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Differential Regulation of the Dioxin-Induced *Cyp1a1* and *Cyp1b1* Genes in Mouse Hepatoma and Fibroblast cell lines

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Abstract

The xenobiotic metabolizing enzymes *Cyp1a1* and *Cyp1b1* can be induced by the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) via the Aryl Hydrocarbon Receptor (AhR). These genes are differentially induced by dioxin in different mouse cell lines. In the mouse hepatoma cell line Hepa1c1c7 (Hepa-1), the *Cyp1a1* gene is induced to very high levels by dioxin, but the levels of *Cyp1b1* mRNA are extremely low and are not inducible by dioxin. The reverse is the case for the mouse embryonic fibroblast cell line C3H10T1/2, in which *Cyp1b1* is induced to very high levels by dioxin, but the levels of *Cyp1a1* mRNA are extremely low and not inducible by dioxin. However, dioxin treatment leads to the recruitment of AhR to the enhancer regions of both genes in both cell lines. Somatic cell hybrid clones generated between the two cell lines display high levels of induction of both genes in response to dioxin. Strong reactivation of the *Cyp1a1* gene was also observed in C3H10T1/2 cell line after treatment with the DNA methyl transferase inhibitor, 5-aza-2'-deoxycytidine (5-AzaC) and the histone deacetylase inhibitor, trichostatin-A (TSA). However, only modest reactivation of *Cyp1b1* was observed in Hepa-1 cells after 5-AzaC or TSA treatment. These data demonstrate that the presence or absence of binding of AhR to regulatory regions is not responsible for determining the differences in levels of induction of the two genes in these cell lines, and indicate that DNA methylation plays a major role in silencing of *Cyp1a1* gene expression in C3H10T1/2 cells, but appears to play only a minor role in silencing *Cyp1b1* gene expression in Hepa-1 cells, which likely occurs principally because Hepa-1 cells lack a factor required for high levels of induction of this gene.

Keywords

Cyp1a1; *Cyp1b1*; AhR; DNA methylation; histone deacetylase

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Introduction

Cytochromes P450 constitute a large multigene family of constitutive and inducible heme-containing enzymes that play major roles in the oxidative metabolism and elimination of a wide range of xenobiotic compounds, including many procarcinogens (Gonzalez and Gelboin, 1994) and anticancer therapeutics (Kivisto et al., 1995). In many cases, however, metabolism by cytochromes P450 can lead to the generation of toxic intermediates that are more deleterious than the parent compound.

Cytochromes P450 in the CYP1 family metabolize environmental procarcinogens, such as polycyclic aromatic hydrocarbons (PAHs) (Hankinson, 1995), aflatoxin B1 (Gonzalez, 1990; Nebert and McKinnon, 1994; Okey, 1990), and arylamines (Hayes et al., 1996b; Shimada et al., 1996). This family consists of three genes, encoding the enzymes CYP1A1, CYP1A2, and CYP1B1 (Guengerich and Turvy, 1991; Sutter et al., 1994). Benzo[a]pyrene, a PAH and major constituent of cigarette smoke, overcooked foods, smoke and diesel exhaust, is bioactivated by these enzymes into toxic intermediates that have been linked to cancer (Morgan and Whitlock, 1992; Okino and Whitlock, 1995). The human CYP1A1 and CYP1B1 enzymes also metabolize certain endogenous compounds, such as estradiol. However, the two enzymes metabolize estradiol into different products. CYP1A1 converts estradiol into 2-hydroxyestradiol, while CYP1B1 metabolism is exemplified by the 4-hydroxyestradiol metabolite, which is mutagenic, and which may be at least partially responsible for the carcinogenic effects of estradiol. (Hayes et al., 1996a; Hayes et al., 1996b).

AhR binds a plethora of PAHs, aromatic amines, indolecarbazoles, and halogenated aromatic hydrocarbons (HAHs), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin). In its unliganded form, the AhR exists in the cytosol as part of a multiprotein chaperone complex (Kazlauskas et al., 2000). Upon binding ligand, the AhR translocates to the nucleus and binds to the aryl hydrocarbon receptor nuclear translocator (ARNT). The dimer so formed, termed the aryl hydrocarbon receptor complex (AhRC), activates transcription of numerous phase I and II xenobiotic- metabolizing genes, including genes of the CYP1 subfamily. Most genes activated by the AhRC contain consensus nucleotide sequences in their 5' untranslated enhancer regions termed Xenobiotic Response Elements (XREs) (Denison et al., 1998). The *Cyp1a1* and *Cyp1b1* enhancers both harbor multiple XREs (Ko et al., 1996; Zhang et al., 1997). The *Cyp1a1* and *Cyp1b1* genes are inducible by PAHs and/or dioxin in several mouse tissues, although the degree of expression of *Cyp1a1* differs from that of *Cyp1b1* in many of these tissues (Hayes et al., 1996b; Murray et al., 2001; Okino and Whitlock, 1995). For example, *Cyp1b1* is particularly highly expressed in fibroblasts and steroidogenic tissues (Christou et al., 1995).

Though a considerable amount of literature is available on the role of transcription factors and coactivators in transcriptional regulation of the *Cyp1a1* and *Cyp1b1* genes, so far very few studies have focused on the possible role of epigenetic mechanisms in the regulation of these genes. The most commonly studied epigenetic mechanisms in reference to transcriptional regulation are histone modifications and DNA methylation. DNA methylation can inactivate genes and suppress gene expression directly by interfering with the binding of transcription factors, or indirectly by attracting methylated DNA binding factors that recruit histone deacetylases, leading to gene silencing (Rivenbark et al., 2006). A variety of genes have been shown to be inactivated in different cancer and immortal epithelial cell lines through methylation-dependent gene silencing, and previous studies have reported that cytochromes P450 can be regulated by DNA hypermethylation or hypomethylation (Nakajima et al., 2003; Rivenbark et al., 2006; Tokizane et al., 2005; Umeno et al., 1988; Vieira et al., 1996) in certain cancerous tissues. Furthermore, CpG islands have been identified in the enhancer and in the promoter regions of the human *CYP1A1* and *CYP1B1* genes, suggesting the potential

role of DNA methylation in the suppression of *CYP1A1* and *CYP1B1* gene expression (Okino et al., 2006; Shen and Whitlock, 1989; Tokizane et al., 2005). Schnakenburger and coworkers (Schnakenburger et al., 2007) also demonstrated the role of epigenetic modifications in inhibition of benzo[a] pyrene induced *Cyp1a1* gene expression by chromium.

In the present study we characterize the induction of the *Cyp1a1* and *Cyp1b1* mRNAs by dioxin in mouse Hepa-1 and C3H10T1/2 cells. In particular we demonstrate a role for both diffusible regulatory proteins and epigenetic modifications in the dioxin-induced differential regulation of the *Cyp1a1* and *Cyp1b1* genes in these two cell lines.

Materials and Methods

Cell culture

Mouse hepatoma cell line Hepa1c1c7 (Hepa-1), its derivative, Hepa-1 OT, and the mouse embryonic fibroblast cell line, C3H10T1/2, were grown as monolayers and maintained in α -minimal essential media containing 10% fetal bovine serum, 5% fungizone, 5% Pen-Strep (Invitrogen, Carlsbad, CA) at 37 ° C and 5 % CO₂. Somatic cell hybrid clones were maintained in 2×10^{-5} hypoxanthine, 2×10^{-6} M aminopterin, 3×10^{-5} M thymidine, and 3mM ouabain (HATO media).

Reverse Transcription and Real Time PCR

The levels of the mRNAs for *Cyp1a1*, *Cyp1b1* and the constitutively expressed ribosomal subunit 36b4, were determined by SYBR Green or Taqman quantitative real-time polymerase chain reaction (QPCR). Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacture's protocol. Reverse transcription was performed using a Taqman reverse transcriptase kit (Applied Biosystems, Foster City, CA) according to the manufacture's protocol. Five micrograms of total RNA was used in a 20 μ l reaction and amplified by cycling between 25 ° C for 10 minutes, 48 ° C for 30 minutes, and 95 ° C for 5 minutes, using the Icycler thermalcycler (BioRad, Hercules, CA). All cDNAs were diluted 10-fold in autoclaved water. Dual labeled Taqman probes for *Cyp1a1* and *36b4* mRNAs were as described previously (Kliwer et al., 2001), and synthesized by Integrated DNA Technologies, Inc (Coralville, IA). A 20x mix of *Cyp1b1* primers and dual labeled probe was purchased from Applied Biosystems Assays-on-Demand (cat # Mm00487229_m1). Taqman assays were performed using an Applied Biosystems 7700 machine. QPCR reaction parameters were 50 ° C for 2 minutes, 95 ° C for 10 minutes, 92 ° C for 15 seconds, 60 ° C for 1 minute, then back to the 92 ° C step 40 times. All gene expressions were reported relative to the house keeping gene, 36b4. We then used the same standard curve generated from cDNA synthesized from 72 hrs dioxin treated mRNA samples allowing us to directly compare the expression of a particular gene across cell lines. The levels of *Cyp1a1* and AHR mRNAs in all samples were compared with a standard RNA sample obtained from Hepa-1 cells treated for 72 hrs with dioxin. The levels of the *Cyp1b1* were compared with a standard RNA sample obtained from C3H 10T1/2 cells treated for 72 hrs with dioxin. In all real-time PCR analyses, three replicates were analyzed for each biological sample, and the standard deviations from those three replicates are reported.

Chromatin Immunoprecipitation (ChIP) assay

Hepa-1 and C3H10T1/2 cells were treated with 10 nM dioxin for the indicated time periods. The ChIP analyses were carried out as described previously by Beedanagari *et al.* (Beedanagari et al., 2009). The human AhR antibody used was from our own laboratory (Zhang et al., 1996). The primers used for the real time PCR of *Cyp1a1* enhancer region were forward 5'-AAGCATCACCCCTTTGTAGCC-3' and reverse 5'-CAGGCAACACAGAGAAGTCG-3'. Primers for the *Cyp1b1* enhancer region were forward 5'-GCTCTGTACGCCAACAAACG-3' and reverse 5'-GCTCTGTACGCCAACAAACG-3'. The ChIP real-time PCR results were

reported relative to that of total inputs and the background, after the background signals represented by the IgG controls were subtracted from all samples. All the ChIP analyses were carried out at least three times, and the data from representative experiments are reported in the manuscript.

Somatic cell hybrid isolation

Hepa-1 OT cells were fused with the C3H10T1/2 cell line, to generate stable somatic cell hybrid clones as described previously (Hankinson, 1983). OT is a derivative of Hepa-1 cells that is resistant to ouabain and 6-thioguanine. 1×10^5 Hepa-1 OT cells and 1×10^5 C3H10T1/2 cells were seeded in 60 mm tissue culture dishes and incubated at 37 ° C. 24 hours later 50% polyethylene glycol-1000 and 5% DMSO was added for 1 minute, and then the new medium was replaced with normal α MEM with 10% FBS. 24 hours later, cells were trypsinized and 1×10^5 cells were seeded into 60 mm dishes containing HATO plus 0.2 μ g/ml 12-O-tetradecanoyl phorbol-13-acetate (TPA) media as described previously (Hankinson, 1983). The presence of TPA in this medium eliminates metabolic cooperation between the two parental cell lines, which can potentially allow the survival of closely apposing parental cells. Cells were grown at 37 ° C for 14 days. On day 14, individual clones were isolated with cloning cylinders and passaged sequentially in HATO medium, HT medium (containing hypoxanthine and guanine), and then α -minimal essential medium without supplements.

Determination of DNA content

The DNA content of the hybrid clones was determined by propidium iodide staining and flow cytometric analysis. 1×10^6 cells were placed into 1.5 ml Eppendorf tubes and centrifuged at $700 \times g$ for five minutes. The pellet was resuspended in 1 ml of buffer (250 mg sodium citrate, 750 ml Triton- 100, 5 mg Rnaselease A). 50 μ l of propidium iodide (500 μ g/ml) was then added to each tube, which were then incubated at 4 ° C for 30 minutes in the dark. Solutions were placed into polystyrene centrifuge tubes (Fisherbrand, Waltham, MA) for flow cytometry analysis.

5-aza-2'-deoxycytidine and Trichostatin-A Treatments

C3H10T1/2 and Hepa-1 cells were plated on day zero and treated with 5 μ M 5-aza-2'-deoxycytidine (5-AzaC; Sigma, St. Louis, MO) starting on day one for three days. Cells were treated with 100 nM Trichostatin-A (TSA; Sigma, St. Louis, MO) and/or dioxin two days after plating for 24 hrs. Control samples were treated with the vehicle, DMSO.

Statistical Analysis

All statistical analyses were done using ANOVA, followed by a post hoc test, Tukey's test to determine the significance of differences in gene expression between samples. Significance is presented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the appropriate controls.

Results

Dioxin selectively induces Cyp1a1 mRNA in Hepa-1 cells and Cyp1b1 mRNA in C3H10T1/2 cells

Cyp1a1, Cyp1b1 and AhR mRNA levels were measured using real time PCR in the Hepa-1 and C3H10T1/2 cell lines at 12hrs, 24hrs and 48 hrs after the initiation of dioxin treatment. Dioxin strongly induced Cyp1a1 mRNA expression levels in Hepa-1 cells by 12 hrs and similar expression levels were observed through 48 hrs of treatment (Fig. 1A). The Cyp1b1 expression levels in Hepa-1 cells were extremely low and were not induced by dioxin, [representing $< 0.2\%$ of the dioxin induced C3H10T1/2 levels]; (Fig. 1B, Note the different scales on the y-axes of the two figures). The levels of Cyp1a1 mRNA in C3H10T1/2 cells were extremely low,

[representing <0.02% of the dioxin-induced Hepa-1 levels] (Fig. 1D) and were not induced by dioxin treatment. Cyp1b1 mRNA levels were high in C3H10T1/2 cells and were induced three fold by dioxin (Fig. 1E). AhR mRNA expression levels remained unchanged with dioxin treatment in both the Hepa-1 and C3H10T1/2 cell lines. However, the expression levels of AhR in Hepa-1 cells were higher than those in C3H10T1/2 cells (Fig. 1C and 1F).

Dioxin induces association of AhR with the Cyp1a1 and Cyp1b1 enhancers in both cell lines

Chromatin immunoprecipitation (ChIP) experiments were performed to determine whether the AhR protein is recruited to the enhancer of the *Cyp1a1* and *Cyp1b1* genes in Hepa-1 and C3H10T1/2 cells. The results demonstrate that dioxin induced rapid association of the AhR to the enhancers of both the *Cyp1a1* and *Cyp1b1* genes in both Hepa-1 and C3H10T1/2 cells (Fig. 2A and 2B). These data suggest that the lack of dioxin induction of *Cyp1a1* gene in C3H 10T1/2 cells and *Cyp1b1* gene in Hepa-1 cells is not due to lack of AhR binding at the XREs, but is likely due to the action of downstream transcriptional regulatory mechanisms.

Somatic cell hybrid clones of Hepa-1 and 10T ½ cells express high levels of both Cyp1a1 and Cyp1b1 mRNAs after dioxin induction

Somatic cell hybrid clones were then constructed to investigate the potential role of diffusible regulatory proteins in determining lack of dioxin induction of the *Cyp1a1* gene in C3H 10T1/2 cells and the lack of induction of the *Cyp1b1* gene in Hepa-1 cells. Hepa-1 OT, a derivative of Hepa-1 cells selected for resistance to both 6-thioguanine and ouabain, was fused with C3H10T1/2 cells. Only hybrid clones arising from fusion of both parental cell lines should survive in HATO + TPA medium. Three individual hybrid clones Hepa*10T1/2 c1, c2, and c3 were isolated and propagated. In order to determine DNA content, cells were stained with propidium iodide and subjected to flow cytometric analysis. C3H10T1/2 and Hepa-1 cells displayed a large G1 peak at 200 arbitrary units on the x-axis (Fig. 3) and another smaller G2 peak at approximately 400 arbitrary units. The Hepa*10T1/2 clones exhibited G1 and G2 peaks at approximately 400 and 800 arbitrary units respectively. These results demonstrate that the Hepa*10T1/2 clones exhibited twice the DNA contents of Hepa-1 and C3H10T1/2 cells, indicating that they are bona fide hybrid clones of the parental cell lines (Fig. 3).

The three hybrid clones were analyzed for Cyp1a1 mRNA and Cyp1b1 mRNA inducibility. The hybrid clones exhibited substantially greater levels of constitutive expression of Cyp1a1 mRNA than the parental Hepa-1 cells, but they were nevertheless highly inducible by dioxin (Compare Figs. 4A and 1A), although the induced Cyp1a1 mRNA levels in the hybrids were approximately half of that in the parental Hepa-1 cells. The three hybrid clones expressed lower constitutive levels of Cyp1b1 mRNA compared with C3H10T1/2 cells (Figs. 4B and 1E), but the levels were inducible by dioxin. These results suggest that diffusible repressors are unlikely to be involved in determining the lack of dioxin induction of the *Cyp1a1* gene in C3H 10T1/2 cells and the *Cyp1b1* gene in Hepa-1 cells, as the somatic hybrids express and are inducible by dioxin for both *Cyp1a1* and *Cyp1b1* genes.

Effect of 5-AzadC and TSA on Cyp1a1, Cyp1b1, and AhR expression

We then investigated the potential role of DNA methylation and histone acetylation in the regulation of these genes. 5-AzadC is a nucleotide analogue that demethylates genomic DNA and is commonly used to study the role of DNA methylation in the regulation of genes (Issa et al., 2005; Karpf and Jones, 2002). We treated both Hepa-1 and C3H10T1/2 cells with 5 μ M 5-AzadC for 72 hrs, (including dioxin or vehicle control in the last 24 hrs), to study its effect on constitutive and dioxin-inducible expression of the *Cyp1a1* and *Cyp1b1* genes. In Hepa-1 cells, 5-AzadC treatment had little or no effect on dioxin-induced expression of the *Cyp1a1* gene (Fig. 5A) and led to only a modest increase in dioxin-induced expression of the *Cyp1b1* gene to levels approximately 10% of those in dioxin-induced C3H10T1/2 cells (Fig.

5B & 1D, note the values on the Y-axes). In C3H10T1/2 cells, 5-AzadC treatment led to a dramatic increase in dioxin-induced expression of the *Cyp1a1* gene, to levels comparable to those in dioxin-induced *Cyp1a1* in Hepa-1 cells (Figs. 6A and 1A). However, 5-AzadC treatment had little or no effect on dioxin-induced expression of the *Cyp1b1* gene in C3H10T1/2 cells (Fig. 6B). 5-AzadC also had little or no effect on the constitutive and dioxin-induced expression of the *AhR* gene in either cell line (Fig 5C and 6C).

Trichostatin-A (TSA) is an inhibitor of histone deacetylation, and thereby enhances the acetylation of histones, thus generally favoring gene expression. To study the potential role of histone acetylation in the regulation of the *Cyp1a1*, *Cyp1b1* and *AhR* genes, we treated the Hepa-1 and C3H10T1/2 cells with 100 nM TSA for 24 hrs. TSA treatment for any periods longer than 24 hrs was cytotoxic to both cell lines, causing approximately 50% cell death (data not shown). Where indicated, the cells were also treated with 100 nM dioxin simultaneously with TSA treatment, to induce the expression of the *Cyp1a1*, and *Cyp1b1* genes. In Hepa-1 cells, TSA caused modest increases in dioxin-induced *Cyp1a1* and *Cyp1b1* mRNA levels (Fig. 5A and 5B), and only a slight increase in the expression of *AhR* (Fig. 5C). However, TSA caused a marked increase in the expression of the *Cyp1a1* gene in C3H10T1/2 cells (although to a lesser extent than 5-AzadC), a modest increase in expression of the *Cyp1b1* gene, and a more marked increase in the expression of the *AhR* gene (Fig. 6A, 6B and 6C). Concomitant 5-AzadC and TSA treatment led to greater reactivation of the *Cyp1b1* gene in Hepa-1 cells and the *Cyp1a1* gene in C3H10T1/2 cells compared with the treatment of each agent on its own (Figure 7). The results from the 5-AzadC and TSA studies indicate that both DNA methylation and histone acetylation play significant roles in the regulation of the *Cyp1a1* gene in C3H10T1/2 cells, but a less significant role in the regulation of *Cyp1b1* in these cells or *Cyp1a1* and *Cyp1b1* in Hepa-1 cells.

Discussion

The *Cyp1a1* and *Cyp1b1* enzymes both oxidize PAHs to begin the process of their metabolism. The *Cyp1a1* and *Cyp1b1* genes are similar in their enhancer structure, and are transcriptionally activated by similar compounds. However, their gene expression patterns are significantly different, especially with regard to tissue distribution. In this study we investigated the expression and dioxin-inducibility of the *Cyp1a1* and *Cyp1b1* mRNAs in a mouse hepatocyte cell line and a mouse embryonic fibroblast cell line. We found that dioxin induced *Cyp1a1* gene expression to markedly high levels in Hepa-1 cells, whereas *Cyp1b1* gene expression was detectable only at extreme low levels using real time PCR, and was not inducible by dioxin. In contrast, in C3H10T1/2 cells, dioxin induced *Cyp1b1* gene to markedly high levels, whereas *Cyp1a1* gene expression was detectable only at extreme low levels using real time PCR and was not inducible by dioxin. Most interestingly we observed the recruitment of *AhR* to the enhancer regions of the *Cyp1a1* gene in C3H 10T1/2 cells and the *Cyp1b1* gene in Hepa-1 cells, despite their lack of induction by dioxin. Eltom and coworkers also reported that Hepa-1 cells express high levels of *Cyp1a1* mRNA but low levels of *Cyp1b1* mRNA, and that the reverse was the case for C3H10T1/2 cells (Eltom et al., 1999). However, their results differ from ours in that they observed dioxin inducibility of the *Cyp1a1* and *Cyp1b1* genes in C3H10T1/2 and Hepa-1 cells, respectively. We have no explanation for the differences in our results from those of Eltom and coworkers.

We utilized somatic cell hybridization to investigate the mechanisms responsible for the lack of dioxin-induced expression of *Cyp1a1* in C3H10T1/2 cells, and *Cyp1b1* expression in Hepa-1 cells. The somatic cell hybrids were found to be inducible for both cytochromes P450, and expressed high levels of both the P450s under dioxin-induced conditions. These results make it unlikely that there exists a diffusible repressor for the *Cyp1b1* gene in Hepa-1 cells and a diffusible repressor for the *Cyp1a1* gene in C3H10T1/2 cells. The observations are however,

compatible with two other potential explanations. (i) C3H10T1/2 and Hepa-1 cells lack specific factors necessary for the transcriptional regulation of the *Cyp1a1* and *Cyp1b1* genes, respectively. These factors could be different transcriptional coactivators that cooperate with AhR/Arnt in a gene specific fashion, or proteins that regulate the expression of the two cytochromes P450 independently of AhR/Arnt. These factors could possibly be silenced in the putatively non-expressing cell line in each case by epigenetic mechanisms. (ii) The *Cyp1b1* gene in Hepa-1 cells and the *Cyp1a1* gene in C3H10T1/2 cells are directly silenced by epigenetic mechanisms, and the corresponding gene from the non-silenced parental cell is expressed in the hybrid clones.

The epigenetic mechanisms that could potentially play a role in silencing are DNA methylation and chromatin (histone) modifications. The results from 5-AzadC experiments looking at the role of DNA methylation in transcriptional regulation of these genes demonstrated that 5-AzadC led to strong reactivation of the *Cyp1a1* gene in C3H10T1/2 cells, but only modest reactivation of the *Cyp1b1* gene in Hepa-1 cells. Since AhR mRNA levels are only slightly increased after 5-AzadC treatment in Hepa-1 cells, and even slightly diminished in C3H10T1/2 cells, the increased levels of the cytochromes P450 in these cell lines after 5-AzadC treatment are not ascribable to changes in the levels of AhR. These data support the hypothesis that DNA methylation is responsible directly or indirectly for silencing the *Cyp1a1* gene in C3H10T1/2 cells, but is only partially responsible for silencing the *Cyp1b1* gene in the Hepa-1 cell line. The results from TSA experiments looking at the role of histone acetylation in transcriptional regulation of these genes demonstrated that TSA treatment markedly increased *Cyp1a1* expression in C3H10T1/2 cells, but had only a modest effect on *Cyp1a1* expression in Hepa-1 cells and on *Cyp1b1* expression in both Hepa-1 and C3H10T1/2 cells. The increase in AhR expression in C3H10T1/2 after TSA treatment may partially contribute to the increased levels of expression of the *Cyp1a1* and *Cyp1b1* genes under these conditions. Nakajima and coworkers (Nakajima et al., 2003) also reported analogous results where both 5-AzadC and TSA reactivated the dioxin-induced expression of *CYP1A1*, *CYP1A2*, and *CYP1B1* genes in the human HeLa cell line. Furthermore, simultaneous 5-AzadC and TSA treatment led to greater reactivation of *Cyp1a1* gene expression in C3H10T1/2 cells and *Cyp1b1* gene expression in Hepa-1 cells compared with treatment of each agent on its own. Together these data strongly suggest that both DNA methylation and histone deacetylation play a significant role in silencing of the *Cyp1a1* gene in C3H10T1/2 cells, but a more modest role in silencing of the *Cyp1b1* gene in Hepa-1 cells. Our results are consistent with recent observations that DNA methylation and histone modifications positively interact during transcriptional regulation of epigenetically regulated genes (Cedar and Bergman, 2009; Fuks, 2005).

Analysis of the proximal promoter and enhancer regions (encompassing all the known functional XREs of the *Cyp1a1* and *Cyp1b1* genes) for CpG sites using the CpG island searcher software (Takai and Jones, 2003), revealed very few CpG sites in either gene. Analysis of –1080 bp upstream of the transcriptional start site (TSS) revealed only 6 CpG sites in the mouse *Cyp1a1* gene, compared to 85 CpG sites in the human *CYP1A1* gene. Similar analysis of –1000 bp upstream of the transcriptional start site revealed only 5 CpG sites in the mouse *Cyp1b1* gene, compared to 92 CpG sites in the human *CYP1B1* gene. Both human *CYP1A1* and *CYP1B1* were previously shown to be transcriptionally silenced in different cancers by DNA methylation, correlating with the abundance of CpG site at the regulatory regions of these genes (Okino et al., 2006; Shen and Whitlock, 1989; Tokizane et al., 2005). Thus the low number of CpG sites at the regulatory regions of the mouse *Cyp1a1* and *Cyp1b1* genes suggests that it is unlikely that 5-AzadC reactivates expression of the *Cyp1a1* gene in C3H10T1/2 cells or the *Cyp1b1* gene in Hepa-1 cells by directly demethylating cytosine residues in the promoter regions of these genes. It should be noted that the XRE sequence contains a CpG site, and methylation of this sequence inhibits binding of AhR/ARNT to the XREs (Shen and Whitlock, 1989). Since we found that dioxin induced recruitment of AhR to the enhancer region of the

Cyp1b1 gene in Hepa-1 cells and the *Cyp1a1* gene in C3H10T1/2 cell, lack of dioxin inducibility of these genes in these cell lines is unlikely to be due to cytosine methylation at the XREs. 5-AzadC therefore likely reactivates *Cyp1a1* expression in C3H10T1/2 cells and partially reactivates *Cyp1b1* expression in Hepa-1 cells by demethylating the gene for specific transcriptional factor(s) or coactivator(s) necessary for their expression. Thus DNA methylation probably indirectly regulates transcription of these genes.

In conclusion, we hypothesize that the lack of expression of *Cyp1a1* in C3H10T1/2 and *Cyp1b1* in Hepa-1 cells is likely ascribable to the lack of expression of specific transcription factors, which in the case of the factor involved in the induction of *Cyp1a1* in C3H10T1/2 cells is silenced by DNA methylation.

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Hepa-1 Cells

C3H10T1/2 Cells

Figure 1A

Figure 1D

Figure 1B

Figure 1E

Figure 1C

Figure 1F

Fig 1. Cell specific induction of Cyp1a1, Cyp1b1 and AhR

Hepa-1 and C3H10T1/2 cells were treated with 10nM dioxin for 12 hrs, 24 hrs, and 48 hrs or with vehicle DMSO (as control) for 48 hrs. RNA was isolated and mRNA levels were assayed by real time PCR. The relative amounts of Cyp1a1, Cyp1b1, and AhR mRNAs from Hepa-1 (grey bars) and C3H10T1/2 (black bars) were corrected against the levels of the mRNA expression for the constitutively expressed ribosomal subunit, 36b4. The levels of the AhR and Cyp1a1 mRNAs in all the samples were compared with a standard RNA sample obtained from Hepa-1 cells treated for 72 hrs with dioxin. The levels of Cyp1b1 in all samples were compared with a standard RNA sample obtained from C3H 10T1/2 cells treated for 72 hrs with dioxin.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with DMSO alone

Figure 2A

Figure 2B

Figure 2C

Figure 2D

Fig 2. AhR associates with the *Cyp1a1* and *Cyp1b1* enhancer region in Hepa-1 and C3H10T1/2 cells
Cells were treated with 10 nM dioxin for the indicated times and subjected to ChIP analysis. AhR recruitment levels measured by real-time PCR of ChIP samples are reported relative to that of total input.

Fig 3. Flow cytometric analysis of propidium iodide stained cells
Intensity of propidium iodide staining is plotted against number of cells.

Cyp1a1 Gene Expression

Figure 4A

Cyp1b1 Gene Expression

Figure 4B

Fig 4. Somatic cell hybrid clones are inducible for both *Cyp1a1* and *Cyp1b1*

Hepa*10T1/2 somatic cell hybrid clones were treated with 10 nM dioxin for 24 hours. RNA was isolated, subjected to reverse transcription, and mRNA levels were assayed by real time PCR. The levels of *Cyp1a1* and AHR mRNAs in all the samples were compared with a standard RNA sample obtained from Hepa-1 cells treated for 72 hrs with dioxin. The levels of the *Cyp1b1* mRNA were compared with a standard RNA sample obtained from C3H10T1/2 cells treated for 72 hrs with dioxin. The mRNA levels of DMSO treated samples were indicated with grey bars and dioxin treated samples were indicated with black bars.

Fig 5. Effect of DNA methylase inhibitors and deacetylase inhibitors on Cyp1a1 and Cyp1b1 expression in Hepa-1 cells

Hepa-1 cells were treated with either 5-AzadC for 72 hrs or TSA for 24 hrs or DMSO (vehicle). The cells were also treated with DMSO (grey bars) or dioxin (black bars), during the last 24 hrs of incubation. Cyp1a1 (Fig. 5A), Cyp1b1 (Fig. 5B) and AhR (Fig. 5C) mRNA levels were then quantified and normalized to the mRNA for the housekeeping gene, 36b4. The levels of Cyp1a1 and AHR mRNAs in all the samples were compared with a standard RNA sample obtained from Hepa-1 cells treated for 72 hrs with dioxin. The levels of Cyp1b1 were compared with a standard RNA sample obtained from C3H 10T1/2 cells treated for 72 hrs with dioxin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with dioxin alone.

Fig 6. Effect of DNA methylase and histone deacetylase inhibitors on Cyp1a1 and Cyp1b1 expression in C3H 10T1/2 cells

C3H10T1/2 cells were treated with either 5-AzaC for 72 hrs, or TSA for 24 hrs or DMSO (vehicle) and the expression levels were reported as described for the legend Figure 5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with dioxin alone.

Cyp1b1 Gene Expression in Hepa-1 cells

Figure 7A

Cyp1a1 Gene Expression in C3H 10T1/2 Cells

Figure 7B

Fig 7. Effect of concomitant treatment of DNA methyl transferase and histone deacetylase inhibitors on Cyp1a1 and Cyp1b1 expression

C3H 10T1/2 cells and Hepa-1 cells were treated with either 5-AzadC for 72 hrs, for the last 24 hrs with TSA and/or dioxin were included. The expression levels were reported as described for the legend of Figure 5. DMSO (grey bars); dioxin (black bars). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with dioxin alone.