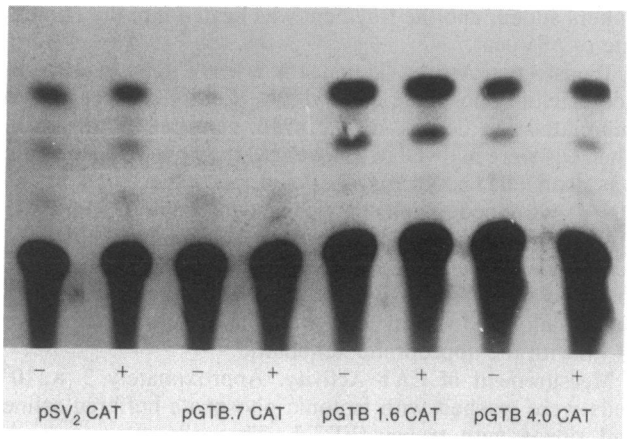

FIG. 4. CAT assay with lysed rat 2M0 cells transfected with glutathione *S*-transferase-CAT chimeric plasmids. Transient assays in the presence (+) and absence (−) of 50 μM β-naphthoflavone were performed in rat 2M0 cells by using pSV2cat, pGTB.7cat, pGTB1.6cat, and pGTB4.0cat. No elevation in CAT activity is seen with β-naphthoflavone treatment. The migration of the substrate and products of the CAT assay is described in Fig. 2.

expressed CAT activity under the direction of the transferase promoter and CAT activity was induced 4-fold by β-naphthoflavone (Fig. 6*B*). By using specific DNA probes for CAT mRNA and neo mRNA, we found that the β-naphthoflavone response produced a comparable elevation in CAT mRNA but no elevation in neo mRNA (data not shown). These data indicate that induction in CAT activity seen in the cell lines after transient transfection is not due to differences in transfection efficiency or protein stability but reflects elevated CAT mRNA levels.

Role of the Dioxin Receptor in Regulation of the Glutathione *S*-Transferase Promoter and β-Naphthoflavone Responsive Element. To assess the role of the dioxin receptor in the regulation of the glutathione *S*-transferase promoter and β-naphthoflavone responsive element, the transferase-CAT constructs were transfected into two mouse cell lines defective in the dioxin receptor. These cell lines, referred to as class I and class II variants, have been isolated and characterized by Whitlock and colleagues (28–30). The class I variants form few TCDD–receptor complexes; however, those that form accumulate within the nucleus. These cells

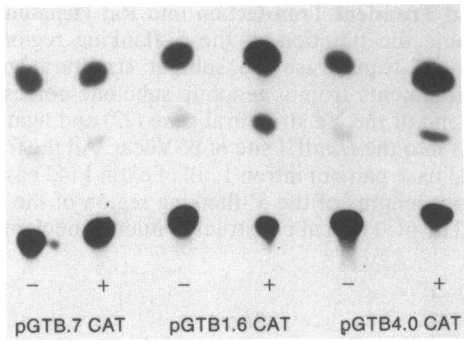

FIG. 5. CAT assay with lysed mouse Hepa 1c1c7 cells transfected with glutathione *S*-transferase-CAT chimeric plasmids. Transient assays in the presence (+) and absence (−) of 15 μM β-naphthoflavone were performed in mouse Hepa 1c1c7 cells by using pGTB.7cat, pGTB1.6cat, and pGTB4.0cat. The extent of induction of CAT activity by β-naphthoflavone in cells transfected with pGTB1.6cat was 3.8 ± 1.3-fold. This value is the mean ± SD of three separate experiments. All assays were done in duplicate. Similarly, transient assays with pGTB4.0cat gave equivalent levels of induction. The migration of substrate and products is described in Fig. 2.

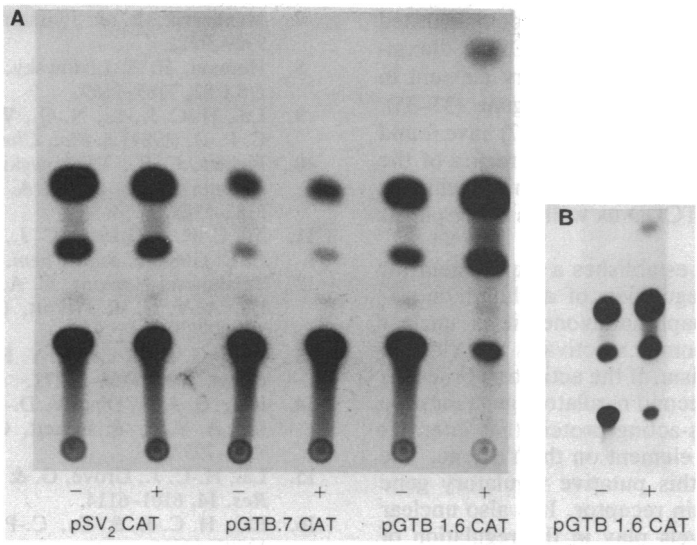

FIG. 6. (*A*) CAT assay with lysed human HepG2 cells transfected with glutathione *S*-transferase-CAT chimeric plasmid. Transient assays in the presence (+) and absence (−) of 50 μM β-naphthoflavone were performed in human HepG2 cells with pSV2cat, pGTB.7cat, and pGTB1.6cat. The extent of induction of CAT activity by β-naphthoflavone in cells transfected with pGTB1.6cat is 7.3 ± 0.5-fold. This value is the mean ± SD of three experiments. All assays were done in duplicate. The migration of substrate and products of the CAT assay on the TLC plate is as described in Fig. 2. (*B*) Effect of β-naphthoflavone on CAT activity in human HepG2 cells with stably integrated pGTB1.6cat chimeric plasmids. The extent of induction of CAT activity in the presence (+) of 50 μM β-naphthoflavone is 4-fold. −, Absence of β-naphthoflavone.

show decreased accumulation of cytochrome P_1-450 mRNA and lower aryl hydrocarbon hydroxylase activity in response to TCDD. Class II variants form a normal number of TCDD–receptor complexes but the complexes do not accumulate in the nucleus. In both of these lines transfected with pGTB1.6cat, we found no elevation in CAT activity after β-naphthoflavone treatment (Fig. 7), whereas the wild-type cell line was responsive to β-naphthoflavone administration (Fig. 5). These data suggest that to obtain regulation of the glutathione *S*-transferase Ya subunit gene by β-naphthoflavone, functional dioxin receptors are required.

DISCUSSION

In this manuscript we have begun to explore the function of the 5′-flanking region of a glutathione *S*-transferase Ya subunit structural gene. We have presented evidence indicating the presence of at least two regulatory elements that function independently of each other in the 5′-flanking sequence of the Ya gene. One element is functional in rat, mouse, and human cells and is required for β-naphthoflavone responsiveness. The second element is required for maximum basal promoter activity. This latter element is functional in rat and human cell lines but not in the mouse cell lines. We have also demonstrated that the β-naphthoflavone regulatory element is responsive only in cells that have functional dioxin receptors.

The regulation of gene expression by planar aromatic compounds, particularly TCDD, has been the subject of several studies. In the mouse Hepa 1c1c7 cell line used in the present study, Whitlock and colleagues (28, 31, 32) have demonstrated that TCDD binds to an intracellular receptor, accumulates in the nucleus, and activates the transcription of the cytochrome P_1-450 gene (P450IA1 gene family), which leads to an increased level of P_1-450 mRNA and increased aryl hydrocarbon hydroxylase activity. By using P_1-450-

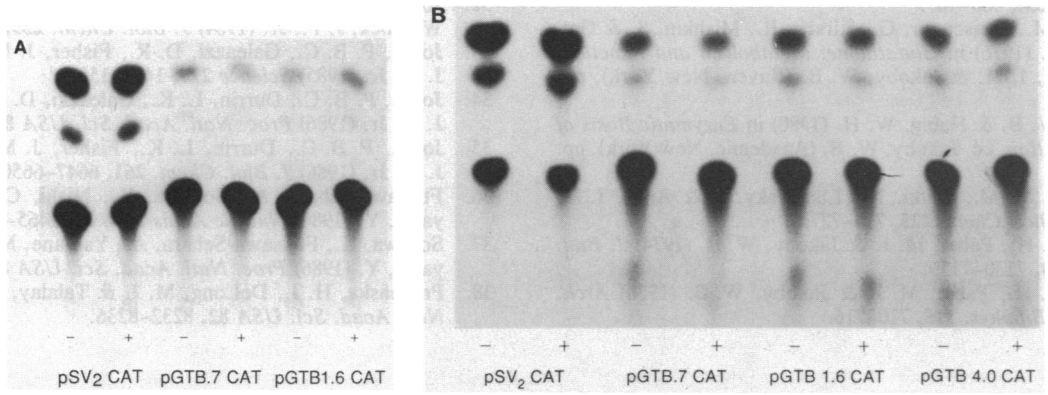

FIG. 7. (*A*) CAT assay with lysed mouse class I variant cells transfected with glutathione *S*-transferase-CAT chimeric plasmids. Transient assays in the presence (+) and absence (−) of 15 μM β-naphthoflavone were performed in mouse class I variants by using pSV2cat, pGTB.7cat, and pGTB1.6cat. No elevation in CAT activity was seen in the presence of inducer. The migration of substrate and products of the CAT assay is described in Fig. 2. (*B*) CAT assay with lysed mouse class II variant cells transfected with glutathione *S*-transferase-CAT chimeric plasmids. Transient assays in the presence (+) and absence (−) of 15 μM β-naphthoflavone were performed in mouse class II variants by using pSV2cat, pGTB.7cat, pGTB1.6cat, and pGTB4.0cat. No elevation in CAT activity was seen in the presence of inducer. The migration of substrate and products of the CAT assay is described in Fig. 2.

CAT fusion genes, these investigators have demonstrated the presence of a core promoter, multiple TCDD or dioxin-responsive elements, and a negative regulatory element in the 5'-flanking region of the mouse P_1-450 gene (33–35). Fujisawa-Sehara *et al.* (36) and Sogawa *et al.* (37) have found similar regulatory elements in the 5'-flanking region of the rat cytochrome P-450c gene (P450IA1 gene family), which is regulated by dioxins such as TCDD as well as other planar aromatic compounds.

Although the present study establishes a requirement for the dioxin receptor in the regulation of a glutathione *S*-transferase Ya gene by β-naphthoflavone, it is unclear whether the receptor–ligand complex activates the Ya gene by a direct or indirect mechanism. If the activation proceeds by an indirect mechanism, a second regulatory gene must be involved that encodes a trans-acting protein that interacts with the cis-acting regulatory element on the Ya gene. The transcriptional activation of this putative regulatory gene must be mediated by the dioxin receptor. It is also unclear what role elevated P_1-450 levels play in the regulation of genes encoding phase II drug metabolizing enzymes. Talalay and colleagues (38) have suggested that the induction of quinone reductase and other phase II enzymes is mediated through altered levels of P_1-450, which metabolizes planar aromatic compounds to redox-labile species. The redox-labile species have been proposed to be the induction signal. Although this mechanism cannot be confirmed or ruled out by the present study, one must still postulate a pathway for the redox-labile species to activate transcription of the genes encoding the phase II enzymes.

In conclusion, the identification of a β-naphthoflavone-responsive element(s) and a basal promoter element(s) in the 5'-flanking region of a glutathione *S*-transferase Ya gene will allow for a detailed structural study to define the minimum DNA sequences that constitute these elements. These studies will provide a fundamental understanding of glutathione *S*-transferase gene expression as well as provide an experimental system to define trans-acting proteins that interact with these cis-acting regulatory elements to control the expression of this important gene.

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