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Role of Epigenetic Mechanisms in Differential Regulation of the Dioxin-Inducible Human *CYP1A1* and *CYP1B1* Genes[S]

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ABSTRACT

The aryl hydrocarbon receptor (AhR) mediates induction of CYP1A1 and CYP1B1 by 2,3,7,8-tetrachlorodibenzo-ρ-dioxin (dioxin) via binding to xenobiotic-responsive elements (XREs) in their enhancer regions. CYP1A1 and CYPIB1 were both inducible by dioxin in human MCF-7 cells. However, only CYP1A1 was inducible in human HepG2 cells. Further experiments focused on providing an explanation for this last observation. Dioxin induced the recruitment of AHR and the transcriptional coactivators p300 and p300/cAMP response element-binding protein binding protein-associated factor (PCAF) to the *CYP1B1* enhancer in HepG2 cells but failed to induce recruitment of RNA polymerase II (polII) or the TATA binding protein (TBP) and acetylations of histones 3 and 4 or methylation of histone 3 at the promoter. Because p300 was required for dioxin induction of the aforementioned histone modifications at the *CYP1B1* promoter and for induction of CYP1B1 transcription (in MCF-7 cells), the recruitments of p300 and AhR, although necessary, are not sufficient for eliciting the above responses to dioxin. Cytosine residues within CpG dinucleotides at the enhancer, including those within the XREs, were partially methylated, whereas those at the promoter were fully methylated. Treatment of HepG2 cells with 5-aza-2′-deoxycytidine led to partial demethylation of the promoter, restored polII and TBP binding, and CYP1B1 inducibility. Thus, the deficiency of CYP1B1 induction in HepG2 cells is ascribable to cytosine methylation at the promoter, which prevents recruitment of TBP and polII. It is noteworthy that our data indicate that stable recruitment of p300 and PCAF to the *CYP1B1* gene does not require their tethering to the promoter and to the enhancer.

Introduction

Dioxin (2,3,7,8-tetrachlorodibenzo-ρ-dioxin), is a common persistent trace environmental pollutant and the most potent carcinogen known (Huff et al., 1994; Mandal, 2005). Dioxin induces a broad range of biological responses, including robust induction of the *CYP1* family of genes, via its binding to the aryl hydrocarbon receptor (AhR). Besides dioxin, AhR binds to a wide spectrum of environmental pollutants and xenobiotics, including polycyclic aromatic hydrocarbons and certain polychlorinated biphenyls (Hankinson, 1995). Unliganded AhR resides in the cytosol, complexed with p23, 90-kDa heat shock protein, and XAP2/ARA9 (Meyer et al., 1998). Upon ligand binding, AhR translocates to the nucleus, discards its associated proteins, and forms a heterodimer with the aryl hydrocarbon nuclear translocator (ARNT) to form the aryl hydrocarbon receptor complex (Reyes et al., 1992; Pollenz et al., 1994). The aryl hydrocarbon receptor complex binds to xenobiotic-responsive elements (XREs) located in the regulatory regions of AhR-responsive genes, leading to the activation of their transcription.

The CYP1 family includes three genes, *CYP1A1*, *CYP1A2*, and *CYP1B1*, all of which are inducible by AhR agonists. A variety of environmental toxicants and xenobiotics are me-

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ABBREVIATIONS: Dioxin, 2,3,7,8-tetrachlorodibenzo-ρ-dioxin; AhR, aryl hydrocarbon receptor; ChIP, chromatin immunoprecipitation; 5-AzadC, 5-aza-2′-deoxycytidine; polII, RNA polymerase II; CBP, cAMP response element-binding protein binding protein; TBP, TATA binding protein; XRE, xenobiotic-responsive element; PCAF, p300/cAMP response element-binding protein binding protein-associated factor; ARNT, aryl hydrocarbon nuclear translocator; HAT, histone acetylase; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; cDNA, complementary DNA; sh, short hairpin; bp, base pair.

tabolized by these three enzymes. CYP1A1 and CYP1B1 are particularly effective at metabolically activating polycyclic aromatic hydrocarbons, some of which represent important carcinogenic components of cigarette smoke, smog, and cooked foods (Nebert et al., 1993). Both the *CYP1A1* and *CYP1B1* genes contain a cluster of XREs approximately 1 kb upstream of their transcription start sites within regions that represent the enhancers of these genes. Although there are many similarities in the modes of dioxin induction of these two genes, there are interesting differences. *CYP1A1* is usually expressed at very low levels in relevant tissues or cells, and dioxin typically induces its expression hundreds- to thousands-fold. In contrast, the constitutive levels of CYP1B1 are usually substantial, and it is generally induced to a lesser degree than CYP1A1 (Tang et al., 1996). Upon dioxin induction, AhR and the histone acetylase (HAT) coactivators p300 and steroid receptor coactivator 2 are recruited to the enhancers of both the *CYP1A1* and *CYP1B1* genes, and they are required for their induction by dioxin. However, although the ATPase-dependent nucleosome remodeling factor Brahma/switch 2-related gene 1 is recruited to the *CYP1A1* gene upon dioxin treatment and is required for its induction, neither is the case for the *CYP1B1* gene (Taylor et al., 2009). Both the *CYP1A1* and *CYP1B1* genes are expressed in a large number of tissues, although there are differences in the tissue-specific expression of the two genes (Bièche et al., 2007). Our understanding of the precise mechanisms involved in the transcriptional regulation of the *CYP1A1* and *CYP1B1* genes is far from complete, and a fuller understanding of the molecular mechanisms underlying the regulation of these genes will be of great importance to delineate the toxic effects mediated by these enzymes.

Epigenetic modifications play a significant role in transcriptional regulation of genes (Kouzarides, 2007; Li et al., 2007). Two of the most important modifications in the context of transcription regulation are covalent chromatin/histone modifications and DNA methylation. Covalent chromatin modifications function either by altering the nucleosomal architecture and/or by affecting the recruitment of nonhistone proteins to chromatin (Kouzarides, 2007; Li et al., 2007). DNA methylation suppresses gene expression either directly by interfering with the binding of transcription factors or indirectly by attracting methylated DNA binding factors that recruit histone deacetylases to generate an inactive heterochromatin structure (Singal and Ginder, 1999; Guo et al., 2002). CpG islands have been identified in the enhancer and in the promoter regions of the human *CYP1A1* and *CYP1B1* genes, and alterations in the DNA methylation status of both the *CYP1A1* and *CYP1B1* genes occur in various types of cancer (Tokizane et al., 2005; Okino et al., 2006; Kang et al., 2008; Habano et al., 2009).

In the current study, we studied the potential role of epigenetic mechanisms in dioxin-induced transcriptional regulation of the *CYP1B1* gene. We demonstrate that the *CYP1B1* gene is silenced in HepG2 cells because of hypermethylation of its promoter, which affects some but not all of the relevant dioxin-induced changes that normally occur at the gene. Our studies provide important insights into both the mechanism of *CYP1B1* induction by dioxin and the mechanisms of gene regulation in mammalian cells in general.

Materials and Methods

Cell Culture and Reagents. The human breast cancer cell line MCF-7, and human hepatic cancer cell line, HepG2, were obtained from the American Type Culture Collection (Manasas, VA) and were grown as monolayers in α-minimal essential media and Dulbecco's modified Eagle's media, respectively, containing 10% fetal bovine serum, 5% Fungizone, 5% penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2. The tissue culture dishes used to grow HepG2 cells were coated with 5 ml of 50 μg/ml poly(L-lysine) and dried before plating the cells. Cells were treated with 100 nM dioxin (Wellington Laboratories, Guelph, ON, Canada) dissolved in dimethyl sulfoxide (DMSO), at a final concentration of 0.1% DMSO in the medium. 5-Aza-2′-deoxycytidine (5-AzadC) was purchased from Sigma-Aldrich (St. Louis, MO). The antibodies used for ChIP analysis are indicated in the supplementary material (Supplementary Table 1).

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 real-time PCR. Total RNA was isolated using RNeasy mini columns (QIAGEN, Valencia, CA) according to the manufacturer's protocol and quantified on a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA). Five micrograms of total RNA was used for complementary DNA (cDNA) synthesis in a 20-μl reaction using Superscript III reverse transcriptase (Invitrogen) and primed with random hexamers (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was performed using incubations at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min, using an iCycler Thermal Cycler (Bio-Rad). cDNAs were diluted 10-fold in autoclaved water. Standard curves were generated using the 72-h dioxin-treated MCF-7 cDNA sample and performing a 10-fold dilution series. The primers for real-time PCR were designed using Primer Express software (Applied Biosystems, Foster City, CA) and were synthesized by Integrated DNA Technologies, Inc (Coralville, IA) (Supplementary Table 2). Real-time PCR assays were performed using an Applied Biosystems 7500 machine. Real-time PCR reaction parameters were 50°C for 2 min, and 40 cycles of 95°C for 10 min, 92°C for 15 s, and 60°C for 1 min. CYP1A1 and CYP1B1 mRNA quantities were normalized to the amount of 36B4 mRNA. The relative expression levels of all genes measured were reported using standard curve generated from MCF-7 cDNA treated for 72 h with dioxin, allowing us to compare HepG2 mRNA expression levels relative to MCF-7 expression levels. 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 Assay. MCF-7 and HepG2 cells were treated with 100 nM dioxin for the indicated amount of time. ChIP analyses were carried out as described previously (Beedanagari et al., 2009). DNA was extracted using DNeasy mini columns (QIAGEN), and finally eluted in a volume of 50 μl using PCR-grade water. The primers used for the housekeeping gene, 36B4, were reported previously (Hsu et al., 2007; Beedanagari et al., 2009). All the ChIP analyses were carried out at least three times, and the data from representative experiments are reported here. The antibodies used in ChIP analyses are listed in Supplementary Table 1.

Sodium Bisulfite Modification and Sequencing. MCF-7 and HepG2 cells were plated on day 0 and treated with 5 μM 5-AzadC (Sigma-Aldrich) starting on day 1 for 72 h. Cells were also treated with 100 nM dioxin on day 3 for 24 h. Control samples were treated with the vehicle, DMSO. The genomic DNA was extracted using the DNeasy kit, (QIAGEN). Bisulfite modification of DNA was performed using the EZ DNA methylation kit according to the manufacturer's protocol (Zymo Research, Orange, CA). The sense strand of bisulfite-modified genomic DNA of the *CYP1B1* enhancer and promoter regions were amplified using two rounds of PCR with specific primers reported previously (Han et al., 2006), followed by quantifi-

cation of CpG methylation by tag-modified bisulfite genomic sequencing as described previously by Han et al., (2006). The amplification products were confirmed and purified from 2% agarose gels and sequenced directly by an outside vendor using the same primer sequences (Genewiz, San Diego, CA). The sequences were analyzed using Sequencher 4.8 version software purchased from Gene Codes Inc. (Ann Arbor, MI).

Statistical Analysis. All the real-time PCR and ChIP analyses were performed at least twice and a representative data set is presented in the figures. All of the real-time PCR samples were run in triplicate, and the standard deviation from these three replicates are presented as standard error bars in the representative graphs.

Results

Effect of Dioxin on the Expression of the *CYP1A1*, *AhR*, and *ARNT* Genes in the MCF-7 and HepG2 Cell Lines. The human breast cancer cell line MCF-7 and human hepatic cancer cell line HepG2 were treated with 100 nM dioxin for 0, 1, 3, 6, 12, 24, 48, and 72 h. This concentration of dioxin maximally induces *CYP1A1* and *CYP1B1* (Taylor et al., 2009) and was not toxic to the cell lines. The CYP1A1, CYP1B1, AhR, and ARNT mRNA levels were quantified using SYBR green real-time PCR. The standard curves for real-time PCR were generated from the corresponding cDNAs derived from MCF-7 cDNAs treated with dioxin for 72 h, allowing us to report the mRNA expression levels of these genes in the two cell lines relative to MCF-7 expression levels. Dioxin treatment had no effect on AhR and ARNT mRNA levels at the different time points tested. However, dioxin markedly induced CYP1A1 mRNA levels in both cell lines. It is noteworthy that dioxin induced CYP1B1 mRNA expression in MCF-7 cells but not in HepG2 cells, in which CYP1B1 mRNA was undetectable both in the presence and absence of dioxin (Fig. 1). Subsequent experiments were directed at determining the basis for the lack of CYP1B1 induction in HepG2 cells.

Effect of Dioxin on Recruitment of AhR and PolII to the Enhancer and Promoter Regions, Respectively, of the *CYP1A1* and *CYP1B1* Genes. We reported previously that, in MCF-7 cells, dioxin induces rapid recruitment of AhR to the enhancer regions of the *CYP1A1* and *CYP1B1* genes, located in each case approximately 1 kb 5′ to the transcriptional start site of the corresponding gene and induced rapid recruitment of RNA polymerase II (polII) to the proximal promoter regions of both genes (Taylor et al., 2009). Here, we investigated the kinetics of AhR recruitment to the enhancers and polII to the promoters of the same genes in HepG2

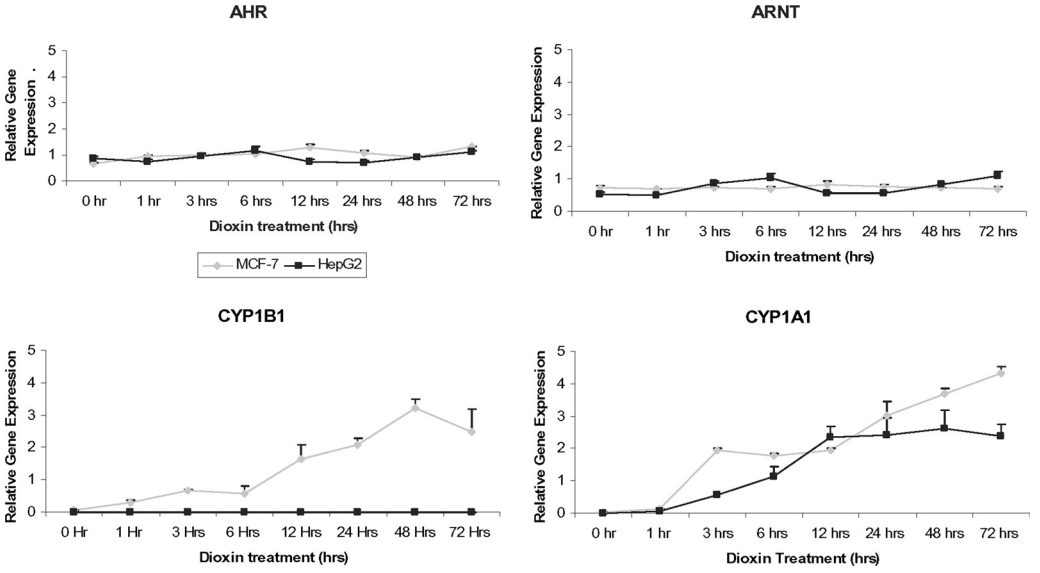

Fig. 1. Effect of dioxin on expression of CYP1A1, CYP1B1, AhR, and ARNT mRNAs. MCF-7 and HepG2 cells were treated with 100 nM dioxin for the indicated times. RNA was isolated, and mRNA levels were assayed by real-time PCR. The relative amounts of AhR, ARNT, CYP1B1, and CYP1A1 mRNAs were corrected against the levels of mRNA expression for the constitutively expressed ribosomal subunit 36B4. All mRNA expression levels were reported using a standard curve generated from a standard cDNA sample obtained from MCF-7 cells treated with dioxin for 72 h.

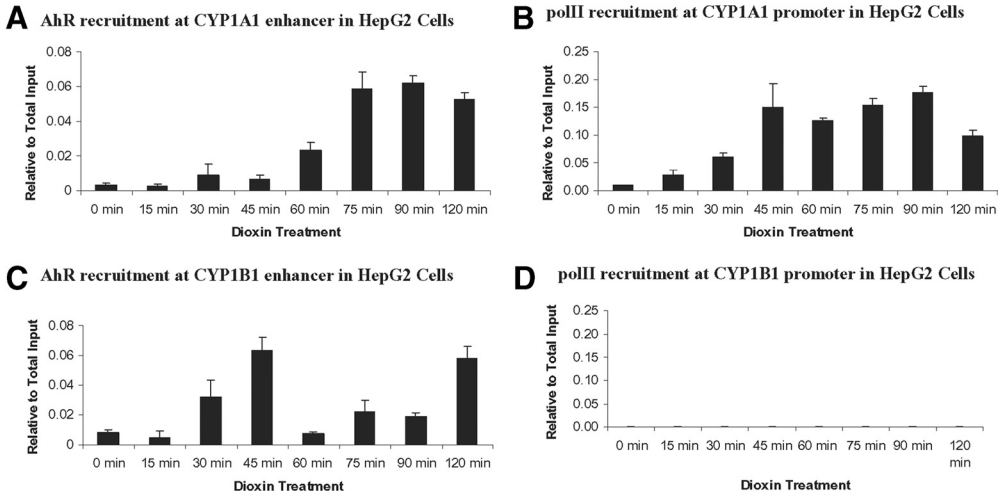

Fig. 2. AhR and polII recruitment to the *CYP1A1* and *CYP1B1* genes in HepG2 cells. HepG2 cells were treated with 100 nM dioxin for the indicated times and were subjected to ChIP analysis. AhR recruitment to the enhancers (A and C) and polII to the promoters (B and D) of the *CYP1A1* and *CYP1B1* genes were measured by real-time PCR and reported relative to total inputs.

cells after dioxin treatment using ChIP analysis. Dioxin rapidly induced AhR recruitment to the enhancers of both the *CYP1A1* and *CYP1B1* genes in HepG2 cells (Fig. 2, A and C). It is noteworthy that AhR binding to the *CYP1B1* enhancer followed a cyclical pattern. Such cycling of AhR binding has been reported previously in some but not all studies on the human *CYP1A1* and *CYP1B1* enhancers after AhR ligand treatment (Hestermann and Brown, 2003; Matthews et al., 2007; Taylor et al., 2009; Wihlén et al., 2009). The basis for this cycling and its potential significance are not clear. Dioxin induced polII recruitment to the *CYP1A1* promoter (Fig. 2B). However, no polII recruitment to the *CYP1B1* promoter was observed in HepG2 cells (Fig. 2D), consistent with the lack of induction of this gene in HepG2 cells. From these studies, we conclude that the loss of dioxin inducibility of *CYP1B1* in HepG2 cells is ascribable to a defect(s) in the induction pathway subsequent to AhR recruitment to the enhancer.

Chromatin Modifications at the Promoter Regions of the *CYP1A1* and *CYP1B1* Genes Correlate with Dioxin Induction. We performed time-course ChIP analyses to investigate whether dioxin induces any changes in the extent of acetylation of histone H3 at lysines 9 and 14 (AcH3K9 and AcH3K14), acetylation of histone H4 (AcH4), and trimethy-

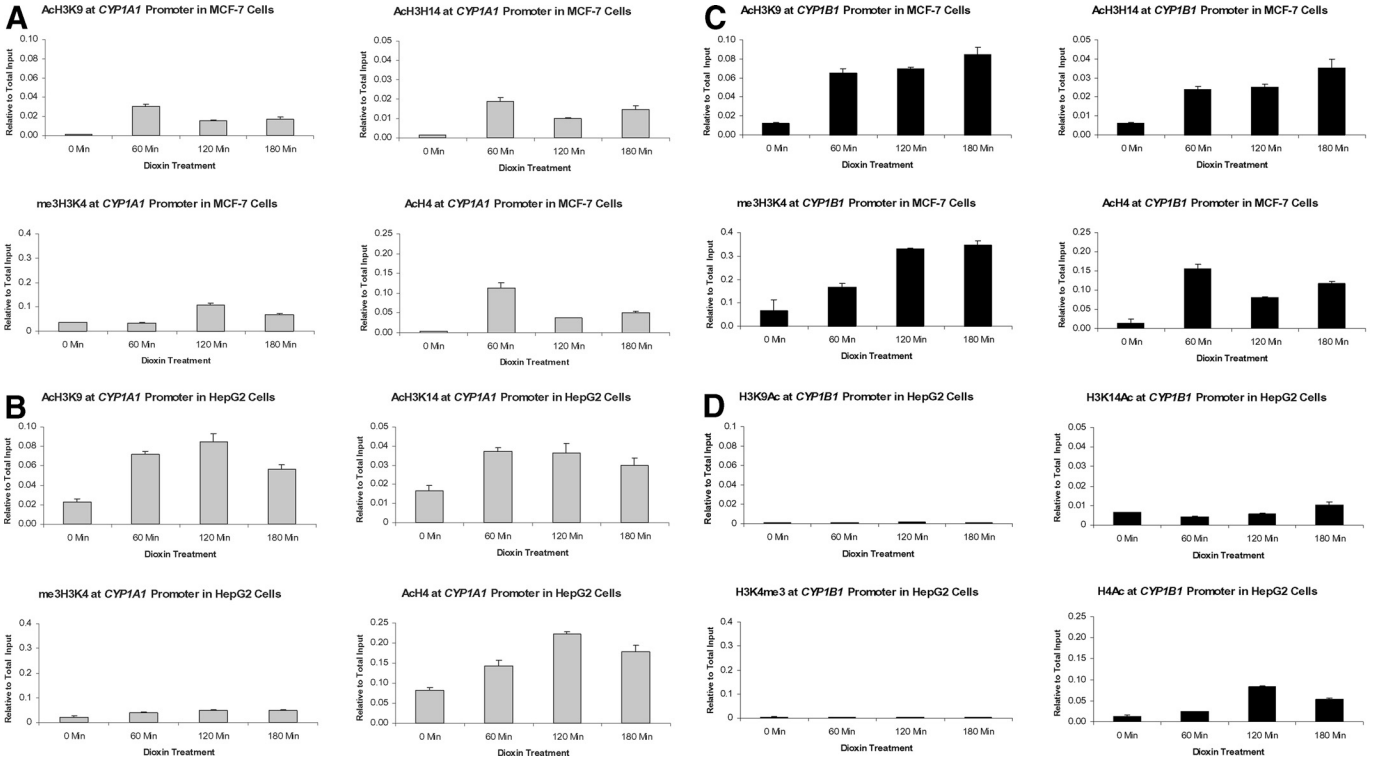

Fig. 3. Characterization of chromatin modifications at the *CYP1A1* and *CYP1B1* promoter regions in MCF-7 and HepG2 Cells. ChIP analyses were carried out using antibodies targeted to four chromatin modifications on MCF-7 and HepG2 cells treated with 100 nM dioxin for the indicated amounts of time at the *CYP1A1* promoter (A and B) and *CYP1B1* promoter (C and D) . The relative levels of the chromatin modifications were measured by real-time PCR and are reported relative to total inputs.

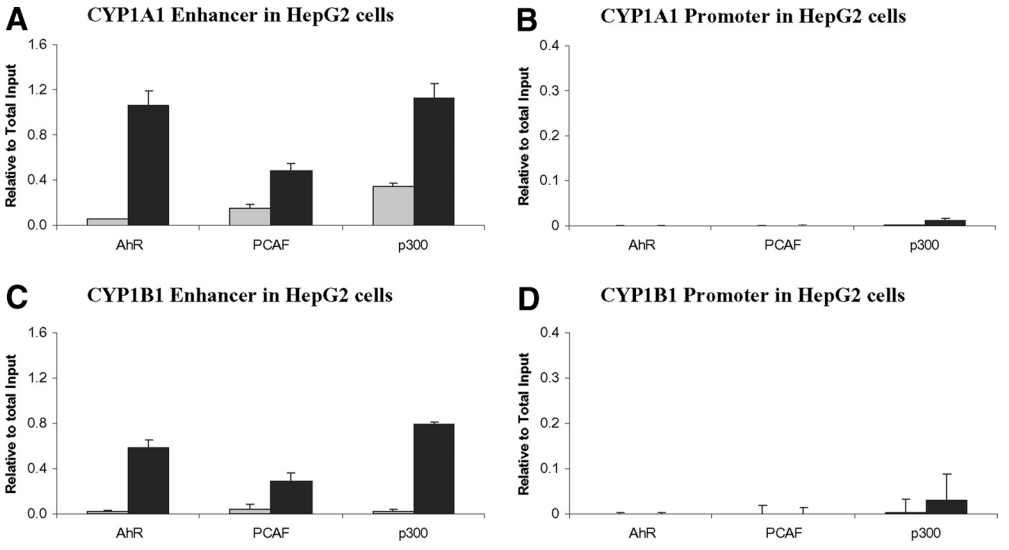

Fig. 4. Coactivator recruitment to the *CYP1B1* and *CYP1A1* enhancer and promoter regions in HepG2 cells ChIP analyses were carried out using antibodies targeted to AhR, PCAF, and p300 in HepG2 cells treated with 100 nM dioxin (■) or with vehicle (DMSO, ▨) for 60 min. AhR, PCAF, and p300 recruitment to the *CYP1B1* and *CYP1A1* enhancer (A and C) and promoter (B and D) regions were measured by real-time PCR and are reported relative to total inputs.

lation of histone H3 at lysine 4 (me3H3K4) at the *CYP1A1* and *CYP1B1* promoters in MCF-7 and HepG2 cells. Dioxin induced increases in these modifications at both of the *CYP1A1* and *CYP1B1* promoters in MCF-7 cells, and these increases reached maximal levels between 60 and 120 min after dioxin treatment (Fig. 3, A and C). The extent of each histone modification at the *CYP1A1* promoter was generally somewhat greater in HepG2 cells than in MCF-7 cells (compare Fig. 3, A and B). In contrast, the extent of the modifications at the *CYP1B1* promoter was much less in HepG2 cells then in MCF-7 cells both before and after dioxin treatment (Fig. 3, C and D). Thus dioxin induction of these chromatin modifications correlates with dioxin inducibility of the *CYP1A1* and *CYP1B1* genes in the two cell lines.

Coactivator Recruitment to the Enhancers and Promoters of the *CYP1B1* and *CYP1A1* Genes in HepG2 Cells. We then studied the recruitment of p300 and the transcriptional coactivator p300/CBP-associated factor (PCAF) to the enhancer and promoter regions of the *CYP1A1* and *CYP1B1* genes in HepG2 cells. Dioxin induced recruitment of p300 and PCAF (and AhR) to the enhancers but not the promoters of the *CYP1A1* and *CYP1B1* genes in HepG2 cells (Fig. 4, A and C). Furthermore, dioxin induced the recruitment of these proteins to the *CYP1B1* enhancer as efficiently as it induced their recruitment to the *CYP1A1* enhancer. Thus, we conclude that the lack of CYP1B1 induction in HepG2 cells is not due to deficiencies in recruitment of either p300 or PCAF to the corresponding enhancer. Furthermore, we conclude that although p300 is required, it is not sufficient for dioxin induction of the above chromatin modifications at the *CYP1B1* enhancer, because these modifications are not induced by dioxin in HepG2 cells. It is noteworthy that the recruitment of coactivators at the promoters of both genes in HepG2 cells was negligible. We made similar observations previously with regard to AhR and p300 recruitment at both genes in MCF-7 cells and furthermore found that polII was recruited to the promoter but not to the enhancer regions of the genes. Thus, under our ChIP protocol conditions, recruitment to the enhancers was distinguishable from recruitment to the promoters.

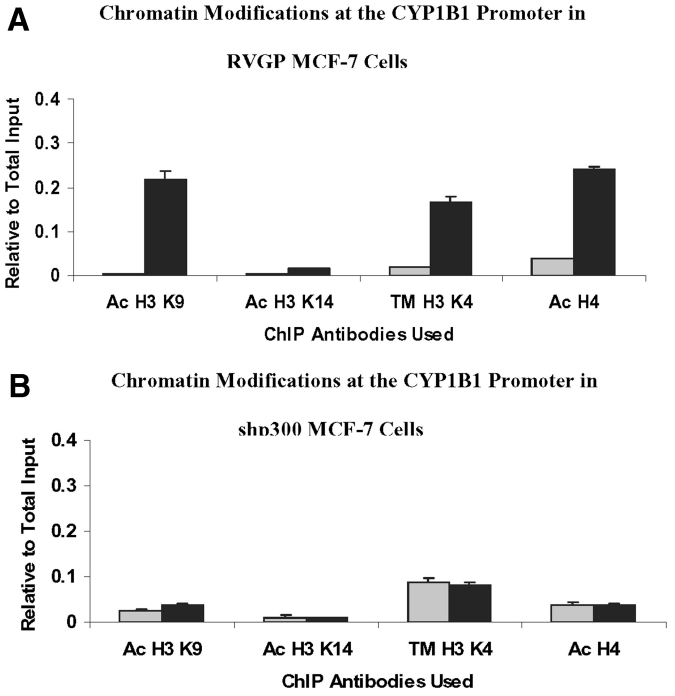

Fig. 5. Effect of p300 knockdown on chromatin modifications at the *CYP1B1* promoter in MCF-7 cells. MCF-7 cells stably knocked down for shp300 or treated with control vector (RVGP MCF-7) were treated with 100 nM dioxin (■) or with vehicle (DMSO, ▨) for 60 min and subjected to ChIP analysis. The chromatin modifications at the *CYP1B1* promoter were measured and reported as described in Fig. 4.

Knockdown of p300 Inhibits Dioxin Induction of Chromatin Modifications at the *CYP1B1* Promoter in MCF-7 Cells. We showed previously that dioxin induces recruitment of p300 to the enhancer regions of the CYP1A1 and CYP1B1 genes in MCF-7 cells. Furthermore, we demonstrated previously that p300 is required for maximal dioxin induction of the CYP1A1 and CYP1B1 mRNAs in this cell line (Taylor et al., 2009). In the current study, we investigated whether p300 is required for dioxin induction of the above chromatin modifications at the *CYP1B1* enhancer. In these experiments, we used MCF-7 cells stably infected with a vector expressing an shRNA for p300 (shp300 MCF-7 cells) that were generated previously in our laboratory. MCF-7 cells stably infected with the empty vector RVGP were used as a control (Taylor et al., 2009). Dioxin treatment increased the levels of the AcH3K9, AcH3K14, AcH4, and me3H3K4 histone modifications at the *CYP1B1* promoter in RVGP MCF-7 cells but failed to induce these modification in shp300 MCF-7 cells (Fig. 5, A and B). We therefore conclude that p300 is required for dioxin induction of these histone modifications at the *CYP1B1* promoter.

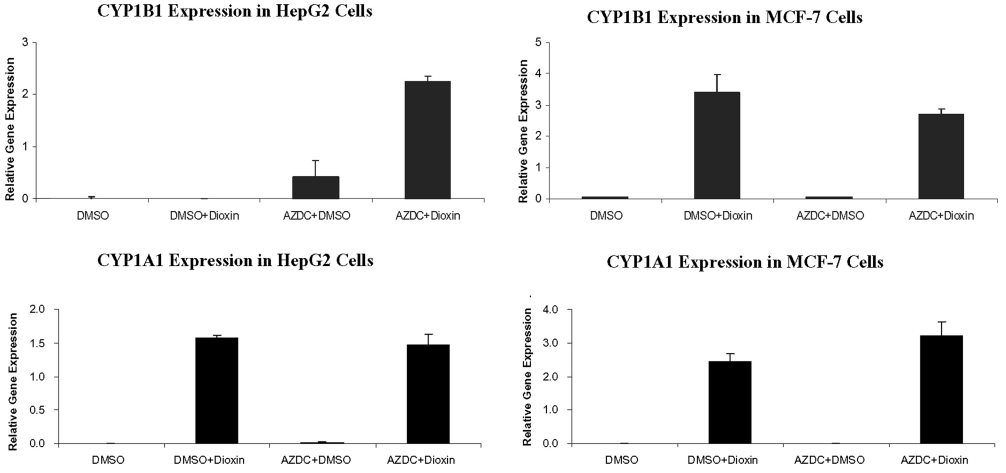

Fig. 6. Effect of 5′-AzadC treatment on CYP1A1 and CYP1B1 expression in MCF-7 and HepG2 cells. MCF-7 and HepG2 cells were treated with 5 μM 5-AzadC or DMSO (vehicle) for 72 h. The cells were also treated with 24 h of either DMSO or dioxin, overlapping with the last 24 h of 5-AzadC treatment. Expression levels of the mRNAs for CYP1B1 and CYP1A1 in HepG2 cells and MCF-7 cells were quantified using real-time PCR and reported relative to the mRNA expression levels of the housekeeping gene, 36B4. All mRNA expression levels were calculated using a standard curve generated from cDNA sample obtained from MCF-7 cells treated with dioxin for 72 h.

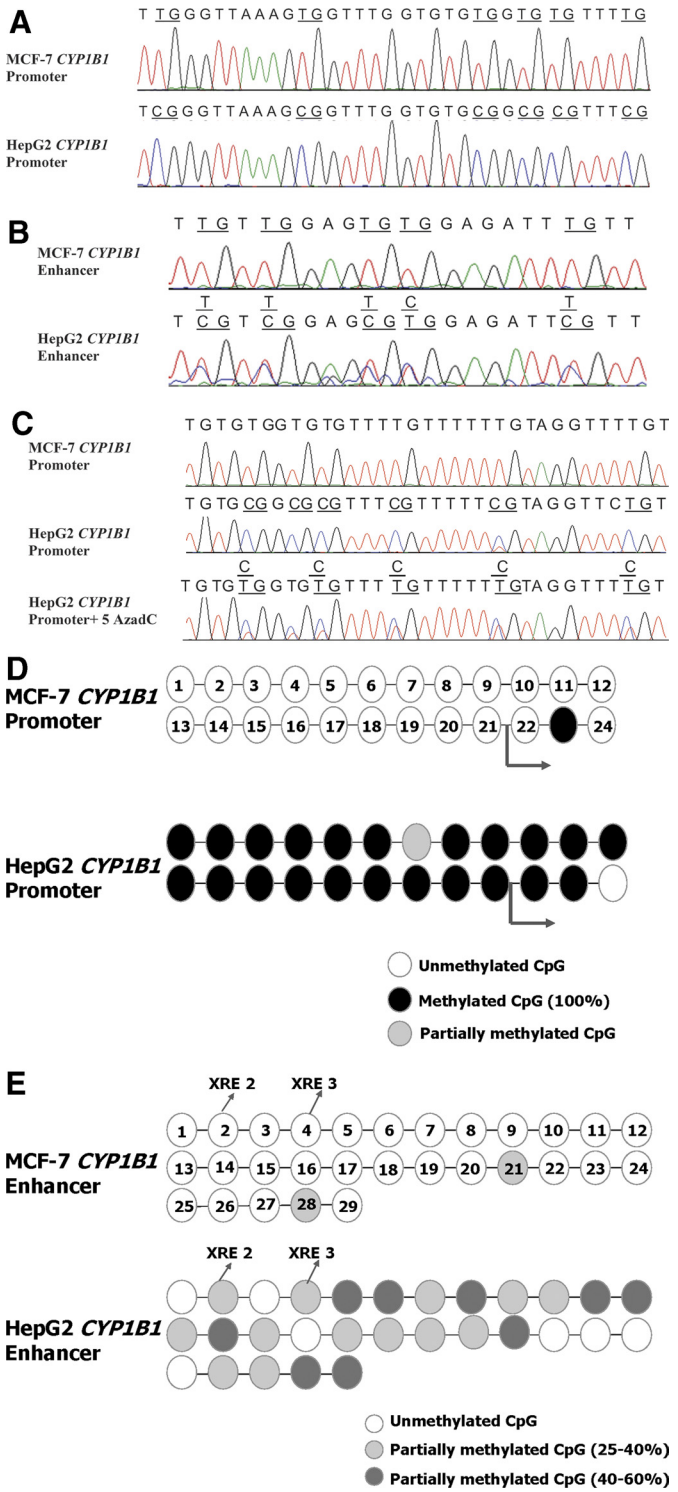

Fig. 7. Bisulfite DNA sequencing of the CYP1B1 enhancer and promoter regions. The MCF-7 and HepG2 enhancer and promoter regions were amplified using bisulfite-converted genomic DNA templates from cells treated or untreated with 5′-AzadC. The bisulfite-converted DNA was amplified using bisulfite sequencing primers, and the amplicons were separated by electrophoresis on 2% agarose gels. The PCR products were excised from the gel, purified, and both the sense and antisense strands were directly sequenced for quantitative analysis of DNA methylation. A representative bisulfite sequencing chromatograms from the *CYP1B1* promoter (A), enhancer (B), and 5-AzadC treated promoter (C) are shown. All of the CpG dinucleotides are underlined. The red peaks of the bisulfite-sequenced chromatogram at CpG dinucleotides represent unmethylated cytosines, and the blue peaks represent methylated cytosines. Overlapping both red and blue peaks indicate a partial methylation status of cytosine at that particular CpG site. The relative heights of the blue versus red peaks from bisulfite sequenced chromatograms were used to calculate the methylation status of cytosines and are represented in terms of the percentage of methylation. The summary of the methylation patterns and percentage of cytosine methylation at each CpG sites of *CYP1B1* enhancer (D) and promoter (E) are represented pictorially.

Reactivation of Dioxin Inducibility of CYP1B1 in HepG2 Cells by 5-AzadC. To examine the role of DNA methylation in the regulation of the *CYP1A1* and *CYP1B1* genes, MCF-7 and HepG2 cells were treated with the DNA methyltransferase inhibitor 5-AzadC for 72 h. Dioxin (100 nM) or the vehicle (DMSO) was included in the medium for the last 24 h before harvest. In HepG2 cells, 5 μM 5-AzadC reactivated dioxin-induced CYP1B1 mRNA expression to a level comparable with that in MCF-7 cells. However, the mRNA expression levels of CYP1A1 in both cell lines and of CYP1B1 in MCF-7 cells were not affected by 5-AzadC treatment (Fig. 6). These results indicate that silencing of the *CYP1B1* gene in HepG2 cells is most likely due to hypermethylation of the *CYP1B1* gene or of a gene encoding a negative regulator of CYP1B1 expression.

DNA Methylation at the CYP1B1 Enhancer and Promoter. High frequencies of CpG dinucleotides occur at both the enhancer and promoter of the human *CYP1B1* gene, and these regions represent "CpG islands." Thus, there are 29 CpG sites within a 360-bp sequence (−560 to −920 bp) encompassing the enhancer, and 24 CpG sites within a 280-bp sequence (−260 to + 20) encompassing the promoter. To study the cytosine methylation status of the *CYP1B1* promoter and enhancer regions, we used the tag-modified bisulfite genomic sequencing (tBGS) procedure as described by Han et al. (2006). Direct bisulfite sequencing was performed on the amplified PCR products of the *CYP1B1* regulatory regions without cloning. Bisulfite treatment of DNA converts unmethylated cytosines to uracils, which will be ultimately converted to thymines during the amplification process, whereas the methylated cytosines remain unaffected. Representative bisulfite sequencing chromatograms are presented in the Fig. 7, A to C. In these figures, red peaks in positions corresponding to cytosines in genomic DNA represent unmethylated cytosines, and blue peaks represent methylated cytosines. Bisulfite sequencing demonstrated that 22 of the 24 CpG sites are fully methylated, 1 is partially methylated, and 1 is unmethylated in the *CYP1B1* promoter in HepG2 cells, whereas only one of these sites is methylated in MCF-7 cells (Fig. 7D). Of the 29 CpG sites, 22 were found to be partially methylated in the *CYP1B1* enhancer in HepG2 cells. In contrast, only two of these sites were partially methylated in MCF-7 cells, the remainder being totally unmethylated (Fig. 7E). The XRE sequence harbors a CpG site. Methylation of the CpG dinucleotide within the XRE prevents binding of the AhR/ARNT dimer in vitro and inhibits the ability of the XRE to mediate dioxin induction of gene transcription (Shen and Whitlock, 1989). We were therefore particularly interested in the methylation status of the CpG dinucleotides in the XRE2 and XRE3 sequences, which are located in the enhancer and play the most significant role in the dioxin-mediated induction of *CYP1B1* (Shehin et al., 2000; Tsuchiya et al., 2003). Bisulfite sequencing revealed that both XREs were approximately 30% methylated in HepG2 cells (Fig. 7E). Thus, either or both XRE2 and XRE3 are unmethylated in a majority of chromosomes, consistent

with the observation that AhR is recruited to the *CYP1B1* enhancer of HepG2 cells after dioxin treatment. In summary, these studies indicate that the loss of dioxin induction of CYP1B1 in HepG2 cells is due to methylation of cytosines at CpG dinucleotides at the gene's promoter. We did not directly analyze the methylation status of the *CYP1A1* gene promoter, because its dioxin induction was unaltered after treatment with 5-AzadC in both MCF-7 and HepG2 cells (Fig. 6), suggesting that DNA methylation does not play an important role in the dioxin-induced transcriptional regulation of this gene in these cell lines.

Partial Demethylation of the CYP1B1 Promoter by 5′-AzadC Treatment in HepG2 Cells, and Restoration of polII Binding at the Promoter. In 5-AzadC-treated HepG2 cells, DNA methylation was partially reversed at the CYP1B1 promoter (Fig. 7C). Furthermore, ChIP analysis demonstrated that dioxin-induced polII recruitment to the *CYP1B1* promoter was restored in these cells (Fig. 8). Both of these observations are consistent with our observation that 5′-AzadC restores dioxin inducibility to the *CYP1B1* gene in HepG2 cells.

Binding of the TATA Binding Protein to the CYP1B1 Promoter in MCF-7 and HepG2 Cells. Because dioxin failed to induce polII recruitment to the *CYP1B1* promoter, we also studied the recruitment of the TATA binding protein (TBP) to this promoter in MCF-7 and HepG2 cells by ChIP analysis. TBP binding generally precedes the binding of other general transcription factors and polII at the promoter and "seeds" their association with the promoter (Kornberg, 2007). TBP presumably binds to the functional TATA-like box sequence located 35 bp upstream of the transcription start site of *CYP1B1* (Wo et al., 1997). Dioxin was found to induce TBP recruitment to the *CYP1B1* promoter in MCF-7 cells but not in HepG2 cells (Fig. 9A). TBP recruitment was restored at the *CYP1B1* promoter after treatment with 5-AzadC. However, the TBP recruitments levels at the *CYP1B1* promoter remain unaltered in MCF-7 cells after treatment with 5-AzadC (Fig. 9B). Thus, the loss of dioxin induction of CYP1B1 in HepG2 cells is attributable to defect(s) occurring before TBP recruitment to the *CYP1B1* promoter.

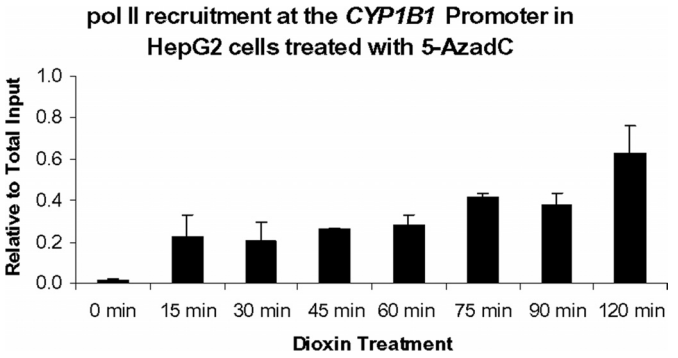

Fig. 8. polII recruitment to the *CYP1B1* gene in 5′-AzadC treated HepG2 cells. HepG2 cells were treated with 5 μM 5′-AzadC for 3 days followed by 100 nM dioxin treatment for the indicated times and subjected to ChIP analysis. polII recruitment to the promoter of the *CYP1B1* gene was measured by real-time PCR and is reported relative to total inputs.

Discussion

We report a detailed analysis of the mechanism responsible for the lack of induction of CYP1B1 by dioxin in HepG2 cells. Our observations provide insights into both mechanisms of gene regulation in general and the regulation of human CYP1B1 in particular.

Whereas CYP1A1 was induced in both MCF-7 and HepG2 cells, and CYP1B1 was induced in MCF-7 cells, CYP1B1 mRNA was neither expressed in the absence of dioxin nor induced by dioxin in HepG2 cells. However, dioxin did induce recruitment of AhR to the enhancer of the *CYP1B1* gene in HepG2 cells, thereby confirming that binding of AhR does not necessarily equate with dioxin inducibility. This conclusion also was made previously by our laboratory and by others based on studies on other cell lines (Yang et al., 2008; Beedanagari et al., 2010). Dioxin induced four different types of histone modifications at the *CYP1A1* promoter in HepG2 cells and at the *CYP1A1* and *CYP1B1* promoters in MCF-7 cells. Other investigators have shown that dioxin induces these same modifications at the promoter of the *Cyp1a1* gene in mouse hepatoma cells (Schnekenburger et al., 2007). These modifications are generally associated with actively transcribed genes (Li et al., 2007). Of interest, these modifications were not induced by dioxin at the *CYP1B1* promoter in HepG2 cells, demonstrating that these cells are blocked in the CYP1B1 induction pathway at a step before the generation of these modifications. We showed previously that the HAT coactivator p300 is required for dioxin induction of CYP1B1 (Taylor et al., 2009), and we now show that p300 is also required for dioxin induction of the above histone modifications at the *CYP1B1* promoter (in MCF-7 cells). The three acetylations we studied, AcH3K9, AcH3K14, and AcH4, are known to be catalyzed by p300 (and by certain other HAT coactivators) (Kouzarides, 2007), and they may therefore represent direct targets of p300 in the *CYP1B1* gene. We show that dioxin induces the recruitment of p300 and the HAT coactivator PCAF to the *CYP1B1* enhancer in HepG2 cells. Thus, the block in the induction pathway in HepG2 cells occurs at a step beyond these recruitments but before the generation of the above chromatin modifications at the promoter.

We show that CpG dinucleotides are fully methylated within the CpG island encompassing the promoter of the

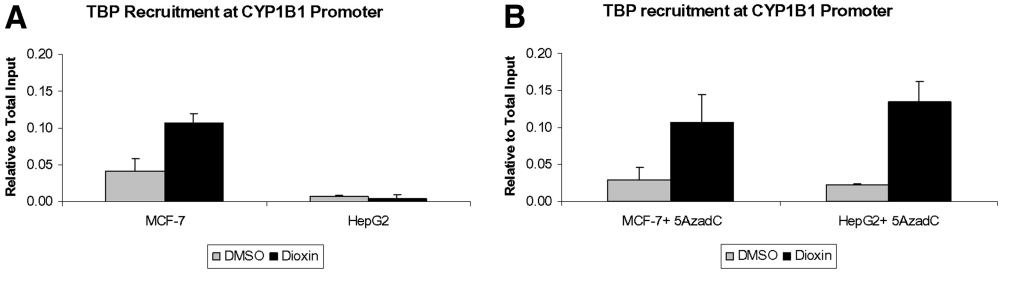

Fig. 9. TBP recruitment to the *CYP1B1* promoter in MCF-7 and HepG2 cells. ChIP analyses were carried out using a TBP antibody in HepG2 and MCF-7 cells treated with a 100 nM dioxin or vehicle (DMSO) for 60 min. TBP recruitment to the *CYP1B1* promoter with or without 5′-AzadC treatment in MCF-7 and HepG2 cells was measured by real-time PCR and is reported relative to that of total inputs.

CYP1B1 gene in HepG2 cells but are only partially methylated within the CpG island encompassing the enhancer. It is noteworthy that the XREs in the enhancer are only partially methylated and are therefore available for binding of the AhR/ARNT dimer. Treatment of HepG2 cells with the DNA methyl transferase inhibitor 5-AzadC partially demethylated the CpG dinucleotides in the *CYP1B1* promoter, restored dioxin induction of the recruitment of RNA polymerase II to the CYP1B1 promoter, and restored induction of CYP1B1 expression. These results indicate that DNA methylation at the *CYP1B1* promoter is directly responsible for silencing the gene's transcriptional response to dioxin. It is noteworthy that Shehin and coworkers also previously observed no induction of *CYP1B1* in HepG2 cells by dioxin, but reported that the DNA-demethylating agent 5-azacytidine failed to restore dioxin induction of CYP1B1 to these cells (Shehin et al., 2000). Because Shehin and coworkers used 5-azacytidine rather than its deoxy analog 5-AzadC that we used in our study, we also tested the former compound in our studies and demonstrated that it is equally as effective in reactivating CYP1B1 mRNA as 5-AzadC (data not shown). We and Shehin and coworkers obtained HepG2 cells from the same source, and we have no explanation for the differences in our results.

Binding of the TBP to gene promoters usually occurs after the binding of sequence-specific transcription factors enhancers and before the association of the general transcription factors and polII to the promoter (Kornberg, 2007). Our observation that dioxin fails to induce TBP binding to the *CYP1B1* promoter in HepG2 cells suggests that one of the critical effects of DNA methylation may be to preclude the association of this factor with the promoter. However, because the TATA-like box sequence in the CYP1B1 promoter does not contain any CpG dinucleotides, DNA methylation may not directly prevent binding of this protein but may inhibit binding of another protein or protein(s) at the promoter required for TBP recruitment. This inhibition could be due to the presence of methylated DNA binding proteins, such as MeCP2, MBDI, MBD2, and/or MBD3 (Szyf, 2009), with the *CYP1B1* promoter. Our conclusions concerning the alterations to the regulatory processes occurring at the *CYP1B1* gene in HepG2 cells are presented in Fig. 10.

It is recognized that transcriptional coactivators are incorporated in large multiprotein complexes straddling both the enhancer and the promoter of responsive genes (O'Malley, 2007). We observed much greater levels of p300 and PCAF recruitment to the *CYP1A1* and *CYP1B1* enhancers than to the corresponding promoters after dioxin treatment. This observation is consistent with the notion that these proteins are in closer proximity to the AhR/ARNT dimer at the *CYP1B1* enhancer than to TBP and the general transcription factors located at the promoter. The lack of detectable binding at the promoter is most likely due to the inefficiency of formaldehyde at cross-linking proteins under the conditions of our ChIP procedure, coupled with the presence of multiple proteins spanning the distance between these coactivators and the promoter. It is therefore of considerable interest that p300 and PCAF are recruited efficiently at the *CYP1B1* enhancer after dioxin treatment in HepG2 cells, despite the fact that TBP and polII are not recruited to the corresponding promoter. These observations strongly imply that p300 and PCAF can be recruited to the enhancer even when they are not incorporated into a multiprotein complex spanning the enhancer and the promoter. The lack of a requirement for coactivators to be tethered at both promoter and enhancer can probably be generalized to other coactivators and other genes, and this represents an important area for future research. It should be noted that several uncommon features of our experimental system, including our ability to distinguish the in vivo binding of proteins to the enhancer from their binding to the promoter and vice versa, and most importantly the fact that the promoter is fully methylated but the enhancer is not, provided us with the opportunity to draw the above conclusion and therefore to provide important insight into the general mechanism of gene transcription in mamma-

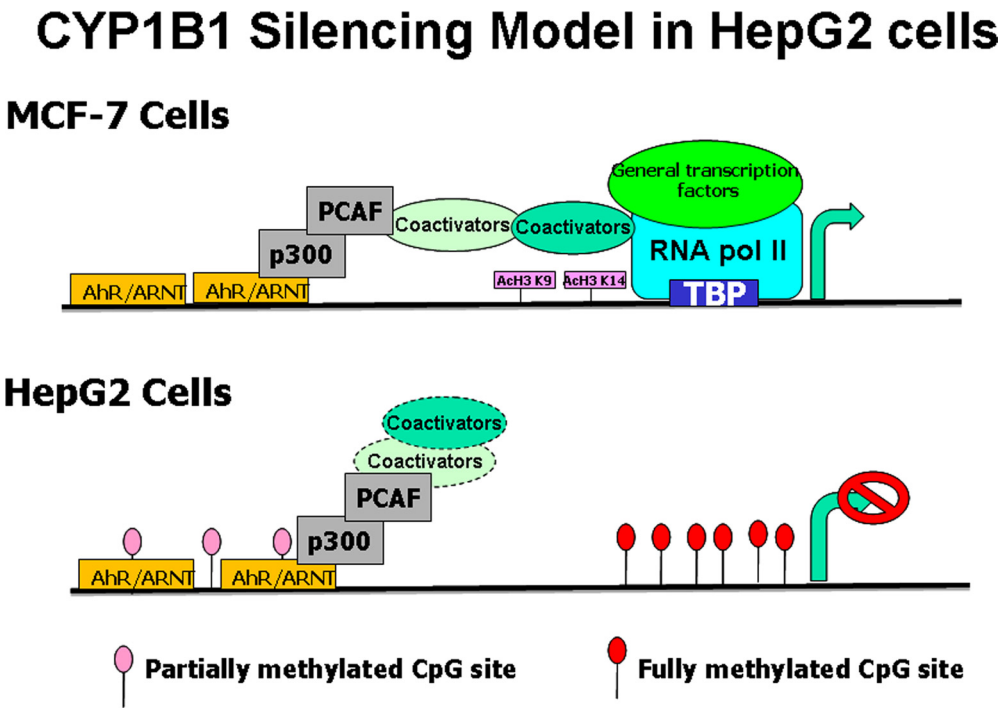

Fig. 10. Proposed silencing model for the CYP1B1 gene in HepG2 Cells: proposed changes in regulatory process occurring at the CYP1B1 promoter in HepG2 cells (compared with MCF-7 cells) after dioxin treatment.

lian cells. Altogether, the *CYP1B1* gene in HepG2 cells provides a valuable experimental system for further experiments focusing on potential regulatory mechanisms affecting the relationship between transcription and epigenetic modifications. Because CYP1B1 has been observed to be inducible by dioxin in other human hepatic cancer cell lines (MacPherson et al., 2009), the mechanism of inhibition of CYP1B1 expression we describe is not universal to such cell lines.

Of considerable importance are the observations that the *CYP1B1* promoter exhibits DNA methylation in a portion of colorectal (Habano et al., 2009) and gastric (Kang et al., 2008) cancers. It is noteworthy that in a portion of colorectal cancers, CpG dinucleotides at the *CYP1B1* promoter are fully methylated, whereas CpG dinucleotides at the enhancer are only partially methylated, just as we observed with HepG2 cells (Habano et al., 2009). Our studies are therefore potentially relevant to the development of these cancers and provide insights into the mechanism of CYP1B1 induction and into the regulation of gene expression in mammalian cells in general.

Acknowledgments

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