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Negative Regulation of Rat GST-Ya Gene via Antioxidant/ Electrophile Response Element Is Directed by a C/EBP-like Site

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The present studies were conducted to evaluate functional interactions between aryl hydrocarbon and antioxidant/electrophile response elements (AhRE and ARE/EpRE, respectively) in transcriptional regulation of the rat (r)GST-Ya gene. Transient transfection of an AhRECAT reporter construct into vascular smooth muscle cells (vSMCs) or HepG2 cells showed that benzo(*a*)pyrene (BaP) (0.3–30 μM) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (0.1–10 nM), but not hydrogen peroxide (H_2O_2) (100–400 μM), increased gene transcription. ARE/EpRE did not mediate gene inducibility by any of the chemicals in vSMCs but increased transcription in HepG2 cells treated with BaP or H_2O_2, but not TCDD. Gene inducibility in response to all chemicals was repressed in both cell types transfected with a 1.6CAT full-length promoter construct containing the AhRE and ARE/EpRE in genomic context. Site-directed mutagenesis of 1.6CAT showed that a CCAAT/enhancer-binding protein (C/EBP)-like site within the ARE/EpRE directed negative regulation of the rGST-Ya gene in vSMCs and HepG2 cells. These results show that ARE/EpRE in rGST-Ya does not function as a positive *cis*-acting regulatory element in all cell types, and that in the context of the full-length rGST-Ya promoter a C/EBP-like site directs negative regulation of the gene by BaP and related chemicals.

Polycyclic aromatic hydrocarbons, such as benzo(*a*)pyrene (BaP), have been implicated as etiologic factors in environmental atherogenesis and carcinogenesis in laboratory animals (1, 2). BaP alters patterns of gene expression directly through the aryl hydrocarbon receptor (AhR) pathway, and indirectly through enzymatic formation of reactive intermediates that cause oxidative stress and DNA damage (3–6). Deregulation of BaP-responsive genes in mammalian cells is mediated at least two distinct cis-acting regulatory DNA elements, namely, the AhRE and ARE/EpRE. The regulatory functions of these elements have been characterized in several genes encoding drug metabolizing enzymes (7, 8), most notably, the rat rGST-Ya gene (9).

The rGST-Ya promoter contains AhRE and ARE/EpRE sequences located at −914 to −882 and −722 to −682, respectively (10). The AhRE contains a 5′-TTGCGTG-3′ consensus sequence which is recognized specifically by the AhR/ARNT protein complex. This element is activated following exposure to planar aromatic compounds such as BaP, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), β-naphthoflavone, and 3-methylcholanthrene (9–13). The ARE/EpRE comprises two adjacent AP-1-like sites (5′-TGGCATTGC-3′ and 5′-TGACAAAGC-3′) that mediate inducibility by some planar aromatic compounds, phenolic antioxidants (e.g. tert-butyl hydroquinone), and prooxidants, such as hydrogen peroxide (H_2O_2) and menadione (9, 13–15). AhRE and ARE/EpRE have been characterized as positive regulatory elements using CAT reporter constructs linked to the minimal promoter region of rGST-Ya in HepG2 cells. To date, little is known about functional interactions between these elements within the rGST-Ya gene.

In this report, we describe for the first time that ARE/EpRE in the rGST-Ya promoter can function as a negative regulatory sequence in both vSMC and HepG2 cells. Site-directed mutagenesis revealed that a CCAAT/enhancer binding protein (C/EBP)-like site within the rGST-Ya ARE/EpRE directs negative regulation of the gene.

MATERIALS AND METHODS

Cell culture. Primary cultures of vSMCs were isolated from mouse aorta and maintained under standard conditions as described previously (16). Cells were grown in Medium 199 containing 10%

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fetal bovine serum (FBS, Atlanta Biologicals), 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 mg/ml amphotericin B (Gibco) at 37°C and an atmosphere of 5% CO_2, 95% air. HepG2 cells were purchased from American Type Culture Collection (Rockville, MD). Cells were maintained in Eagle's Minimal Essential Medium (Sigma) with supplements as described above.

Plasmids. Four CAT constructs kindly provided by C. B. Pickett (Schering Plough Research Institute, Lafayette, NJ) were employed to evaluate xenobiotic inducibility profiles. The 1.6CAT construct contains the rGST-Ya promoter region from bases −1651 to +66, including AhRE and ARE/EpRE sites at −914 to −882 and −722 to −682, respectively (Fig. 1A), while the −164CAT contains the minimal promoter region of the gene. ARE/EpRECAT contains the ARE/EpRE (from bases −722 to −682) linked to the minimal promoter region, while AhRECAT contained the AhRE (from bases −914 to −882) linked to the minimal promoter region.

Transfection, chemical treatments, and CAT/β-galactosidase measurements. vSMCs were transfected at 80% confluence by a liposome-mediated transfection method as described previously (17). Briefly, cells were incubated with transfection solution at 37°C/5% CO_2 for 5 h and then treated with BaP at a final concentration of 0.3, 3 or 30 μM, TCDD at 0.1, 1 or 10 nM, or H_2O_2 at 100, 250 or 400 μM for 24 h. HepG2 cells were transfected at 60% confluence by a modified calcium phosphate precipitation method (18). Cells were incubated with transfection solution for 5 h and then challenged with specific agents. CAT activity was determined as described (17), using 20–120 μg of protein in an overnight incubation at 37°C. β-galactosidase activity was determined using a commercially available ELISA kit (Boehringer Mannheim) according to manufacturer's instructions. CAT activities were expressed as fold induction relative to control activities.

Site-directed mutagenesis. Site-directed mutagenesis was performed using a Morph site-specific plasmid DNA mutagenesis Kit from 5 Prime-3 Prime, Inc. (Boulder, CO) according to manufacturer's instructions. All mutations were confirmed by DNA sequencing.

Statistics. The response profiles for the different rGST-Ya CAT constructs was analyzed by Friedman's Test at the 0.01 level of significance. Data were expressed as means ± standard errors. Three determinations were carried out for each measurement.

RESULTS

rGST-Ya induction profiles in vSMCs or HepG2 cells challenged with BaP, TCDD, and H_2O_2. Previous reports by Pickett and coworkers have shown that the AhRE or ARE/EpRE independently activate the rGST-Ya gene in HepG2 cells following BaP exposure (9). These findings implicate both AhR and oxidative signaling in the mammalian response to this hydrocarbon. To determine if comparable responses are elicited in vSMCs, 1.6CAT, AhRECAT, ARE/EpRECAT and −164CAT (Fig. 1A) were transiently transfected into cells as described under Materials and Methods. As shown in Fig. 1B, induction profiles for vSMCs treated with BaP (0.3. 3, 30 μM) were AhRECAT > 1.6CAT = ARE/EpRECAT = −164CAT. AhRECAT was strongly induced by BaP, while 1.6CAT and ARE/EpRECAT were unaffected by BaP. For comparison, we also examined the effects of BaP in HepG2 cells. Results indicated that induction of AhRECAT = ARE/EpRECAT > 1.6CAT = −164CAT (Fig. 1C). Both AhRECAT and ARE/EpRECAT were significantly induced by BaP in HepG2 cells, while CAT activity was unaffected by BaP in cells transfected with the full-length (1.6CAT) reporter construct.

Next, we monitored induction profiles by TCDD (0.1, 1, 10 nM) and H_2O_2 (100, 250, 400 μM) to better understand the mechanisms of BaP-induction in vSMCs and HepG2 cells, and to define the basis for loss of xenobiotic inducibility of the 1.6CAT construct. TCDD and H_2O_2 are known to activate transcription of the rGST-Ya promoter in HepG2 cells via the AhRE and ARE/EpRE, respectively (9, 13–14). The activation patterns by TCDD in vSMCs were AhRECAT > 1.6CAT = ARE/EpRECAT = −164CAT (Fig. 1D), while those in HepG2 cells were AhRECAT > 1.6CAT = ARE/EpRECAT = −164CAT (Fig. 1E). In both cell types, AhRECAT was highly induced by TCDD, while 1.6CAT and ARE/EpRECAT were not. For HepG2 cells, this finding was unexpected in light of a previous report by Rushmore and Pickett showing 2.7-fold induction of 1.6CAT by TCDD in HepG2 cells (13). Because the TCDD concentration used in their studies was never defined, we conducted additional experiments to determine if higher TCDD concentrations could activate 1.6CAT in HepG2 cells. Challenge of the cells with TCDD at 50 and 100 nM was associated with a 1.3 ± 0.1 and 1.1 ± 0.02 fold reporter activity, respectively (n = 3). These results demonstrated that even at extremely high concentrations 1.6CAT is uninducible by TCDD in HepG2 cells. Treatment with H_2O_2 (100, 250, 400 μM) in vSMCs showed no inducibility, with AhRECAT = 1.6CAT = ARE/EpRECAT = −164CAT (Fig. 1F). In contrast, the induction profile in HepG2 cells was ARE/EpRECAT > AhRECAT = 1.6CAT = −164CAT (Fig. 1G). Thus, H_2O_2 activated ARE/EpRECAT in HepG2 cells, but not in vSMCs. As expected, 1.6CAT and AhRECAT were unaffected by H_2O_2 in both cell types. These data suggest that when examined in genomic context negative regulatory mechanism(s) are operative in vSMCs and HepG2 cells that preclude induction of rGST-Ya via either AhRE or ARE/EpRE.

A C/EBP-like site within ARE/EpRE directs negative regulation of rGST-Ya gene. Multiple mechanisms may account for low inducibility of 1.6CAT in vSMCs and HepG2 cells. Our next set of experiments was designed to test the hypothesis that the ARE/EpRE functions as a negative regulatory sequence within the rGST-Ya promoter. Site-directed mutagenesis was conducted to mutate or delete the C/EBP-like site within the ARE/EpRE in the 1.6CAT construct as described by other investigators (10, 19–20) (Fig. 2). These experiments were designed based on previous reports showing that C/EBP can function as a negative regulator of gene transcription (21). The induction patterns of the mutated or deleted constructs (referred to as 1.6CAT-EpREm and 1.6CAT-EpREd, respectively) elicited by 3 μM BaP, 1 nM TCDD

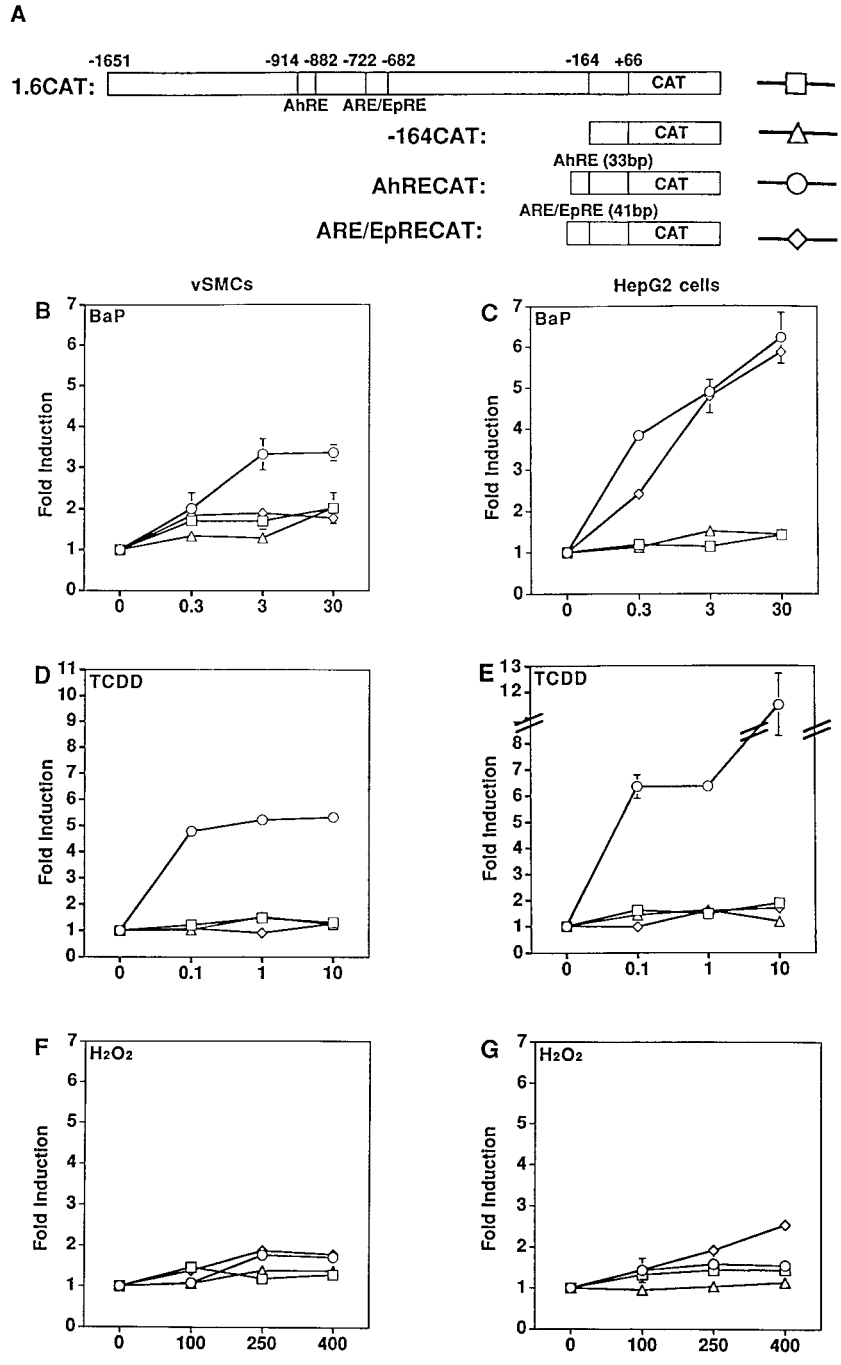

FIG. 1. Comparative activation profiles of rat GST-Ya CAT constructs by BaP, TCDD, and H_2O_2 in vSMCs or HepG2 cells. vSMCs were transfected by lipofection with rGST-Ya CAT constructs and a β-galactosidase plasmid as an internal control to correct for transfection efficiency. HepG2 cells were transfected by calcium phosphate precipitation. Cells were challenged with individual agents for 24 h. Cell extracts were prepared and analyzed for CAT activities and β-galactosidase activities as described under Materials and Methods. Data are expressed as fold induction relative to respective controls. Values represent the mean ± S.E. from three separate experiments. ($p < 0.01$) (A) Diagram of the rat GST-Ya gene CAT constructs. (B), (C) responses of vSMCs and HepG2 cells, respectively to BaP (0.3–30 μM) (D), (E) responses of vSMCs and HepG2 cells to TCDD (0.1–10 nM), (F), (G) responses of vSMCs and HepG2 cells to H_2O_2 (100–400 μM).

or 400 μM H_2O_2 in vSMCs are shown in Fig. 3. The 1.6CAT-EpREm and 1.6CAT-EpREd exhibited 6.7- and 4.0-fold induction in BaP-treated vSMCs, respectively, as compared to a 1.4-fold induction in the 1.6CAT construct. In contrast, in HepG2 cells 1.6CAT-EpREm and 1.6CAT-EpREd exhibited 10.4- and 15.1-fold induction, respectively, in response to BaP, as compared to 1.2-fold induction of 1.6CAT.

Gene:	Sequence:	Reference:
Rat GST-Ya EpRE	ATTGCTAATG	10
C/EBP high affinity sequence	ATTGCGCAAT	19
Rat GST-Ya AhRE	GTTGCGTGCA	10,19
Alcohol dehydrogenase	ATTGCACATA	20
α-fetoprotein	ATTGCCTAAC	20
Apolipoprotein B	ATTGCAAAAG	20
Cholesterol ester transfer protein	ATTGCTTAAT	20
Tyrosine aminotransferase	ATTGCAAATA	20
T kininogen	ATTGCCCAAC	20
Serum amyloid A1	ATTGCAAAAC	20
Serum amyloid A3	ATTGCTCCAT	20

FIG. 2. Comparison of rGST-Ya EpRE, C/EBP high affinity sequence, rGST-Ya AhRE, and other C/EBP binding sequences. The rGST-Ya EpRE contains a C/EBP-like site homologous to the C/EBP high affinity sequence, rGST-Ya AhRE, and other C/EBP binding sequences.

Treatment with 1 nM TCDD induced 1.6CAT-EpREm and 1.6CAT-EpREd 3.0- and 3.6-fold, respectively, relative to a 1.7-fold activation of 1.6CAT in vSMCs, while in HepG2 cells TCDD induced 1.6CAT-EpREm and 1.6CAT-EpREd 10.1- and 9.6-fold induction, respectively, relative to 1.1-fold activation of 1.6CAT. In contrast, induction of mutated/deleted constructs was not observed in response to H_2O_2 in either cell type. Thus, in the absence of the C/EBP-like site within the ARE/EpRE sequence of the full-length promoter, inducibility by BaP or TCDD is selectively recovered in both cell types. Taken together, these results suggest that a C/EBP-like site within the rGST-Ya mediates negative regulation of the gene in vSMCs and HepG2 cells by aromatic hydrocarbons.

DISCUSSION

AhRE and ARE/EpRE are cis-acting regulatory elements involved in coordinate regulation of mammalian gene expression by BaP and related xenobiotics (7–9). Activation of AhRE signaling is mediated by the AhR, while transacting factors involved in ARE/EpRE signaling have yet to be identified. Although the AhRE and ARE/EpRE function as positive regulatory elements when linked to the minimal rGST-Ya promoter, functional interactions between these elements have not yet been rigorously examined. On the basis of gene inducibility profiles and site directed mutagenesis we present evidence that: 1) in genomic context ARE/EpRE does not function as a positive regulatory element in rGST-Ya promoter, and 2) xenobiotic inducibility of rGST-Ya is lost when both AhRE and ARE/EpRE are present in genomic context within the full-length promoter.

Activation of rGST-Ya via AhRE was observed in both vSMCs and HepG2 cells treated with aromatic hydrocarbons. The integrity of AhRE signaling in these cell systems is consistent with previous reports for other hydrocarbon-regulated promoters (7–9). Cell- and/or species-specific patterns of rGST-Ya regulation exist since by itself ARE/EpRE elicited a positive response in HepG2 cells challenged with BaP or H_2O_2, but not vSMCs (Fig. 1, B–C and F–G). The inability of BaP or H_2O_2 to activate transcription via the ARE/EpRE in vSMCs was unexpected in view of previous work showing that ARE/EpRE mediates transactiva-

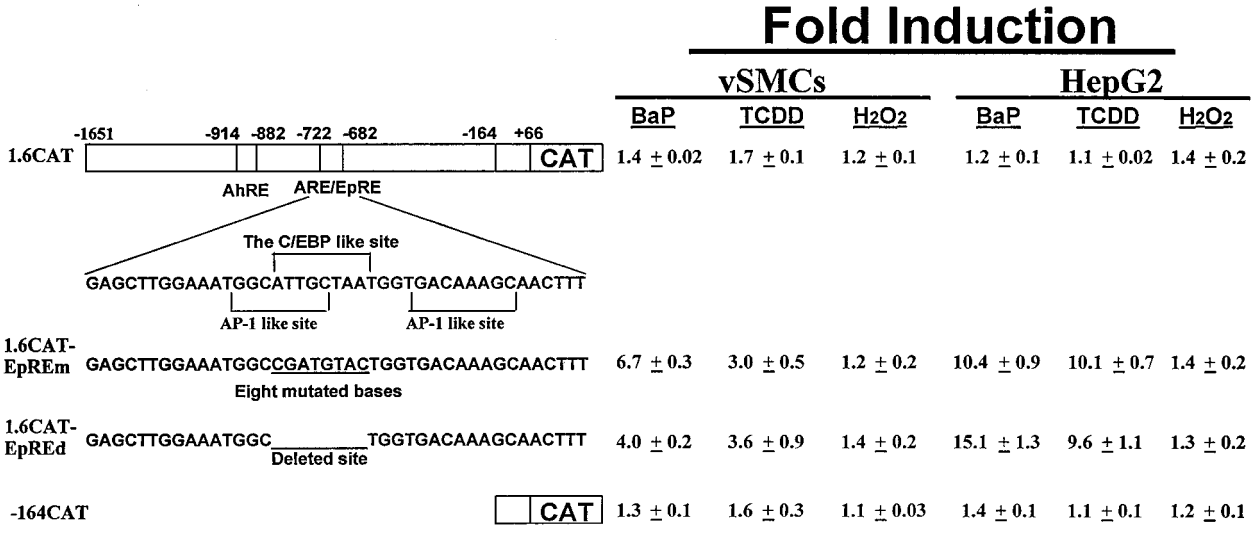

FIG. 3. Activation profiles of 1.6CAT, 1.6CAT-EpREm, 1.6CAT-EpREd, and 164CAT constructs by BaP, TCDD and H_2O_2. The C/EBP-like site in rGST-Ya promoter was mutated or deleted by site-directed mutagenesis as indicated in Materials and Methods. The 1.6CAT with mutation or deletion in the C/EBP-like site was referred to as 1.6CAT-EpREm and 1.6CAT-EpREd, respectively. vSMCs were transfected by lipofection with rGST-Ya CAT constructs with a β-galactosidase plasmid as internal control to correct for transfection efficiency. HepG2 cells were transfected by calcium phosphate precipitation. Cells were challenged with 3 μM BaP, 1 nM TCDD or 400 μM H_2O_2 for 24 h. Cell extracts were prepared and analyzed for CAT activities and β-galactosidase activities as described under Materials and Methods. Data are expressed as fold induction relative to respective controls. Values represent the mean ± S.E. from three separate experiments.

tion of the Ha-ras promoter in vSMCs by BaP and related oxidative intermediates (17). The absence of ARE/EpRE inducibility of rGST-Ya in vSMCs may reflect differential expression of transcription factors that activate ARE/EpRE-like sequences within this promoter. This suggestion is consistent with preliminary studies showing cell type- and promoter-specific differences in complex assembly on the ARE/EpRE in vSMCs and HepG2 cells. Thus, the positive regulatory functions of ARE/EpRE appear to be cell type- and promoter-specific.

Despite cell type-specific differences in patterns of ARE/EpRE-mediated gene regulation, functional interactions between the AhRE and ARE/EpRE were observed in both vSMCs and HepG2 cells when these elements were examined in genomic context. This interpretation is consistent with the finding that: 1) hydrocarbon inducibility by AhRECAT was lost in the 1.6CAT construct in vSMCs and HepG2 cells (Fig. 1, B–E), 2) ARE/EpRECAT was inducible in HepG2 cells by BaP or H_2O_2, but induction was lost in the 1.6CAT construct (Fig. 1, C and G), and 3) ARE/EpRE was unresponsive to H_2O_2 in the context of 1.6CAT (Fig. 1G). Loss of xenobiotic inducibility in 1.6CAT suggests that a negative regulatory mechanism is operative within the context of the full-length rGST-Ya promoter. Site-directed mutagenesis confirmed this hypothesis and showed that a C/EBP-like site within the consensus ARE/EpRE core sequence is responsible for the negative regulatory function of ARE/EpRE in response to BaP and TCDD. As noted above for cell type-specific patterns of xenobiotic inducibility, differential protein binding to the C/EBP-like site within the rGST-Ya promoter may account for variable transcriptional regulatory activity. In the case of H_2O_2, loss of transactivation potential in 1.6CAT in HepG2 cells further strengthens the view that reciprocal interactions between the two xenobiotic responsive cis-acting elements exist.

The finding that only aromatic hydrocarbons were affected by mutation of the C/EBP-like site suggests that AhR itself, or AhRE participate in negative regulation of rGST-Ya via the C/EBP-like site. In this regard it is interesting to note that C/EBP-α interacts with the AhRE (5′-AGGCATGTTGCGTGCATCC-3′) of the rGST-Ya gene in HepG2 cells (C/EBP site is underlined) (19). Puga and coworkers have suggested that a 35–40 kDa protein that binds AhRE3 (5′-CTTCTCACGCAACTCCGGG-3′) (C/EBP site is underlined) in the murine Cyp1a1 gene in wild-type and mutant murine hepatoma cells might be C/EBP-α, and provided evidence that bacterially expressed C/EBP-α interacts with AhRE3 (22). Thus, cross interactions of C/EBP members with the C/EBP-like site and AhRE may contribute to negative regulation of rGST-Ya in both vSMCs and HepG2 cells. Studies are now in progress to identify transcription factors that bind to the C/EBP-like site of the rGST-Ya promoter.

The inability of TCDD to activate transcription in the 1.6CAT construct was unexpected in view of previous reports by Rushmore and Pickett showing 2.7 fold induction in HepG2 cells (13). Because the TCDD concentration used in their studies was not defined, direct comparison of the two data sets is not possible. In a separate report, however, Hayes and Pulford (23) reported that TCDD could only activate transcription of 1.6CAT two-fold at 5 μM, a concentration that far exceeds limits of hydrocarbon solubility and is known to activate non-AhR-mediated events that interfere with the overall transcriptional response. Thus, the mechanisms that govern reciprocal activation of AhRE and ARE/EpRE in mammalian cells are complex and require further investigation.

In summary, our results show that ARE/EpRE can function as a negative regulatory sequence within the rGST-Ya promoter and implicate a C/EBP-like site as the critical sequence responsible for xenobiotic inducibility of the gene.

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