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The Human Cytochrome Cyp1A2 Gene Contains Regulatory Elements Responsive to 3-Methylcholanthrene

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SUMMARY

The regulation of the human cytochrome Cyp1A2 gene by 3-methylcholanthrene was studied through the transfection of 5′-flanking sequences into human cells. The Cyp1A2 promoter sequence and 3700 bases 5′ to the cap site were linked to the procaryotic chloramphenicol acetyltransferase gene. Transfection of this construct into HepG2 cells generated a 2–3-fold increase in Cyp1A2-directed chloramphenicol acetyltransferase activity when the cells were treated with 3-methylcholanthrene. Deletion of flanking sequence to −1079 resulted in a loss of 3-methylcholanthrene-induced chloramphenicol acetyltransferase activity. When 5′-flanking sequences of the Cyp1A2 gene were inserted into a plasmid containing the chloramphenicol acetyltransferase gene under control of the simian virus 40 promoter, 3-methylcholanthrene-enhanced chloramphenicol acetyltransferase activity was observed. The strongest 3-methylcholanthrene-induced chloramphenicol acetyltransferase activity, a 4-fold increase, was observed for a DNA fragment located at −3202 to −1595. When this Cyp1A2 responsive element was transfected into human breast carcinoma MCF-7 cells, 3-methylcholanthrene did not stimulate chloramphenicol acetyltransferase activity. In comparison, when a DNA fragment that contained a copy of the human Cyp1A1 xenobiotic-responsive element was analyzed for enhancer activity, 3-methylcholanthrene initiated chloramphenicol acetyltransferase activity in both HepG2 cells and MCF-7 cells. These results suggest that the 3-methylcholanthrene-responsive Cyp1A2 element may be regulated in a tissue-specific manner.

PAHs are some of the most ubiquitous environmental contaminants. In animals, these agents have been shown to initiate a series of events that lead to the induction of the cytochrome P450IA monooxygenases, resulting in the metabolism and bioactivation of the PAHs to carcinogenic and tumorigenic metabolites (1). Two forms of cytochromes, termed P450IA1 and P450IA2, are induced following exposure to PAHs such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3MC. The induction of the Cyp1A1 gene by PAHs follows association of inducer with a cytosolic receptor (2), nuclear transport (3, 4), and recognition of the PAH-receptor complex by *cis*-acting elements (5–9). Consensus sequences have been proposed for these elements and they are termed XREs (10) and DREs (11, 12). In the human Cyp1A1 gene, the 5′-flanking region contains these sequences. A construct containing these elements has been shown in transfection assays to be responsive to transcriptional activation in the presence of 3MC (13–15).

With few exceptions (16), the expression of P450IA2 is limited in extrahepatic tissues (17–22). Genetic analysis of hepatic Cyp1A2 gene products in PAH-responsive and -non-responsive mice implicates a regulatory role for the dioxin receptor (23) in the induction process, but involvement of transcriptional control has not been conclusively demonstrated. Kimura *et al.* (24) have indicated that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin enhances the transcriptional rate of the mouse Cyp1A2 gene approximately 7-fold, while contributing substantially to the posttranscriptional stability of the mRNA. In rats, the 70-fold increase in liver P450IA2 mRNA by β-naphthoflavone resulted primarily from mRNA stabilization, with only a 2-fold increase in the transcriptional run-on levels (25). Although transcriptional control through receptor binding has been suggested as a mode of regulation following exposure to PAHs, the properties of tissue-specific expression and differential regulation at the molecular level suggest that the expression of P450IA2 is susceptible to control by multiple alleles.

Our laboratory has isolated and characterized the human Cyp1A2 structural gene (26, 27). In the work presented here, the transcriptional control of this gene was assessed through functional analysis of the 5′-flanking sequences. The activity of transfected Cyp1A2 gene constructs in human cell lines was compared with a DNA enhancer element that controls the human Cyp1A1 gene. We report that the transfection of 5′-flanking sequences of the human Cyp1A2 gene fused to the bacterial CAT gene results in tissue-specific CAT expression that is regulated by 3MC.

Experimental Procedures

Identification of the human Cyp1A2 and Cyp1A1 genes. Human cDNA clones that encode human P450 6 and P450 4 (26),

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designated P450IA1 and P450IA2 (28), respectively, were used to isolate their corresponding structural genes designated Cyp1A1 and Cyp1A2 (29) from an EMBL-3 human genomic library (27, 30). Characterization of the Cyp1A2 gene has been previously described (27). Restriction enzyme analysis and partial DNA sequence analysis confirmed that the human Cyp1A1 genomic clone was the same as the previously isolated human P450c gene (13, 14).

DNA sequence analysis. The nucleotide sequences of the 5′-flanking regions of the Cyp1A2 and Cyp1A1 genes were determined by the dideoxy chain-termination method (31) from selected fragments inserted into M13mp18 and M13mp19 vectors (32). Subclones were generated from restriction enzyme analysis, as well as from a random shotgun approach using the insertion of blunt-ended DNA fragments (33). When selective 5′-flanking regions were subcloned into the appropriate plasmid expression vectors, each insert was characterized by double-stranded DNA sequence analysis.

Construction of expression plasmids. To construct a plasmid containing the Cyp1A2 5′-flanking sequences, a 3700-base pair *Bam*HI-*Sal*I fragment, which includes exon 1 and 35 base pairs of intron 1, was purified from an EMBL-3 genomic clone (27) and subcloned into the *Bam*HI-*Sal*I site of pUC19. The promoterless CAT gene was removed from the pUCCAT vector as a *Bam*HI-*Sma*I fragment, *Bam*HI linkers were added to the *Sma*I end, and the fragment was inserted 3′ to the Cyp1A2 promoter in the *Bam*HI site of the pUC19 recombinant. This construct is identified as pH4CAT1.

To test for enhancer activity, the enhancerless SV40 early promoter, which is fused to the CAT gene in clone pUCAT2 (34), was removed as a *Pst*I-*Bam*HI fragment and inserted into these same sites in pBluescript M13+ (Stratagene, La Jolla, CA). This construction, termed pSVCAT, allowed for the efficient transfer of Cyp1A1 and Cyp1A2 DNA fragments into a number of unique sites in locations either 5′ or 3′ to the CAT gene. Both pUCCAT and pUCAT2 plasmids were kindly provided by Michael Karin (Department of Pharmacology, University of California, San Diego).

Cell culture and DNA transfection. HepG2 cells were obtained from American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Twenty four hours before transient transfections, cells were trypsinized and plated at 10^6 cells/10-cm dish. 3MC was dissolved in dimethyl sulfoxide and added to a final concentration of 2 μM. The final concentration of dimethyl sulfoxide in the culture medium was 0.1%. Control plates received dimethyl sulfoxide at the same concentrations. Transient transfections were performed by the calcium phosphate-DNA precipitation method (35). Approximately 24 hr after transfection, cells were treated with 3MC for 18–20 hr.

CAT assays. Cellular extracts from transfected cells were prepared and assayed for CAT enzyme activity (36). Protein content was measured as described by Bradford (37), and equal amounts of protein were used for each assay. CAT activities were evaluated by autoradiography and liquid scintillation counting of radioactive spots.

Results

Expression of human Cyp1A2 fusion genes in HepG2 cells. Northern blot analysis of mRNA isolated from HepG2 cells that were treated with 3MC demonstrated that the P450IA2 mRNA was not detectable in these cells when probed with a P450IA2-specific cDNA probe.[1] However, transfected genes can be expressed in cells that lack the ability for endogenous regulation such that the regulatory regions within the 5′-flanking sequences can be delineated (38, 39). In order to identify regulatory regions in the Cyp1A2-flanking gene, the DNA sequence was determined from the *Kpn*I to the *Bam*HI site (Fig. 1). Analysis of the DNA sequence shows the presence of common promoter elements such as a TATA and CCAAT box, as well as other *trans*-acting DNA elements (40–45) known to play important roles in transcriptional control. In addition, comparison of this sequence with the National Institutes of Health Genbank database revealed three regions that contained repetitive sequences (46) found in other primate sequences.

To determine whether the 5′-flanking sequences are responsive to 3MC, the Cyp1A2 promoter and 3700 bases of flanking DNA were placed upstream of the reporter CAT gene and were transfected into HepG2 cells. As shown in Fig. 2, inducible CAT activity was observed when clone pH4CAT1 was transfected and the cells were treated with 2 μM 3MC. In five separate experiments, the effects of 3MC resulted in a 2–3-fold enhancement of CAT activity. When a deletion to −1079 was placed in front of the CAT gene (clone pH4CAT2), 3MC had no stimulatory effect on CAT activity, demonstrating that the Cyp1A2 gene contained a responsive element from −1079 to approximately −3700 bases. Primer extension analysis, using an oligonucleotide specific to the CAT gene (34) to detect the start of transcription initiated on the pH4CAT1 construct, was not successful. It is felt that the level of expression from pH4CAT1 was below our ability to detect the RNA transcripts, because the proper size transcripts (34) were detected from RNA preparations prepared from cells transfected with pSV2CAT, which generates >10-fold the level of CAT activity.[1]

PAH-inducible elements in the Cyp1A2 gene were identified by testing different regions of the gene for the ability to enhance transcription from a heterologous promoter in the presence of 3MC. The viral enhancer sequences are deleted in pSVCAT, making it possible to examine enhancer activity from the inserted Cyp1A2 gene fragments. Insertion of a Cyp1A2 DNA fragment (clone p4SVCAT1) that extended from the *Kpn*I to the *Pst*I site (−3202 to −1595) resulted in stimulation of 3MC-inducible CAT activity when transfected into HepG2 cells. In four separate experiments, expression of this construct in the presence of 3MC was 3–6-fold greater than constitutive levels. This activity in HepG2 cells occurred when the fragment was inserted into pSVCAT in either orientation. The smaller *Pst*I fragment (−1595 to −1079) was slightly responsive (1.5-fold) in HepG2 cells. Further studies were not conducted with this fragment. When the Cyp1A2-flanking sequence was analyzed for homology to the Cyp1A1 enhancer elements (10, 12), a region at −2903 contained the hexamer sequence TCACGC, which is present in the rat Cyp1A1 XRE1 and XRE2 (10), as well as the mouse Cyp1A1 DRE2 and DRE3 (12). This exact hexamer is also found in the human Cyp1A1 XRE (−986 and −1058). The location of this hexamer in Cyp1A2 and the fact that it is perfectly conserved in both XREs and DREs may indicate that this and the surrounding sequence participate in 3MC enhancement of CAT activity from the p4SVCAT1 construct (see Discussion).

Plasmid pSV2CAT, which contains the SV40 early promoter and enhancer elements linked to the CAT sequences, and pUCCAT, the promoterless CAT gene, were used as positive and negative control plasmids for transfection, respectively. The levels of CAT expression from pSV2CAT and pUCCAT were not affected by treatment with 3MC.

Analysis of Cyp1A2 responsive element in other human cell lines. P450IA2 is expressed primarily in hepatic

[1] L. C. Quattrochi and R. H. Tukey, unpublished observations.

PAH, polycyclic aromatic hydrocarbon; CAT, chloramphenicol acetyltransferase; DRE, dioxin responsive element; 3MC, 3-methylcholanthrene; XRE, xenobiotic responsive element.

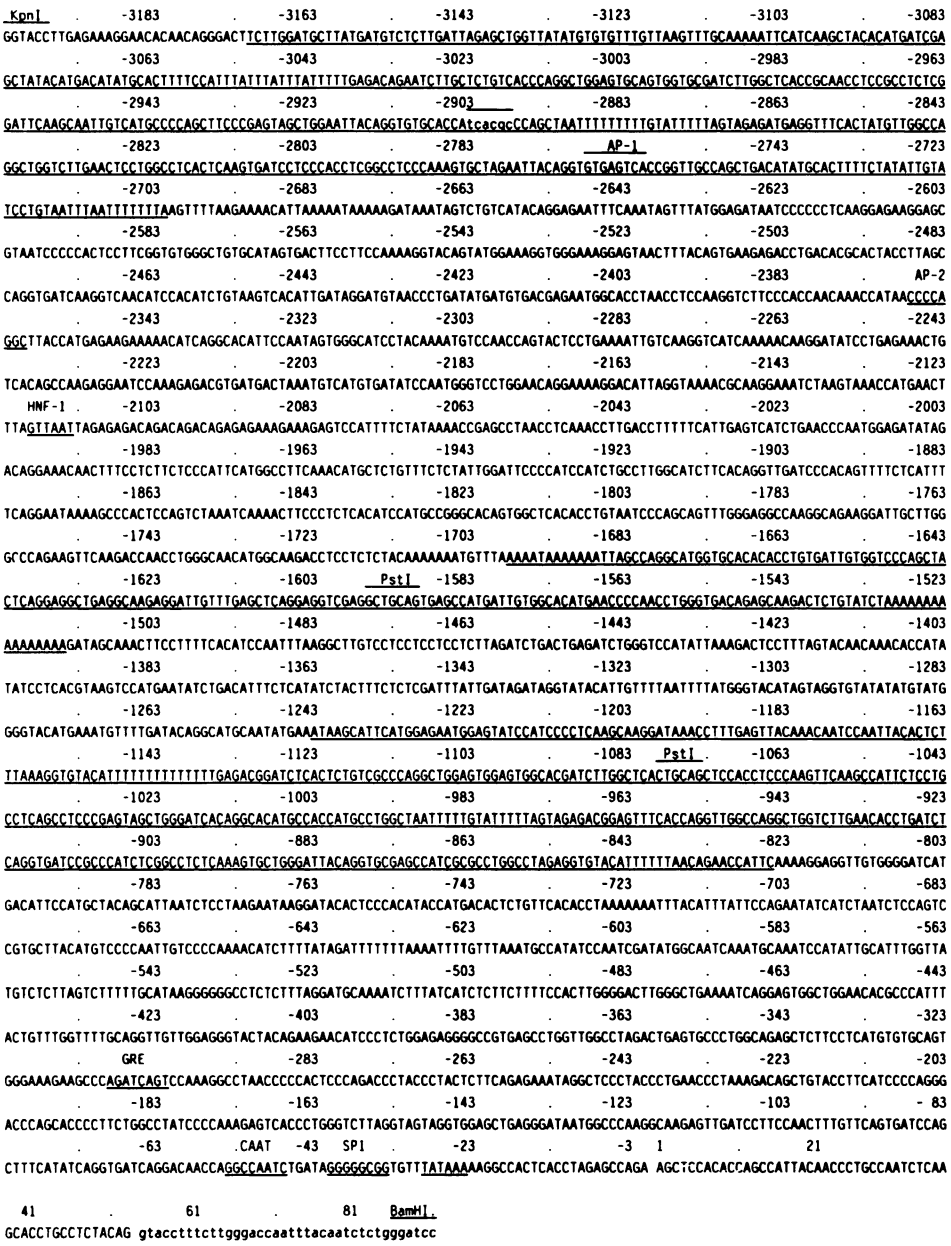

Fig. 1. Nucleotide sequence of the 5′-flanking region of the human Cyp1A2 gene. The nucleotide sequence of the 3293-base pair *Kpn*I-*Bam*HI fragment was determined by the dideoxynucleotide sequencing method (30). The start of transcription (indicated with a *1*) was determined previously by primer extension analysis (27). Nucleotides 5′-ward of the transcription start site are numbered consecutively with negative numbers, whereas nucleotides 3′-ward of the transcription start site are designated by consecutive positive numbers. The consensus TATA box (−24 to −29) is *underlined*, whereas the upstream promoter CCAAT (−52 to −46) (60) box is *labeled* and *underlined*. Analysis of the DNA sequence revealed AP1 (42), AP2 (43), GRE (40), Sp1 (44), and HNF1 (45) transcriptional elements (labeled above the DNA sequence). The TCACGC consensus core sequence (−2888 to −2903) is *overlined* and shown in *lower case letters*. Three repetitive stretches of DNA are *underlined*. The 5′-flanking sequence and exon 1 are represented by *upper case letters*, whereas intron 1 sequence is represented by *lower case letters*. Restriction endonuclease sites used to subclone portions of the DNA into expression vectors are indicated above the sequence.

tissue. To determine whether the 3MC-responsive element identified in the 5′-flanking sequences of the Cyp1A2 gene is expressed in a tissue-specific fashion, clone p4SVCAT1 was transfected into the human breast carcinoma cell line MCF-7. For comparison, an *Alu*I fragment (−890 to −1304) from the human Cyp1A1 gene, which contains one copy of the consensus XRE enhancer sequence (11), was cloned into pSVCAT (clone p6SVCAT) and transfected into these cells. As shown in Fig. 3, the enhancer region in the human P450IA1 gene showed inducible CAT activity that was approximately equal in both HepG2 and MCF-7 cells. In contrast, the putative enhancer region in the human Cyp1A2 gene showed the 3–6-fold induction when transfected into HepG2 cells but no enhancement of CAT activity when transfected into MCF-7 cells. A similar series of experiments were performed with human lung and ovarian carcinoma cell lines, and a similar pattern of expression was observed.[1] 3MC enhanced CAT transcriptional activity driven by the Cyp1A1 enhancer element in each cell line but had no effect on the Cyp1A2 enhancer sequence. These results indicate that the 3MC-responsive element in the human Cyp1A2 gene may help to control the patterns of tissue-specific expression observed with this gene.

Discussion

In animal studies, P450IA1 and P450IA2 mRNA are inducible after exposure to PAHs such as 3MC and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (23, 47–49). In hepatic tissue, genetic analysis of mRNA that was isolated from 3MC-responsive and -nonresponsive mice has demonstrated that the *Ah* locus plays a regulatory role in coordinating the molecular events that underlie the induction response of these two genes by PAHs (2, 23, 47). The induction of P450IA1 and P450IA2 in mice has been shown by nuclear run-on analysis to be in part an activation of transcription (50). A significant contribution to post-

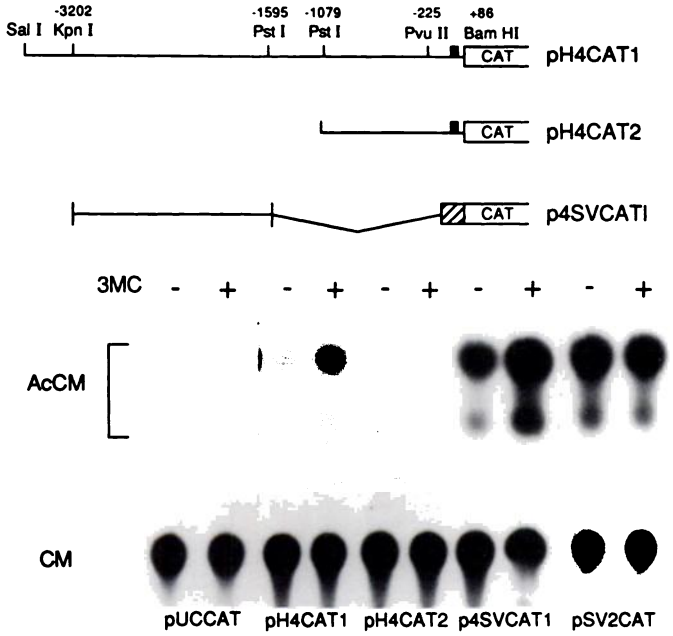

Fig. 2. Expression of CAT activity in HepG2 cells. The 5′-flanking sequences of the Cyp1A2 gene were cloned in front of the promoterless CAT-coding sequences (pUCCAT) or the SV40 early region, which contains the enhancerless promoter (*hatched bar*). Exon 1 is indicated by a *solid bar* (27). CAT activities were measured in extracts from uninduced (*−3MC*) and induced (*+3MC*) HepG2 cells. A representative autoradiogram shows the conversion of [^{14}C]chloramphenicol (*CM*) to its acetylated products (*AcCM*). CAT activity was quantitated by measuring the amount of unreacted and acetylated [^{14}C]chloramphenicol. The fold induction by 3MC is expressed as the ratio of induced/noninduced CAT activity for the following: pUCCAT, no response; pH4CAT1, 1.9 ± 0.3; pH4CAT2, no response; p4SVCAT1, 4.4 ± 0.4; and pSV2CAT, no response. Each value represents the mean and standard error for five experiments.

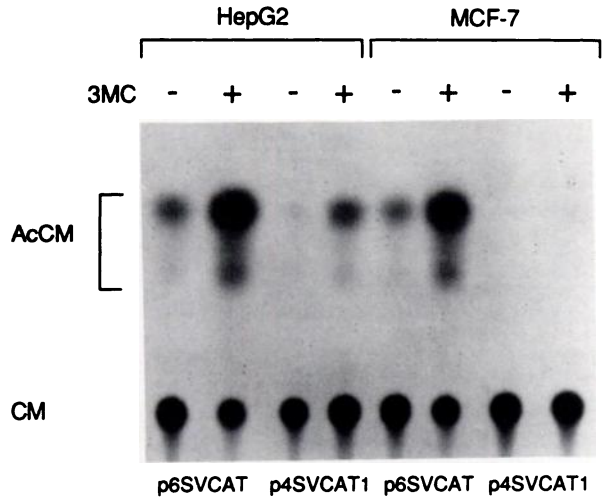

Fig. 3. Expression of 3MC-responsive regions within the human Cyp1A2 and Cyp1A1 genes in HepG2 and MCF-7 cells. Enhancer constructs were transfected into both HepG2 cells and the human breast carcinoma cell line MCF-7. CAT activities were measured in extracts from noninduced (*−3MC*) and induced (*+3MC*) cells. The fold induction by 3MC following transfection with the various constructs are as follows: in HepG2 cells, p6SVCAT, 6.4 ± 0.9; and p4SVCAT1, 3.8 ± 0.5; in MCF-7 cells, p6SVCAT, 6.3 ± 0.7; and p4SVCAT1, no response. Each value represents the mean and standard error for three experiments. *CM*, chloramphenicol; *AcCM*, acetylated chloramphenicol.

transcriptional control has been implicated in the rat (25). Studies on the transcriptional control of the Cyp1A1 gene have identified enhancer sequences 5′ to the cap site that are thought to associate with the dioxin-receptor complex (5–7, 9, 10, 51, 52). In this communication, we present evidence that there are regions in the human Cyp1A2 gene that are capable of enhancing transcription in the presence of 3MC.

With few exceptions (16, 24), previous work has demonstrated that P450IA2 is induced primarily in hepatic tissue (17–22). Results presented in this report indicate that the tissue-specific expression of P450IA2 may be linked to the 3MC-responsive region of the structural gene. Other enhancers have been shown to be expressed in a tissue-specific manner (53–56). Clone p4SVCAT1 elicits 3MC-inducible CAT activity in HepG2 cells and yet is unable to support this response in MCF-7 cells. The lack of inducible CAT activity from clone p4SVCAT1 in MCF-7 cells indicates that certain regulatory elements may not be expressed in these cells. These results also suggest that the molecular events involved in the 3MC induction of P450IA1 and P450IA2 in HepG2 cells may not be coordinated through similar transcriptional elements. It could be assumed that, because the Cyp1A1 enhancer supports 3MC-induced transcriptional activation in both HepG2 and MCF-7 cells whereas the Cyp1A2 element is responsive only in HepG2 cells, different cellular processes underlie the induction of these two genes.

Several studies have shown that the level of enhancer activity decreases as the distance between the promoter and enhancer is increased (57, 58). It may be that 3MC inducibility depends on the relative distance between the promoter and enhancer. The XREs identified for the Cyp1A1 gene have been mapped to regions on the gene that are approximately 1.5 kilobases and less. Results presented in this report demonstrate that the 3MC-responsive region in the human Cyp1A2 gene may be located as far 5′-ward as 2.9 kilobases. In a recent review (59), it was reported from unpublished data that the mouse Cyp1A2 promoter and 1.8 kilobases of 5′-flanking DNA could not support transcriptional activation after transfection into mouse Hepa cells. Given that the human Cyp1A2 responsive element identified in this report is located approximately 3 kilobases upstream of the start site of transcription, it was not surprising that the mouse Cyp1A2 element was not responsive to PAHs.

One responsive fragment in the P450IA2 gene, located between bases −3202 and −1595, contained the hexamer TCACGC, which was also found to be conserved in the Cyp1A1 enhancer elements (10, 11). However, important base pair homology of the sequences surrounding this hexamer was not strong between the human Cyp1A2 gene and the reported Cyp1A1 XREs (11). If the TCACGC and flanking sequences facilitate the association of the dioxin-receptor complex, the surrounding sequences may be important in dictating the affinity of the binding and the resulting intensity of the response. As observed recently, the induction of the Cyp1A2 gene in rats by PAHs occurs through a posttranscriptional mechanism, with weak contribution from the transcriptional component (25). The weak transcriptional response of the rat Cyp1A2 gene, when compared to the Cyp1A1 gene (25), mirrors the results that have been observed in this report. The PAH-responsive DNA fragment from the human Cyp1A2 gene is not as efficient in driving the heterologous SV40 promoter as the DNA fragment that contains the Cyp1A1 enhancer sequences. In addition, the

weaker Cyp1A2 gene response in the HepG2 cells appears to be facilitated in a tissue-specific fashion, because PAHs are unable to promote this activity in several non-hepatic-derived human cell lines.

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