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# A Dioxin-Responsive Enhancer 3' of the Human *CYP1A2* Gene<sup>S</sup>

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### **ABSTRACT**

The human *CYP1A* genes *CYP1A1* and *CYP1A2* are in a head-to-head orientation on chromosome 15. Both *CYP1A* genes and *CYP1B1* are transcriptionally induced by the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that binds 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin). Although the TCDD-responsive enhancers for *CYP1A1* and *CYP1B1* are well characterized, a similar *CYP1A2* enhancer has not been identified. In the human prostate cell line RWPE-1, CYP1A2 mRNA expression is dramatically induced by TCDD. Therefore, analysis of the native *CYP1A2* gene in these cells can provide insight into its induction mechanism. To identify sites that may bind AhR on the *CYP1A* locus, we scanned 75 kilobases of chromosome 15 sequence for high-affinity AhR binding sites. We then analyzed most of the sites for TCDD-inducible AhR interaction by chromatin immunoprecipitation.

As expected, the *CYP1A1* and *CYP1B1* enhancers bind AhR in TCDD-treated cells. It is noteworthy that we identify a region 3' of *CYP1A2* that also binds AhR in response to TCDD. We cannot detect AhR binding at other sites on the *CYP1A* locus. In vivo footprinting demonstrates that two AhR binding sites in the *CYP1A2* 3' region are occupied in TCDD-treated cells. Reporter-gene studies show that these sites confer TCDD-responsiveness to a heterologous promoter. AhR also binds to the *CYP1A2* 3' region in TCDD-treated LS180 cells but not in HepG2 and ND-1 cells. In the latter cell lines, the *CYP1A2* 3' region is extensively methylated. In summary, we identify a novel TCDD-responsive enhancer for *CYP1A2*. We were surprised to find that this enhancer is not conserved across species and is primarily human-specific.

The CYP1 gene family, CYP1A1, CYP1A2, and CYP1B1, encode cytochrome P450s that metabolize a wide variety of structurally diverse chemicals and are implicated in both their detoxification and bioactivation into carcinogenic and toxic compounds. All members of the CYP1 gene family are transcriptionally induced by the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that binds polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons with high affinity. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, dioxin), a notorious environmental contaminant, has the highest affinity for AhR and is the most potent inducer of the CYP1 genes (Poland and Knutson,

1982; Schmidt and Bradfield, 1996; Whitlock et al., 1997; Nebert et al., 2004; Shimada and Fujii-Kuriyama, 2004; Nebert and Dalton, 2006).

The most well-studied dioxin response is the transcriptional induction of *CYP1A1*. TCDD induces *CYP1A1* by binding to and activating AhR, which then translocates to the nucleus and interacts with its partner protein ARNT to form an active heteromeric transcription factor. The AhR complex then interacts with DNA binding sites, termed dioxin-response elements (DREs), located on the *CYP1A1* enhancer to mediate TCDD-inducible gene expression (Hankinson, 1995; Whitlock, 1999). Like *CYP1A1*, the TCDD-responsive *CYP1B1* enhancer contains DRE sites upstream of its transcriptional start site that bind AhR and mediate TCDD-inducible gene expression (Zhang et al., 1998; Tsuchiya et al., 2003).

Unlike the other *CYP1* genes, the mechanism by which TCDD-induces *CYP1A2* is not well understood. A nonconsensus DRE that binds AhR in vitro and confers TCDD-responsiveness to a reporter gene has been identified upstream of

**ABBREVIATIONS:** PAH, polycyclic aromatic hydrocarbon; AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; DRE, dioxin-response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; DMS, dimethyl sulfate; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; ARNT, aryl hydrocarbon receptor nuclear translocator; kb, kilobase(s); bp, base pair(s).

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the CYP1A2 promoter (Quattrochi and Tukey, 1989; Postlind et al., 1993; Quattrochi et al., 1994). However, the in vivo functionality of this site has not been established. In addition, because the CYP1A1 and CYP1A2 genes are positioned in a head-to-head orientation, they share a common 5' upstream region. Thus, the CYP1A1 enhancer might also control CYP1A2 expression (Corchero et al., 2001). Indeed, a reporter gene study demonstrates that the CYP1A1 enhancer confers TCDD-inducibility on the distant CYP1A2 promoter (Ueda et al., 2006). In addition, transgenic mice containing 85 kb of human DNA containing both CYP1A genes induce CYP1A2 in response to TCDD. In contrast, a 50-kb transgene containing CYP1A2 but lacking the CYP1A1 enhancer region did not (Jiang et al., 2005). Together, these findings imply that the TCDD-responsive CYP1A1 enhancer also controls CYP1A2 expression. However, a significant caveat of these studies is that CYP1A2 was analyzed outside of its native setting, either as a reporter plasmid or as a transgene. Here, we analyze the endogenous CYP1A2 gene in several human cell lines. We identify a novel TCDD-responsive, AhR-dependent enhancer 3' of the human CYP1A2 gene.

# **Materials and Methods**

Cells and Cell Culture. RWPE-1, HepG2, and LS-180 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured as directed. RWPE-1 cells are normal human adult epithelial prostate cells that have been immortalized by transfection with a plasmid containing the human papilloma virus. HepG2 cells are a human hepatocellular carcinoma cell line. LS-180 cells are a human colonic adenocarcinoma cell line. ND-1 cells, a human primary prostatic adenocarcinoma cell line, were developed in our laboratory and were cultured as described previously (Narayan and Dahiya, 1992). Cells were treated with TCDD (10 nM; Wellington Laboratories Inc., Ontario, Canada) for 30 min to 18 h.

Quantitation of mRNAs. Total RNA was isolated from 90% confluent plates of cultured cells using the RNeasy mini kit (QIA-GEN, Valencia, CA) according to the manufacturer's directions. Human liver and prostate total RNA was purchased from Clontech (Mountain View, CA). The liver RNA sample was from a 51-year-old white man. The prostate RNA sample was from a pool of 32 white men aged 21 to 50 years. RT-PCR was performed using the Titanium One-Step RT-PCR Kit (BD Biosciences, San Jose, CA) following the manufacturer's directions. For conventional PCR, mRNA was amplified using 30 cycles (unless otherwise specified) of 30 s at 94°C, 30 s at 63°C, and 1 min at 72°C. CYP1A1 mRNA was amplified using the following primers: forward, (5'-ATCCCAGGCTCCAAGAGTCCACCCT-3'); reverse, (5'-GCGGGT-TCTTTCCCAGGGTCAGCAT-3'). CYP1A2 mRNA was amplified using the following primers: forward, (5'-CCACACCAGCCATTA-CAACCCTGCC-3'); reverse, (5'-TGCGCTGGCTCATCCTTGACA-GTGC-3') (35 cycles). CYP1B1 mRNA was amplified using the following primers: forward, (5'-CTGGCACTGACGACGCCAAGA-GACT-3'); reverse, (5'-TGGTCTGCTGGATGGACAGCGGGTT-3'). AhR mRNA was amplified using the following primers: forward, (5'-CCACAGCAACAGCTGTGTCAGAAGATG-3'); reverse, (5'-CGGATGATGAAGTGGCTGAAGATGTGT-3'). Arnt mRNA was amplified using the following primers: forward, (5'-GCTGG-GAGATCAGAGCAAC GCTACAA-3'); reverse, (5'-TGTTTCTTTCCA-GAGGGACTGCTCACA-3'). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified using the following primers: forward, (5'-TTGGTCGTATTGGGCGCCTGGTCAC-3'); reverse, (5'-AGACGCCAGTGGACTGGCCGACGTA-3') (28 cycles). The amplified DNA was electrophoresed on a 2.5% agarose gel and visualized by staining with ethidium bromide.

Quantitative PCR was performed using CYP1A1, CYP1A2, and GAPDH TaqMan primers on a 7500 Fast Real-Time System as directed by the manufacturer (Applied Biosystems, Foster City, CA). In most cases, two independent samples were analyzed. For the prostate and liver RNA samples, one sample was analyzed. Each sample was analyzed in quadruplicate. GAPDH served as an internal control to normalize CYP1A mRNA expression data.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) analysis was performed using the protocol recommended for use with the EZ-ChIP kit (Upstate Biotechnology, Char-

TABLE 1 Primers used in the ChIP analysis

Forward Reverse Forward Forwar		
Reverse 5'-TGAAGTCTGTTCATATTCTTTGTCC-3' +8 kb Forward 5'-GAGACGGAGTTTCGATCTTTGTTGT-3' Reverse 5'-CCGAGATCACCTGAGGACAGGAGTT-3' +6 kb Forward 5'-CAGCACAGTGATTAGGAGTCTTGTC-3' Reverse 5'-TCCACCTCCTGGGCTCACACGATTC-3' 0 kb Forward 5'-CCGCCACCTTTCTCTCCAATCCCAG-3' Reverse 5'-ATAGGCGGGCTTGTACCTGTGCCCA-3' -0.5 kb Forward 5'-CAGGCTGGGGTCGCAGCGCTTCT-3' Reverse 5'-GCTACAGCTTGCCCCTAGAGGGATGCCA-3' -1 kb Forward 5'-TGACCTCTGCCCCCTAGAGGGATGT-3' Reverse 5'-TTGGCAGAGAAATCCGCGGG-3' -1 kb Forward 5'-GATACTGGCGCCGTAGAGGGATGT-3' Reverse 5'-TTAGTCCTCTGCCAGTTGCAGTCT-3' Reverse 5'-TACTTGGCCTAGAGGATTCGAGGGGGGTTC-3' -6 kb Forward 5'-CATACTGGCGCAGTTGCAGTCT-3' Reverse 5'-GCTACAGCATTTCAGTACATAG-3' Reverse 5'-ACCTTGGCCTCTGGAGTGTGGGGGTTG-3' Reverse 5'-GCTACAATATTAGCCGGCATGGTGG-3' -1 kb Forward 5'-ACAGAATATTAGCCGGCATGGTGG-3' Reverse 5'-CCTACTCTGTCAGTACTACGCAG-3' Reverse 5'-CCTACTCTGTCAGAGACCTCCA-3' -19 kb Forward 5'-ACAGAATATTAGCCGGCATGGTGG-3' Reverse 5'-CCTACTCTGTCAGGAGACACTC-3' -23 kb Forward 5'-ACTGGTCAATCCAGGAGACTCA-3' Reverse 5'-CCTACTCTGTCAGGAGACACTC-3' Reverse 5'-CCTACTCTGGTCAGGAGCATCT-3' -33 kb Forward 5'-ACTCAGTCTAGGCAACCAGGCTC-3' Reverse 5'-GCTAAAATCAGGAGACTCCAC-3' Reverse 5'-GCTAAATCTCAGGCAACCAGGCTC-3' Reverse 5'-GCTAAACACCGGAGCACCAGGCTC-3' Reverse 5'-ACCGGGTGAAACCCGAGGACACCAGGCTC-3' Reverse 5'-GCTAGACACCGGAGAACACTCC-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTC-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTC-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTC-3' Reverse 5'-GCAAAGTATGGCAACCAGGACACCAGGCTC-3' Reverse 5'-GCAAAGTATGGCAACCCAACCAGGCTC-3' Reverse 5'-GCAAAGTATGGCAACCCAACCAGGCTC-3' Reverse 5'-GCAAAGTATGGCAACCCAACCAGGCTC-3' Reverse 5'-GCAAAGTATGGCAACCCAACCAGGCACAGGAACATGGC-3' Reverse 5'-CCAACCAGGAAAACCCCAATCTCTAC-3' Reverse 5'-CCAACCAGGAAAACCCCAATCTCTAC-3' Reverse 5'-GCAAAGGAAACACCCAATCTCTAC-3' Reverse 5'-CAATCACCAGGAAAACCCCAATCTCTAC-3' Reverse 5'-CAATCACCAGGAAAACCCCAATCTCTAC-3' Reverse 5'-CAACTGACCAGGAAAACACCCAACCCAACCAGTCTTT-3' -37 kb Forward 5'-CACACCAGGAAAACCCCAACCAGGATTAGACCCA' Reverse 5'-CCAACTCACCAGGAAAACCCCAACAGGAAACACCCAACCAGCAGAGAACACCCAACCAGCAG	+15 kb	
Forward 5'-GAGACGGAGTTTCGATCTTTGTTGT-3' Reverse 5'-CCGGAGATCACCTGAGGACAGGAGTT-3' Reverse 5'-CAGCACAGTGATTAGGAGTCTAGC-3' Reverse 5'-TCCACCTCTCGGCTCACACGATTC-3' Reverse 5'-ATAGGCGGCTTGTACCTGAGCACAGATC-3' Reverse 5'-ATAGCGGGCTTGTACCTGGCCA-3' Reverse 5'-GCTACAGCCTACAGGAGCGCA-3' Reverse 5'-GCTACAGCCTACCAGGACCGATC-3' Reverse 5'-GCTACAGCCTACCAGGACCGCAG-3' -1 kb Forward 5'-TGACCTCTGCCCCCTAGAGGGATGT-3' Reverse 5'-TTGGCAGAGCACGATCGCAG-3' -2 kb Forward 5'-GATACTGGGCGGTGAGGGGGTTC-3' Reverse 5'-TTGGCAGAGAAATCCGGCGG-3' -2 kb Forward 5'-GATACTGGGCGGTGAGGGGGTTT-3' Reverse 5'-TATTCCCTCTGCCAGTTGCAGTCT-3' Reverse 5'-ACCTTGGCCCTTAGAGGATTC-3' Reverse 5'-ACCTTGGCCTGGAGTGAGGGGGTT-3' Reverse 5'-ACCTTGGCCTGGAGTAGACACTC-3' -12 kb Forward 5'-ACCTTGGCCTGGAGTAGACACTC-3' Reverse 5'-ACCTTGGCCTGGAGTAGACACTC-3' -15 kb Forward 5'-ACCTTGGCCTGGAGTGGGGGGTG-3' Reverse 5'-CCTACTCTGGTAATGACCAGC-3' Reverse 5'-CCTACTCTGGTAATCACCCAGC-3' Reverse 5'-CCTACTCTGGTCAGAGACACTC-3' -19 kb Forward 5'-ACCCTGTAATCTCAGCAACCAGCCTC-3' -23 kb Forward 5'-ACCCTGTAAATCAGACCAGCCTC-3' Reverse 5'-CCTAGTCTAGGCAACCAGGCTC-3' -33 kb Forward 5'-ACCCTGTAAAATCAGGAGAGTTGG-3' Reverse 5'-GCAAAAGTATGGCAACCAGGCTC-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTC-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTC-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTCC-3' Reverse 5'-GCAAAGTATGGCAACCAGGAGATTG-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTCC-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTCC-3' Reverse 5'-GCAAAGTATGGCAACCAGAGATTGG-3' -33 kb Forward 5'-GCGCTCTGGGTCAGTGCCTCCACCA-3' Reverse 5'-GCAAAGTATGGCAACCCAACCGAGCAGAGATTGG-3' Reverse 5'-GCAAAGTATGGCAACCCAACCGAGCACAGGGTTC-3' -37 kb Forward 5'-GCGCTCTGGCCAGGAACAATGGG-3' Reverse 5'-GCAAAGTATGGCAACCAGGGTTCC-3' Reverse 5'-GCAAAGTATGGCAACCCAACCGAGCAGTTTG-3' -37 kb Forward 5'-GCGCTCTGGCCAGGAAACCCCATCTTACT-3' -37 kb Forward 5'-GCGACGCAGGAGAATGGCTTCCACCA-3' Reverse 5'-CCAAGGGAGAACACGGGGGGGGGGGGGGGGGGGGGGGG	Forward	5'-AAATACAAAAATTAGCCAGGCATGG-3'
Forward Reverse 5'-GAGACGGAGTTTCGATCTTTGTTGT-3' Reverse 5'-CCGAGATCACCTGAGGACAGGAGTT-3'+6 kb Forward 5'-CAGCACAGTGATTAGGAGTCTGTC-3' Reverse 5'-TCCACCTCTGGGCTCACAGGATTC-3' 0 kb Forward 5'-CAGCACAGTGATTAGGAGTCTTGTC-3' Reverse 5'-ATAGGCGGGCTTGTACGTGTGCCA-3' Reverse 5'-ATAGGCGGGTTGTACGTGTGCCA-3' -0.5 kb Forward 5'-CAGAGCCTTGCCAGCGAGCGGTTCT-3' Reverse 5'-GCTACAGCCTACCAGGACTCGCA-3' -1 kb Forward 5'-TAGCCTGCCCCCTAGAGGGATGT-3' Reverse 5'-TTGGCAGAGACACGAGAATCCGCAG-3' -2 kb Forward 5'-GATACTGGCGCGTGAGGGGGTTA-3' Reverse 5'-ACCTTGCCAGTTGCAGTCTCAGTCAGGA' -1 kb Forward 5'-CCTGCTCTGCCAGTTGCAGTCT-3' -6 kb Forward 5'-ACCTTGGCCTGGAGAGAATCCGCAGG-3' -12 kb Forward 5'-ACCTTGGCCTGGAGACACTCA-3' Reverse 5'-ACCTTGGCCTGAGAGGACACTCA-3' -12 kb Forward 5'-ACGAGAATATTAGCCGGGCATGGG-3' Reverse 5'-CCTAGAGAGAGAGAATCCAGCAG-3' Reverse 5'-CCTAGTGCAGAGACACTCATGG-3' Reverse 5'-CCTAGTGCAGAGCACCTCCTCC-3' -19 kb Forward 5'-ACCTTGTCAGGAGCAGCACCAGGA-3' Reverse 5'-CCTAGGCTGAAAATCAGGAGCTCA-3' Reverse 5'-CCTAGGCTGAAAATCAGGAGCATCT-3' -33 kb Forward 5'-ACCTGTAACACCAGGCTCA-3' Reverse 5'-GCAAAGTATGCCAAGCAGCTCA-3' Reverse 5'-GCTTGAGCAAACACGGCTCA-3' Reverse 5'-GCAAAGTATGCCAAGCAGGTTG-3' Reverse 5'-GCAAAGTATGCCAAGCAGGTTC-3' Reverse 5'-GCAAAGTATGCCAAGCAGGTTC-3' Reverse 5'-GCAAAGTATGCCAAGCAGGTTC-3' Reverse 5'-GCAAAGTATGCCAAGGAGAGTTTG-3' Reverse 5'-GCAAAGTATGCCAAGCAGGGGAACAATGG-3' Reverse 5'-GCAAAGTATGCCAAGGAGAGAGGAGTTTG-3' Reverse 5'-GCAAAGTATGCCAAGGAGAGAGGAGAGAGAGAGAGAGAGA	Reverse	5'-TGAAGTCTGTTCATATTCTTTGTCC-3'
Reverse   5'-CCGAGATCACCTGAGGACAGGAGTT-3' +6 kb   Forward   5'-CAGCACAGTGATTAGGAGTCTTGTC-3'   Reverse   5'-TCCACCTCCTGGGCTCACACGATTC-3'   0 kb   Forward   5'-CCGCCACCTTTCTCTCCAATCCCAG-3'   Reverse   5'-ATAGGCGGGCTTGTACGTGTGGCCA-3'   -0.5 kb   Forward   5'-CAGGCTGGGTCCACAGCGCTTCT-3'   Reverse   5'-GCTACAGCCTACCAGGACTCGGCAG-3'   -1 kb   Forward   5'-GCTACAGCCTACCAGGACTCGGCAG-3'   -1 kb   Forward   5'-TGACCTCTGCCCCCTAGAGGGATGGCAG-3'   -2 kb   Forward   5'-TTGGCAGAGCACAGAAATCCGCGG-3'   -2 kb   Forward   5'-GATACTGGGGCGTGAGGGGGTT-3'   Reverse   5'-TTATTCCCTCTGCCAGTTGCACTCT-3'   -6 kb   Forward   5'-CCTGCTCCTGGTATTGATCACTAG-3'   Reverse   5'-ACCTTGGCCTGGAGCTGAACACTCA-3'   -12 kb   Forward   5'-ACAGAATATTAGCCGGGCATGGTGG-3'   Reverse   5'-CATGATGGTGCAGCTGAACACTCA-3'   -15 kb   Forward   5'-CATGATGGTGCAGCTAGAGCAGCAGCAGCAGAATGACACTCCAGG-3'   -19 kb   Forward   5'-CATGATGGTGCAGCTAGCAGGCAGC-3'   Reverse   5'-ACCTTGGTCAGAGCACACGCAGCAGC-3'   -23 kb   Forward   5'-ACCTGTAATCTCAGGAACACTCCATC-3'   -23 kb   Forward   5'-ACCTGTGAACACAGGCTCA-3'   Reverse   5'-CTTGGGCTGAACACAGGCTCC-3'   -39 kb   Forward   5'-GCTTGAACACCAGGCTCAC-3'   Reverse   5'-CTTGGGCTGAACCCAGCTGCTTGC-3'   -39 kb   Forward   5'-GCTTGAACACCAGGCTCCAC-3'   Reverse   5'-ACCGCGTGAACCCAGCTCTTCC-3'   -38 kb   Forward   5'-GCTTGACCAGCAACAGGCTCCAC-3'   Reverse   5'-ACGCGGTGAACCCCACCAGCTTTGACC-3'   Reverse   5'-TTGGACCAGCAAGAACACCCCACCA-3'   Reverse   5'-TGGACCAGGAACAACCCCACTCTCTACC-3'   -47 kb   Forward   5'-GCTTGACCAGGAACAACGCGG-3'   Reverse   5'-CCAGCCAGGAACAACGCGG-3'   Reverse   5'-CCAGCCCAGGAACAAGCGGG-3'   Reverse   5'-CCCAGGAGACAACGCGCCACG-3'   Reverse   5'	+8 kb	
Reverse   5'-CCGAGATCACCTGAGGACAGGAGTT-3' +6 kb   Forward   5'-CAGCACAGTGATTAGGAGTCTTGTC-3'   Reverse   5'-TCCACCTCCTGGGCTCACACGATTC-3'   0 kb   Forward   5'-CCGCCACCTTTCTCTCCAATCCCAG-3'   Reverse   5'-ATAGGCGGGCTTGTACGTGTGGCCA-3'   -0.5 kb   Forward   5'-CAGGCTGGGTCCACAGCGCTTCT-3'   Reverse   5'-GCTACAGCCTACCAGGACTCGGCAG-3'   -1 kb   Forward   5'-GCTACAGCCTACCAGGACTCGGCAG-3'   -1 kb   Forward   5'-TGACCTCTGCCCCCTAGAGGGATGGCAG-3'   -2 kb   Forward   5'-TTGGCAGAGCACAGAAATCCGCGG-3'   -2 kb   Forward   5'-GATACTGGGGCGTGAGGGGGTT-3'   Reverse   5'-TTATTCCCTCTGCCAGTTGCACTCT-3'   -6 kb   Forward   5'-CCTGCTCCTGGTATTGATCACTAG-3'   Reverse   5'-ACCTTGGCCTGGAGCTGAACACTCA-3'   -12 kb   Forward   5'-ACAGAATATTAGCCGGGCATGGTGG-3'   Reverse   5'-CATGATGGTGCAGCTGAACACTCA-3'   -15 kb   Forward   5'-CATGATGGTGCAGCTAGAGCAGCAGCAGCAGAATGACACTCCAGG-3'   -19 kb   Forward   5'-CATGATGGTGCAGCTAGCAGGCAGC-3'   Reverse   5'-ACCTTGGTCAGAGCACACGCAGCAGC-3'   -23 kb   Forward   5'-ACCTGTAATCTCAGGAACACTCCATC-3'   -23 kb   Forward   5'-ACCTGTGAACACAGGCTCA-3'   Reverse   5'-CTTGGGCTGAACACAGGCTCC-3'   -39 kb   Forward   5'-GCTTGAACACCAGGCTCAC-3'   Reverse   5'-CTTGGGCTGAACCCAGCTGCTTGC-3'   -39 kb   Forward   5'-GCTTGAACACCAGGCTCCAC-3'   Reverse   5'-ACCGCGTGAACCCAGCTCTTCC-3'   -38 kb   Forward   5'-GCTTGACCAGCAACAGGCTCCAC-3'   Reverse   5'-ACGCGGTGAACCCCACCAGCTTTGACC-3'   Reverse   5'-TTGGACCAGCAAGAACACCCCACCA-3'   Reverse   5'-TGGACCAGGAACAACCCCACTCTCTACC-3'   -47 kb   Forward   5'-GCTTGACCAGGAACAACGCGG-3'   Reverse   5'-CCAGCCAGGAACAACGCGG-3'   Reverse   5'-CCAGCCCAGGAACAAGCGGG-3'   Reverse   5'-CCCAGGAGACAACGCGCCACG-3'   Reverse   5'	Forward	5'-CACACCCACTTTCCATCTTTCTTCT-3'
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Forward Reverse Okb Forward Forward Forward Forward Forward Reverse S'-TCCACCTCCTGGGCTCACACGATTC-3' Okb Forward Reverse S'-ATAGGCGGCTTTCTCCCAATCCCAG-3' Reverse S'-ATAGGCGGCTTGTACGTGTGGCCA-3' -0.5 kb Forward Reverse S'-GCTACAGCCTACCAGGACTCGGCAG-3' 1 kb Forward Reverse S'-GCTACAGCCTACCAGGACTCGCAG-3' 1 kb Forward Reverse S'-TTGGCAGAGCCACGAGATCCGCAG-3' 2 kb Forward S'-GATACTGGGCGGGTGAGGGGATG-3' Reverse S'-TTATTCCCTCTGCCCCTAGAGGGATG-3' Reverse S'-TATTCCCTCTGCCAGTTGCAGTC-3' Reverse S'-ACCTTGGCCTGGAGGGGGGTT-3' Reverse S'-ACCTTGGCCTGGAGGGGGGTT-3' Reverse S'-ACCTTGGCCTGGAGGGGGGGT-3' Reverse S'-ACCTTGGCTGGAGTGAACACTCA-3' -12 kb Forward S'-ACAGAATATTAGCCGGGCATGGTG-3' Reverse S'-GCTGCAAATGACAGGATCTCATTGG-3' -15 kb Forward S'-CATGATGGTGCACTATACGCCAGC-3' Reverse S'-CCTACTCTGGTCAGAGCCTCCTTCC-3' -19 kb Forward S'-ACCTTGGTCAGAGCCTCCTTCC-3' -19 kb Forward S'-ACCTTGGTCAGAGCCTCCTCCC-3' -23 kb Forward S'-ACTCAGTCTAGGCCAACCAGGCTCA-3' Reverse S'-CTTGGGCTGAAAATCAGGAGAGTTTG-3' -33 kb Forward S'-GCTTGAACACCGGAGGCAGAGTTTG-3' Reverse S'-ACGCGGTGAAACCCCATCTCTACT-3' -39 kb Forward S'-GCAAAGTATGGCAATGCCTGCCTCCACCA-3' Reverse S'-ACGCGGTGAAACCCCATCTCTACT-3' -37 kb Forward S'-GCAAAGTATGGCAATGCCTGCTTGC-3' -38 kb Forward S'-TGGTAGCGTGCCAGGGAACAAGGGG-3' Reverse S'-TGGTAGCAGCAGGAGAAGAGACACCCATCTTACT-3' -38 kb Forward S'-TGGTAGCCAGCCAGGAGAAGAGCCC-3' Reverse S'-CCAGCCAGGAAAACCCAGCCTGAACCC-3' Reverse S'-CCAGCCAGGAAAACCCAGCCTGAACCC-3' Reverse S'-CCAGCCAGGAAAACCCAGCCTGAACCC-3' Reverse S'-CCAGCCAGGAGAAACCCCAGCTGGATTGAT-3' -44 kb Forward S'-GCAGGCCCAGGGAATAGACGGGG-3' -47 kb Forward S'-GCAGGCCCAGGGAATAGACGCGG-3' -47 kb Forward S'-CCCAGCAGGAGAAGCCGCCACG-3' -47 kb Forward S'-CCCAGCAGGAGAGAGCCGCCAC-3' -47 kb Forwa		5 CCCAGATCACCTGAGGACAGGAGTT 5
Reverse 5'-TCCACCTCCTGGGCTCACACGATTC-3' 0 kb Forward 5'-CCGCCACCTTTCTCTCCAATCCCAG-3' Reverse 5'-ATAGGCGGGCTTGTACGTGTGGCCA-3' -0.5 kb Forward 5'-TCAGGCTGGGTCGCAGCGCTTCT-3' Reverse 5'-GCTACAGCCTACCAGGACTCGCAG-3' -1 kb Forward 5'-TGACCTCTGCCCCCTAGAGGGATGT-3' Reverse 5'-TTGGCAGAGCAGAAATCCGGCGG-3' -2 kb Forward 5'-GATACTGGGCGGTGAGGGGGTTT-3' Reverse 5'-TTATTCCCTCTGCCAGTTGCAGTCT-3' -6 kb Forward 5'-CCTGCTCCTGGTATTGATCACTAG-3' Reverse 5'-ACCTTGGCCTGGAGCTGAGCGGTT-3' -12 kb Forward 5'-ACAGAATATTAGCCGGGCATGGTGG-3' Reverse 5'-GCTGCAAATGACAGGATCTCA-3' -12 kb Forward 5'-CATGATGGTGCCACTATACGCCAGC-3' Reverse 5'-GCTGAAATGACAGGATCTCATTGG-3' -19 kb Forward 5'-ACCTTGGTCAGGAGCCTCCTTCC-3' -19 kb Forward 5'-ACCTTGATCTCAGGAGCATCCTCC-3' -23 kb Forward 5'-ACCTGATATCTCAGCTACTCAGGA-3' Reverse 5'-CTTGGGCTGAAAATCAGCAGGCTCA-3' Reverse 5'-CTTGGGCTGAAAATCAGGAGATCTCAT' -33 kb Forward 5'-ACTCAGTCTAGGCCAACCAGGCTCA-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTCA-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTCA-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTCA-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTCA-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTCA-3' Reverse 5'-GCAAAGTATGGCAACCAGGCTCA-3' Reverse 5'-ACCCGGTGAAAACCCAGGCTCACCA-3' Reverse 5'-ACGCGGTGAAAACCCCATCTCTACC-3' -37 kb Forward 5'-GCGCTCTGGGTCAGGGAGACACAGG-3' Reverse 5'-ACGCGGTGAAAACCCCATCTCTACC-3' Reverse 5'-ACGCGGTGAAAACCCCATCTCTACC-3' Reverse 5'-ACGCGGTGAAAACCCCATCTCTACC-3' Reverse 5'-ACGCGGTGAAAACCCCATCTCTACC-3' Reverse 5'-ACGCAGGAGAAACCCCATCTCTACC-3' Reverse 5'-ACGCAGGAGAAACCCCATCTCTACC-3' Reverse 5'-ACGCAGGAGAAACACCCATCTCTACC-3' Reverse 5'-ACGCAGGAGAAACACCCATCCTACCC-3' Reverse 5'-ACGCAGGAGAAACACCCAGGCTC-3' Reverse 5'-ACGCAGGAGAAACACCCAGCTTGACCC-3' Reverse 5'-ACGCAGGAGAAACACCCAGCTTGACCC-3' Reverse 5'-CCAGCCAGGAGAAACACCCAGCATTGACCC-3' Reverse 5'-CCAGCAGGAGAAACACCCAGCCCAGGATTAGACCC-3' Reverse 5'-CCAGCAGGAGAAACACCCAGCCCAGGATTAGACCCC-3' Reverse 5'-CCAGGAGAGAACCCCAGCAGGAGACACAGGGG-3' Reverse 5'-CCAGCAGGAGAAGAGCCCCACGAGAACCCAGCAGAGAGACACAGCGCCAGAGAGACACAGCGCCCAGGATTAGACCCGAGAACAGCGGCCCAGAGAACACCCAGCAGAGAGAGACCCCAGCAG		5/
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- 12 kb Forward Reverse - 5'-ACAGAATATTAGCCGGGCATGGTGG-3' Reverse - 15 kb Forward Reverse - 5'-CATGATGGTGCCACTATACGCCAGC-3' Reverse - 19 kb Forward Reverse - 19 kb Forward Reverse - 23 kb Forward Reverse - 5'-ACTCAGTCTAGGCACTACTCAGGA-3' Reverse - 5'-ACTCAGTCTAGGCCACCTACTCC-3' - 23 kb Forward Reverse - 5'-CTTGGGCTGAAAATCAGGAGCTCA-3' Reverse - 5'-CTTGGGCTGAAAATCAGGAGTTGG-3' - 33 kb Forward Reverse - 39 kb Forward Reverse - 5'-GCAAAGTATGGCAATGCCTCCTCC-3' - 37 kb Forward Reverse - 5'-AACGCGGTGAAAACCCGAGCTCA-3' Reverse - 5'-AACGCGGTGAAACCCCATCTCTACT-3' - 37 kb Forward Reverse - 5'-TTGAGCAGCATGGGGTCTCCAGCA-3' Reverse - 5'-TGGTAGCGTGCCAGGGAACAATGGG-3' - 38 kb Forward Reverse - 5'-TGGTAGCGTGCCAGGGAACAATGGG-3' Reverse - 5'-CCAGCCAGGTATGTCGTTTTGTA-3' - 44 kb Forward Reverse - 5'-CAGTCACTCATCAGCAAAGCCGGG-3' Reverse - 5'-CAGTCACTCATCAGCAAAGCCGG-3' Reverse - 5'-CAGTCACTCATCAGCAAAGCGGG-3' Reverse - 5'-AGTTGAACCCCAGCAGGGAATTGGG-3' Reverse - 5'-CCAGCCAGGTATTGCGTTTTTTTA-3' - 47 kb Forward Reverse - 5'-CCAGCCAGGTATTGCGTTTTTTTA-3' - 47 kb Forward Reverse - 5'-CCAGCCAGGAAATCGCTTGAACCC-3' Reverse - 5'-CCAGCCAGGAAATCGCTTGAACCC-3' Reverse - 5'-CCAGCCAGGAGAATCGCTTGAACCC-3' Reverse - 5'-CCAAGTGACCAGCCAGCAGTGTTT-3' - CYP1B1 promoter Forward - 5'-CCACTCCAGAGTCAAAGCCGCCCAT-3' Reverse - 5'-CCACTCCAGAGTCAAAGCCGCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCCACGT-3' - 5'-CCACTCCAGAGTCAAAGCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCGCCACT-3' - 5'-CCACTCCAGAGTCAAAGCCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3' - 5'	Forward	5'-CCCTGCTCCTGGTATTGATCACTAG-3'
- 12 kb Forward Reverse - 5'-ACAGAATATTAGCCGGGCATGGTGG-3' Reverse - 15 kb Forward Reverse - 5'-CATGATGGTGCCACTATACGCCAGC-3' Reverse - 19 kb Forward Reverse - 19 kb Forward Reverse - 23 kb Forward Reverse - 5'-ACTCAGTCTAGGCACTACTCAGGA-3' Reverse - 5'-ACTCAGTCTAGGCCACCTACTCC-3' - 23 kb Forward Reverse - 5'-CTTGGGCTGAAAATCAGGAGCTCA-3' Reverse - 5'-CTTGGGCTGAAAATCAGGAGTTGG-3' - 33 kb Forward Reverse - 39 kb Forward Reverse - 5'-GCAAAGTATGGCAATGCCTCCTCC-3' - 37 kb Forward Reverse - 5'-AACGCGGTGAAAACCCGAGCTCA-3' Reverse - 5'-AACGCGGTGAAACCCCATCTCTACT-3' - 37 kb Forward Reverse - 5'-TTGAGCAGCATGGGGTCTCCAGCA-3' Reverse - 5'-TGGTAGCGTGCCAGGGAACAATGGG-3' - 38 kb Forward Reverse - 5'-TGGTAGCGTGCCAGGGAACAATGGG-3' Reverse - 5'-CCAGCCAGGTATGTCGTTTTGTA-3' - 44 kb Forward Reverse - 5'-CAGTCACTCATCAGCAAAGCCGGG-3' Reverse - 5'-CAGTCACTCATCAGCAAAGCCGG-3' Reverse - 5'-CAGTCACTCATCAGCAAAGCGGG-3' Reverse - 5'-AGTTGAACCCCAGCAGGGAATTGGG-3' Reverse - 5'-CCAGCCAGGTATTGCGTTTTTTTA-3' - 47 kb Forward Reverse - 5'-CCAGCCAGGTATTGCGTTTTTTTA-3' - 47 kb Forward Reverse - 5'-CCAGCCAGGAAATCGCTTGAACCC-3' Reverse - 5'-CCAGCCAGGAAATCGCTTGAACCC-3' Reverse - 5'-CCAGCCAGGAGAATCGCTTGAACCC-3' Reverse - 5'-CCAAGTGACCAGCCAGCAGTGTTT-3' - CYP1B1 promoter Forward - 5'-CCACTCCAGAGTCAAAGCCGCCCAT-3' Reverse - 5'-CCACTCCAGAGTCAAAGCCGCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCCACGT-3' - 5'-CCACTCCAGAGTCAAAGCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCGCCACT-3' - 5'-CCACTCCAGAGTCAAAGCCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3' - 5'	Reverse	5'-ACCTTGGCCTGGAGCTGAACACTCA-3'
Forward Reverse  -15 kb  Forward S'-GCTGCAAATGACAGGATCTCATTGG-3' Reverse -19 kb  Forward Reverse -23 kb Forward Reverse -33 kb Forward S'-ACTCAGTCTAGGCCAGCTATACGCCAGCAGCTAGTAGGCAGCAGCTGCTTCCAGAGCCTGCTTCCAGAGCCTGCTGCAGAGCCTCCTTCCAGAGCCTGCTGCAGAGCCTCCTTCCAGAGCCTGCTGCAGGAGCAGAGAGAG		
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- 15 kb Forward Reverse - 19 kb Forward Reverse - 19 kb Forward Reverse - 23 kb Forward Reverse - 23 kb Forward Reverse - 23 kb Forward Reverse - 5'-ACTCAGTCTAGGCCACCTATACGCCAGC-3' Reverse - 23 kb Forward Reverse - 5'-CTTGGGCTGAGACCAGGCTCC-3' Reverse - 5'-CTTGGGCTGAAAATCAGGAGCTCC-3' Reverse - 5'-CTTGGGCTGAAAATCAGGAGTTGG-3' Reverse - 5'-GCAAAGTATGGCAAAGCCTGCTTGC-3' Reverse - 5'-GCAAAGTATGGCAATGCCTGCTTGC-3' Reverse - 5'-AACGCGTGAAAACCCCATCTCTACT-3' - 37 kb Forward Reverse - 5'-TTGAGCAGCATGGGGTCACCACA-3' Reverse - 5'-TTGAGCAGCATGGGGTCTCCAGCA-3' Reverse - 5'-TTGAGCAGCATGGGGTCTCCAGAC-3' Reverse - 5'-TGGTAGCTGCCAGGAACAATGGG-3' Reverse - 5'-GATTCTCACGGCAAGAAGGACTCTC-3' Reverse - 5'-CCAGCCAGGTATGTGCTTGTA-3' - 44 kb Forward Reverse - 5'-CAGCCAGGTATGTGCTTGAACCC-3' Reverse - 5'-AGTTGAACTCCTGGCAGGAGATGCCTGTTA-3' - 47 kb Forward Reverse - 5'-ATCCTCACTCATCAGCAAAGCGGG-3' Reverse - 5'-CCAAGTGACCAGCAGAAAGCGGG-3' Reverse - 5'-CCAAGTGACCAGCCAGCAGTGTTT-3' - 5'-CCAAGTGACCAGCCAGCAGTGTT-3' - 5'-CCAAGTGACCAGCCAGCAGTGTT-3' - 5'-CCAAGTGACCAGCCAGCAGTGTT-3' - 5'-CCCACTCCAGAGTCAAAGCCGCCAT-3' - 5'-CCCACTCCAGAGTCAAAGCCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCCGCCACGT-3' - 5'-CCACTCCAGAGTCAAAGCCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCCGCCACGT-3' -		
Forward Reverse  -19 kb  Forward Reverse  -19 kb  Forward Reverse  -23 kb  Forward Reverse  -23 kb  Forward Reverse  -33 kb  Forward Reverse  -33 kb  Forward Reverse  -34 kb  Forward Reverse  -35'-ACTCAGTCTAGGCCAACCAGGCTCA-3'  -36 kb  Forward Reverse  -37 kb  Forward Reverse  -37 kb  Forward Reverse  -37 kb  Forward S'-GCGCTCTGGGTCAGGAGCATCTC-3'  -37 kb  Forward Reverse  -37 kb  Forward S'-GCGCTCTGGGTCAGTCCTCACCA-3' Reverse  -38 kb  Forward S'-TTGAGCAGCATGGGGTCTCCACCA-3' Reverse  -38 kb  Forward S'-TTGAGCAGCATGGGGTCTCCAGAC-3' Reverse  -38 kb  Forward S'-GATTCTCACGGCAAGAAGGACTCTC-3' Reverse  -38 kb  Forward S'-GATTCTCACGGCAAGAAGAGACTCTC-3' Reverse  -44 kb  Forward S'-GATTCTCACGGCAAGAAGAGACTCTC-3' Reverse  -47 kb Forward S'-TGAGGCAGGAGAATCGCTTGAACCC-3' Reverse  -47 kb Forward S'-ATCCTCACTCATCAGCAAAGCGGG-3' Reverse  -47 kb Forward S'-ATCCTCACTCATCAGCAAAGCGGG-3' Reverse  -47 kb Forward S'-ACCTCACTCATCAGCAAAGCGGG-3' Reverse  -47 kb Forward S'-CCAAGTGACCAGCCAGCAGTGTTT-3'  CYP1B1 enhancer Forward Reverse  5'-CCAAGTGACCAGCCAGGAATAGACCGGG-3' Reverse  5'-CCAAGTGACCAGCCAGGAATAGACCGGA-3' Reverse  5'-CCAAGTGACCAGCCAGGAATAGACCGGA-3' Reverse  5'-CCAAGTGACCAGCCAGGAATAGACCGGA-3' Reverse  5'-CCAAGTGACCAGCCAGGAATAGACCGGA-3' Reverse  5'-CCAAGTGACCAGCCAGGAATAGACCGGA-3' Reverse  5'-CCAAGTGACCAGCCAGGAATAGACCGGA-3' Reverse  5'-CCAAGTGACCAGCCAGCCAAT-3'  CYP1B1 promoter Forward S'-TCCCATGAAAGCCTGCTGGTAGAGC-3' Reverse  5'-CCACTCCAGAGTCAAAGCGGCCCAT-3'  GAPDH promoter Forward  5'-TCCCATGAAAGCCTGCTGGTAGAGC-3' Forward S'-TCCCATCCAGAGTCAAAGCGGCCCAT-3'  Forward S'-TACTAGCGGTTTTACGGGCGCACGT-3'		5 -GCIGCAAAIGACAGGAICICAIIGG-5
Reverse 5'-CCTACTCTGGTCAGAGCCTCCTTCC-3' -19 kb Forward 5'-AGCCTGTAATCTCAGCTACTCAGGA-3' Reverse ATGAGCCCTGCTGTCAGGAGCATCT-3' -23 kb Forward 5'-ACTCAGTCTAGGCCAACCAGGCTCA-3' Reverse 5'-CTTGGGCTGAAAATCAGGAGTGGCT-3' -33 kb Forward 5'-GCTTGAACACCGGAGGCAGAGTTTG-3' Reverse 5'-GCAAAGTATGGCAATGCCTGCTTGC-3' -39 kb Forward 5'-GCGCTCTGGGTCAGTGCCTCCACCA-3' Reverse 5'-AACGCGGTGAAAACCCCATCTCTACT-3' -37 kb Forward 5'-TTGAGCAGCATGGGGTCTCCAGCA-3' Reverse 5'-TGGTAGCGTGCCAGGAGACAATGGG-3' -38 kb Forward 5'-TTGAGCAGCATGGGGAACAATGGG-3' Reverse 5'-CCAGCCAGGTATGTCGTTTTTA-3' -44 kb Forward 5'-GATTCTCACGGCAAGAAGGACTCTC-3' Reverse 5'-CCAGCCAGGTATGTCGTTTTTTA-3' -47 kb Forward 5'-TGAGGCAGGAGAATCGCTTGAACCC-3' Reverse 5'-AGTTGAACTCCTGGCAGGGAGTTAG-3' -47 kb Forward 5'-TGAGGCAGGAGAATCGCTTGAACCC-3' Reverse 5'-AGTTGAACTCCTGGCAGGGAGTTAG-3' -47 kb Forward 5'-TCCCATCACTCATCAGCAAAGCGGG-3' Reverse 5'-CCAAGTGACCAGCCAGCAGTGTTT-3'  CYP1B1 enhancer Forward 5'-GGCAGCCCCAGGGATATGACTGGA-3' Reverse 5'-CCGAGGGAGTATGACTGGA-3' Reverse 5'-CCAAGTGACCAGCCAGCAGTGTTT-3'  CYP1B1 promoter Forward 5'-TCCCATGAAAGCCTGCTGGTAGAGC-3' Reverse 5'-CCACTCCAGAGTCAAAGCGGCCCAT-3'  Forward 5'-TCCCATGAAAGCCTGCTGGTAGAGC-3' Reverse 5'-CCACTCCAGAGTCAAAGCGGCCCAT-3'  CYP1B1 promoter Forward 5'-TACTAGCGGTTTTACGGGCGCACGT-3'		-1
- 19 kb Forward Reverse - 23 kb Forward Reverse - 23 kb Forward Reverse - 5'-ACTCAGTCTAGGCCAACCAGGCTCA-3' Reverse - 5'-CTTGGGCTGAAAATCAGGAGGCTCA-3' Reverse - 5'-CTTGGGCTGAAAATCAGGAGTGGCT-3' - 33 kb Forward Reverse - 5'-GCAAAGTATGGCAATGCCTGCTTGC-3' - 39 kb Forward Reverse - 5'-GCGCTCTGGGTCAGTGCCTCCACCA-3' Reverse - 5'-AACGCGGTGAAAACCCCATCTCTACT-3' - 37 kb Forward Reverse - 5'-TTGAGCAGCATGGGGTCTCCAGCA-3' Reverse - 5'-TGGTAGCGTGCCAGGAACAATGGG-3' Reverse - 5'-TGGTAGCGTGCCAGGGAACAATGGG-3' - 38 kb Forward Reverse - 5'-CCAGCCAGGTATGTGCGTTTGTA-3' - 44 kb Forward Reverse - 5'-CCAGCCAGGTATGTGCTTGAACCC-3' Reverse - 5'-AGTTGAACTCCTGGCAGGGAGTTAG-3' - 47 kb Forward Reverse - 5'-ATCCTCACTCATCAGCAAAGCGGG-3' Reverse - 5'-ATCCTCACTCATCAGCAAAGCGGG-3' Reverse - 5'-CCAAGTGACCAGCCAGCAGTGTTT-3'  CYPIBI enhancer Forward Reverse - 5'-CCAAGTGACCAGCCAGCAGTGTTT-3' - 5'-CCAAGTGACCAGCCAGCAGTGTGTT-3' - 5'-CCAAGTGACCAGCCAGCAGTGTGTT-3' - 5'-CCAAGTGACCAGCCAGCAGTGTGTT-3' - 5'-CCAAGTGACCAGCCAGCAGTGTGTT-3' - 5'-CCAAGTGACCAGCCAGCAGTGTGTT-3' - 5'-CCAAGTGACCAGCCAGCAGTGTGTT-3' - 5'-CCCATCCAGAGTCAAAGCCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCCGCCCAT-3' - 5'-CCACTCCAGAGTCAAAGCCGCCCACT-3' - 5'-CCACTCCAGAGTCAAAGCCCACTCTCAGAGTCAAAGCCCACTCATCATCAGAAGCCCAC		
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$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	-47 kb	
$\begin{array}{lll} CYP1B1 \text{ enhancer} \\ Forward & 5'-\text{GGCAGCGCCCAGGGATATGACTGGA-3'} \\ Reverse & 5'-\text{CGGAGAGTGGCAGGAGGAGGCGAAT-3'} \\ CYP1B1 \text{ promoter} \\ Forward & 5'-\text{TCCCATGAAAGCCTGCTGGTAGAGC-3'} \\ Reverse & 5'-\text{CCACTCCAGAGTCAAAGCGCGCCAT-3'} \\ GAPDH \text{ promoter} \\ Forward & 5'-\text{TACTAGCGGTTTTACGGGCGCACGT-3'} \\ \end{array}$		
Forward 5'-GGCAGCGCCCAGGGATATGACTGGA-3' Reverse 5'-CGGAGAGTGGCAGGAGGAGGCGAAT-3' $CYPIBI$ promoter Forward 5'-TCCCATGAAAGCCTGCTGGTAGAGC-3' Reverse 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3' $GAPDH$ promoter Forward 5'-TACTAGCGGTTTTACGGGCGCACGT-3'	Reverse	5'-CCAAGTGACCAGCCAGCAGTGTGTT-3'
	CYP1B1 enhancer	
$\begin{array}{lll} CYP1B1 \text{ promoter} \\ Forward & 5'-TCCCATGAAAGCCTGCTGGTAGAGC-3' \\ Reverse & 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3' \\ GAPDH \text{ promoter} \\ Forward & 5'-TACTAGCGGTTTTACGGGCGCACGT-3' \\ \end{array}$	Forward	5'-GGCAGCGCCCAGGGATATGACTGGA-3'
$\begin{array}{lll} CYP1B1 \text{ promoter} \\ Forward & 5'-TCCCATGAAAGCCTGCTGGTAGAGC-3' \\ Reverse & 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3' \\ GAPDH \text{ promoter} \\ Forward & 5'-TACTAGCGGTTTTACGGGCGCACGT-3' \\ \end{array}$	Reverse	5'-CGGAGAGTGGCAGGAGGAGGCGAAT-3'
Forward 5'-TCCCATGAAAGCCTGCTGGTAGAGC-3' Reverse 5'-CCACTCCAGAGTCAAAGCGCGCCAT-3'  GAPDH promoter Forward 5'-TACTAGCGGTTTTACGGGCGCACGT-3'		
		5'-TCCCATGAAAGCCTCCTCCTAGAGC-3'
GAPDH promoter Forward 5'-TACTAGCGGTTTTACGGGCGCACGT-3'		
Forward 5'-TACTAGCGGTTTTACGGGCGCACGT-3'		J CCACICCAGAGICAAAGCGCGCCAI-3
		E/ ma cma occommma coccocca com c/
neverse 5'-TCGAACAGGAGGAGCAGAGAGCGAA-3'		
	neverse	5 -TCGAACAGGAGGAGCGAA-3'

lottes ville, VA) with the following modifications: the cellular extract in ChIP dilution buffer was precleared with protein A agarose/salmon sperm DNA (Upstate Biotechnology; 60  $\mu$ l per milliliter of extract) for 18 h and then again for 1 h at 4°C. The cleared cellular extract was then incubated with antibody (10  $\mu$ l per milliliter of

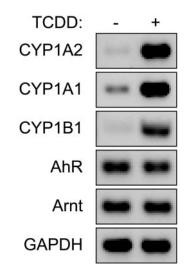
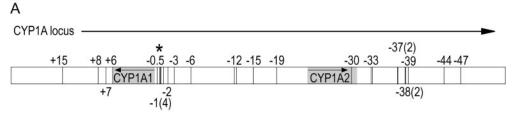
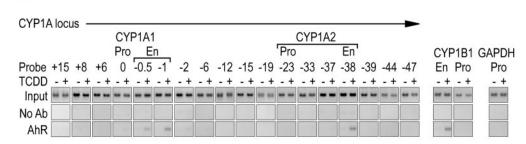


Fig. 1. TCDD induction of CYP1 mRNAs in RWPE-1 cells. RNA isolated from untreated or TCDD treated (10 nM, 18 h) RWPE-1 cells was analyzed by RT-PCR to assess the level of CYP1A2, CYP1A1, CYP1B1, AhR, Arnt, and GAPDH RNA. CYP1A2 RNA was amplified for 35 PCR cycles; CYP1A1, CYP1B1, AhR, and Arnt RNA were amplified for 30 PCR cycles; and GAPDH RNA was amplified for 28 PCR cycles.

extract) for 1 h at 4°C. The antibody was then precipitated with protein A agarose/salmon sperm DNA (60 µl per milliliter of extract) for 1 h at 4°C. The protein A agarose-antibody/chromatin complex was then transferred to a small spin column (QIAGEN) for washing. Three 400-µl washes were performed with each of the following buffers at room temperature: low-salt immune complex wash buffer, high-salt immune complex wash buffer, LiCl immune complex wash buffer, high-salt LiCl immune complex wash buffer (1 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, and 1 mM Tris, pH 8.1), and Tris-EDTA buffer. After the last Tris-EDTA wash, the small spin columns were transferred to a fresh collection tube, and the protein-DNA complexes were eluted as indicated in the EZ-ChIP protocol. After the cross-link reversal and RNase A/proteinase K digestion steps, the DNA was purified using the QIAquick PCR purification kit (QIAGEN) as indicated by the manufacturer. The immunoprecipitated DNA was eluted in a total volume of 200 μl. DNA (10 µl) was analyzed in a 20-µl PCR reaction using the following conditions: 3 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C followed by a 5-min 72°C extension step. The amplified DNA was electrophoresed on a 2.5% agarose gel and visualized by staining with ethidium bromide. The AhR antibody used in the immunoprecipitation was from Santa Cruz Biotechnology (H-211X; Santa Cruz, CA). The acetyl histone H4 antibody was purchased from Upstate Biotechnology. The sequences of the primers used in the ChIP analysis are shown in Table 1.

In Vivo Footprinting. In vivo footprinting using dimethyl sulfate (DMS; Sigma, St. Louis, MO) was performed as described previously (Okino and Whitlock, 1995). To analyze the the *CYP1A2* 3' enhancer, we used the following primer set: primer 1, (5'-AT-GGGCAGATGTGGCCTGGA-3'); primer 2, (5'-CTGGAGTGGTCAG-GTGGATGCCTGT-3'); and primer 3, (5'-GTGGTCAGGTGGATGC-





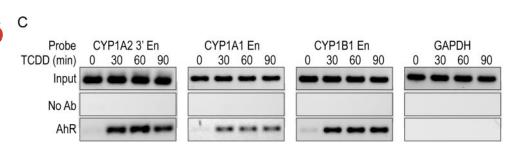


Fig. 2. AhR interaction with the CYP1A locus. A, a schematic depiction of a 75-kb region that encompasses the human CYP1A locus. The coding regions of CYP1A1 and CYP1A2 are in gray, and the direction of transcription is indicated. High-affinity AhR binding sites are indicated (black lines) along with the approximate distance, in kilobases (kb), from the CYP1A1 transcriptional start site. For closely spaced AhR binding sites, the number of sites is indicated in parenthesis. The location of the CYP1A1 enhancer is also indicated (\*). B, AhR binding on the CYP1A locus, the CYP1B1 gene, and the GAPDH gene was assessed in untreated and TCDD-treated (10 nM, 1 h) RWPE-1 cells by ChIP. Enhancer (En) and promoter (Pro) regions are indicated. C, AhR binding on the CYP1A2 3' enhancer, the CYP1A1 enhancer, the CYP1B1 enhancer, and the GAPDH promoter was assessed in RWPE-1 cells treated with TCDD (10 nM) for the indicated length of time by



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Construction of Reporter Genes. The pGL3–38k-wt reporter vector was constructed by cloning 112 bp of human chromosome 15 DNA sequence into the Nhe-I site of the pGL3-promoter vector (Promega, Madison, WI). pGL3–38k-mut was identical with pGL3–38k-wt, except that the sequence of the DRE sites was changed to prevent AhR binding.

Transient Transfections and Luciferase Activity Assays. RWPE-1 and HepG2 cells were plated at a density of approximately  $1.5 \times 10^5$  and  $1.3 \times 10^5$  cells/well, respectively, in 24-well plates. Transfections were performed the following day using Fugene6 transfection reagent following the manufacturer's protocol (Roche Applied Science, Indianapolis, IN). The pDRE12 plasmid, a gift from Dr. Judy Raucy (Puracyp Inc., Carlsbad, CA), contains three copies of the consensus DRE subcloned into the pGL3-promoter vector and was used as a positive control for TCDD-inducible reporter gene activity (Yueh et al., 2005). For each transfection assay, 100 ng of luciferase reporter and 10 ng of pRL-SV40 (for transfection normalization; Promega) were transfected into each well. The culture media were removed after incubation for 24 h with the transfection reagent-DNA complexes, and the cells were then treated for 24 h with 10 nM TCDD dissolved in DMSO. Control cells received media containing 0.1% DMSO. After treatment, cells were rinsed with phosphatebuffered saline, and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity of cellular lysates was quantified with a Packard LumiCount luminometer (PerkinElmer Life and Analytical Sciences, Waltham, MA). Firefly luciferase activity was determined from three independent transfections, and normalized against Renilla reniformis luciferase activities of the pRL-SV40 vector obtained from the same culture.

**Statistics.** Statistics were performed using InStat Instant Statistics (Prism 4; GraphPad Software, San Diego, CA). Statistical differences between values were determined by two-tailed t test for comparing means from two groups. A p < 0.05 is considered statistically significant.

Bisulfite DNA Sequencing. Genomic DNA was isolated from cultured cells using the AllPrep DNA/RNA Mini Kit (QIAGEN) following the manufacturer's directions. Bisulfite modification of genomic DNA was performed using the EpiTect Bisulfite Kit (QIA-GEN) following the manufacturer's directions. Bisulfite-modified DNA was amplified using two rounds of PCR using nested primers that recognize the bisulfite modified DNA region 3' of CYP1A2. All primer sequences lack CpG sites and thus amplify methylated and unmethylated DNA equivalently. Sequences of the primers are as follows: CYP1A2-3'-1F (5'-CTAACCCTACCCTAAACCTTACTAA-3'); CYP1A2-3'-2F (5'-ACCTTACTAACCTAAAATAACCCAA-3'); and CYP1A2-3'-4R (5'-GGAAATGAGGGAAAAGGAGATAGAG-3'). PCR conditions were 3 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C followed by a 5-min 72°C extension step. First-round PCR was with the CYP1A2-3'-1F and CYP1A2-3'-4R primers. Second-round PCR was performed using 1 µl of the firstround PCR product in a total volume of 50 μl with the CYP1A2-3'-2F and CYP1A2-3'-4R primers. The amplification product was confirmed by electrophoresis on a 2% agarose gel and sequenced directly



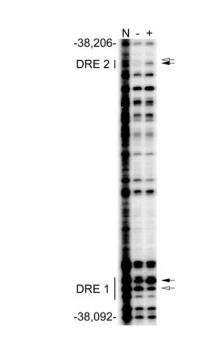


Fig. 3. TCDD-inducible occupancy of AhR binding sites on the CYP1A2 3' enhancer. A, sequence of the human CYP1A2 3' enhancer region. High-affinity DRE sites are in boldface type. The DNA regions used to design primers used for ChIP and ligation-mediated polymerase chain reaction primer 3 are indicated (boxed). The DNA region cloned into pGL3-38k-wt is indicated (italics), and the base changes used in the construction of pGL3-38k-mut are shown (below sequence). Filled arrows indicate sites hypersensitive to DMS modification after TCDD treatment. Open arrows indicate sites hyposensitive to DMS modification after TCDD treatment. The distance, in base pairs, from the CYP1A1 transcriptional start site is also indicated. B, untreated or TCDD-induced (10 nM, 1 h) RWPE-1 cells were treated with 0.1% DMS for 90 s. Genomic DNA was isolated, and the pattern and extent of DMS modification was assessed by ligation-mediated PCR. N, naked genomic DNA treated with DMS in vitro; -, genomic DNA isolated from untreated cells; +, genomic DNA isolated from TCDD-induced cells. Filled arrows indicate sites hypersensitive to DMS modification after TCDD treatment. Open arrows indicate sites hyposensitive to DMS modification after TCDD treatment. DRE sites and the distance in base pairs from the CYP1A1 transcriptional start site are also indicated.

with the *CYP1A2-3'-2F* primer by an outside vendor (McLab, South San Francisco, CA).

Cross-Species Comparison of DNA Homology. The cross-species comparison of DNA homology was performed using the vertebrate multiz alignment and conservation track on the University of California at Santa Cruz genome browser (http://genome.ucsc.edu/) using the March 2006 human genome assembly (Kent et al., 2002).

## Results

A Cell Culture Model System to Study TCDD Induction of CYP1A2. The dioxin-responsive enhancers of CYP1A1 and CYP1B1 are well characterized; they are both located 5' of the promoter and contain multiple DRE sites that bind AhR and confer TCDD-inducibility on a reporter gene (Hankinson, 1995; Zhang et al., 1998; Whitlock, 1999; Tsuchiya et al., 2003). In contrast, the location of the CYP1A2 enhancer is not clear due, at least in part, to the lack of a cell culture model system that expresses CYP1A2 well. To identify a model system to analyze CYP1A2 regulation, we screened several human prostate cell lines for their ability to respond to dioxin. We find that in RWPE-1 cells, a normal human prostate epithelial cell line, the level of CYP1A2 mRNA is dramatically increased by TCDD exposure; CYP1A1 and CYP1B1 are also strongly induced. In addition, the main components of the TCDD-response system, AhR and Arnt, are expressed constitutively (Fig. 1). From these results we infer that analysis of the native CYP1A2 gene in RWPE-1 cells can provide mechanistic insight into its induction by TCDD.

TCDD-Inducible AhR Binding 3' of *CYP1A2*. To identify sites that might bind AhR on the *CYP1A* locus, we scanned 75 kb of human chromosome 15 DNA sequence for the high-affinity DRE site: 5'-TNGCGTG-3' (Swanson et al., 1995). We find that in addition to the well-characterized DRE sites on the *CYP1A1* enhancer, many other DRE sites exist (Fig. 2A). We then analyzed most of these sites for the ability to interact with AhR by chromatin immunoprecipitation (Fig. 2B). Some DNA regions have high sequence similarity with

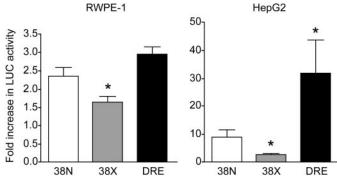


Fig. 4. Reporter gene analysis of the CYP1A2 3' enhancer. Expression of pGL3–38k-wt (38N) and pGL3–38k-mut (38X) in RWPE-1 and HepG2 cells. CYP1A2 3'-flanking sequences containing AhR binding sites and mutations of those sites were cloned into the pGL3-promoter plasmid and transfected into cell lines as described under Materials and Methods. pDRE12 (DRE), containing three copies of the consensus DRE subcloned into the pGL3-promoter vector, was used as a control. DMSO and TCDD treatments were performed 24 h after transfection; the cells were harvested and assayed for luciferase activity 24 h after chemical treatment. The amount of Firefly luciferase activity was normalized against R. reniformis luciferase activities of the pRL-SV40 vector obtained from the same culture. Data are presented as the ratio of luciferase activity of treated cells to DMSO control cells and represent the mean  $\pm$  S.D. from three experiments performed in triplicate. \*, denotes statistical significance (p < 0.05) of each reporter construct versus 38N.

other genomic regions and were thus refractory to PCR analysis. As expected, the CYP1A1 and CYP1B1 enhancers exhibit strong TCDD-inducible interaction with AhR. We also found that a region 3' of the CYP1A2 gene (approximately −38 kb relative to the *CYP1A1* transcription start site) exhibits TCDD-inducible AhR binding. We cannot detect AhR binding at other sites on the CYP1A locus. A time course experiment reveals that the CYP1A2 3' region binds AhR with the same kinetics as the CYP1A1 and CYP1B1 enhancers, indicating that binding there, like on CYP1A1 and CYP1B1, is a primary response to TCDD (Fig. 2C). These findings indicate that only two regions within the CYP1A locus have the requisite in vivo AhR-binding property expected of a dioxin-inducible AhR-dependent enhancer, the previously characterized CYP1A1 enhancer, and a novel region 3' of CYP1A2.

TCDD-Inducible Protein Binding at DREs 3' of **CYP1A2.** Two high-affinity DRE sites are contained within the AhR-binding CYP1A2 region. These sites are separated by 87 bp and are in opposite orientations (Fig. 3A). We conducted an in vivo footprinting experiment to verify that these sites are indeed occupied by protein in response to dioxin (Fig. 3B). We treated control and TCDD-induced RWPE-1 cells with DMS, a reagent that modifies DNA in living cells. We then isolated genomic DNA and assessed the pattern and extent of DMS modification by ligation-mediated PCR. We observe a TCDD-inducible change in the DMS modification pattern at both of the DRE sites in the CYP1A2 3' region. Within each DRE, one base exhibits hyposensitivity, and another base exhibits hypersensitivity to DMS modification. This TCDD-inducible change in the pattern of DRE modification is consistent with changes identified previously analyzing CYP1A1 enhancer DREs in mouse and human cells (Wu and Whitlock, 1993; Kress et al., 1998; Okino et al., 2006). This implies that DRE sites 3' of CYP1A2 are occupied in response to TCDD exposure. This finding, together with our data analyzing AhR-DNA interaction (Fig. 2B), strongly suggests that AhR binds to two DRE sites located 3' of the endogenous CYP1A2 gene.

Reporter-Gene Analysis. We next performed a reportergene analysis to determine whether the CYP1A2 3' region can function as a TCDD-responsive enhancer. We constructed two reporter vectors using the pGL3 promoter backbone, one vector, termed pGL3-38k-wt, contains 112 bp of genomic DNA sequence, including both DRE sites. The other vector, termed pGL3-38k-mut, was identical with the first except that the sequence of both DRE sites was changed to prevent AhR binding (Fig. 3A). We then assayed the TCDDresponsiveness of these vectors and pDRE12, a TCDD-responsive control plasmid (Yueh et al., 2005), by transient transfection into RWPE-1 cells and HepG2 cells. Our results (Fig. 4) reveal that the pGL3-38k-wt reporter construct is inducible by TCDD in both cell lines, although the magnitude of induction is greater in HepG2 cells (mean  $\pm$  S.D., 2.33  $\pm$ 0.25 and  $8.90 \pm 2.75$ , respectively). When the DRE sites were mutated, the TCDD-responsiveness decreased considerably. It is interesting that the decrease in inducibility of the reporter construct harboring the mutated sites was not as pronounced in the RWPE-1 cells relative to HepG2 cells (30 versus 72% decrease, respectively). The low level of TCDDresponsiveness observed in the pGL3-38k-mut reporter construct is probably due to a low-affinity DRE site (5'-GT-

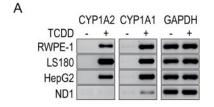


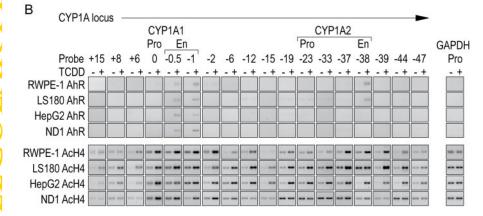
GCGTG-3') centered at -38,116 relative to the *CYP1A1* transcriptional start site. These findings, together with the results of our ChIP and in vivo footprinting experiments (Figs. 2 and 3), indicate that the *CYP1A2* 3' region can function as a TCDD-responsive AhR-dependent enhancer.

Analysis of Other Human Cell Lines. We extended our analysis of the native *CYP1A* locus by studying three additional human cell lines. We analyzed LS180 cells (colon adenocarcinoma) and HepG2 cells (hepatocellular carcinoma), which are inducible for *CYP1A2* (Li et al., 1998), as well as ND-1 cells (prostate adenocarcinoma), which represent a cancerous counterpart to the noncancerous RWPE-1 prostate cells. Analysis of RNA induction by both conventional PCR and real-time PCR reveals that *CYP1A2* is strongly induced in RWPE-1, LS180, and HepG2 cells, whereas induction is barely detectable in ND-1 cells (Fig. 5A and Table 2). All of the cell lines induce CYP1A1 RNA; however, in ND-1 cells, the fold induction and level of induced CYP1A1 RNA is significantly lower than that found in the other cell lines (Fig. 5A and Table 2).

ChIP analysis of the *CYP1A* locus reveals that AHR binds to the *CYP1A2* 3' enhancer in TCDD-treated RWPE-1 and LS180 cells; thus, the activity of the *CYP1A2* 3' enhancer is not restricted to a single cell line. In contrast, in HepG2 and ND-1 cells, AhR does not bind to the *CYP1A2* 3' region (Fig. 5B). These findings imply that AhR binding to the *CYP1A2* 3' region is associated with *CYP1A2* induction but is not required. In all cell lines, AhR binds to the *CYP1A1* enhancer after TCDD treatment. We do not detect AhR binding at other *CYP1A* DRE sites.

We also analyzed histone H4 acetylation on the *CYP1A* locus by ChIP. Previously, we and others demonstrated that TCDD increases histone H4 acetylation on the *CYP1A1* regulatory region (Ke et al., 2001; Hestermann and Brown, 2003; Okino et al., 2006). Our results show that in the three cell lines that strongly induce *CYP1A1* and *CYP1A2* (RWPE-1, LS180 and HepG2), TCDD treatment increases histone H4 acetylation along the entire 75-kb *CYP1A* locus. In contrast, in ND-1 cells in which *CYP1A1* but not *CYP1A2* is inducible, only the chromatin in the immediate vicinity of





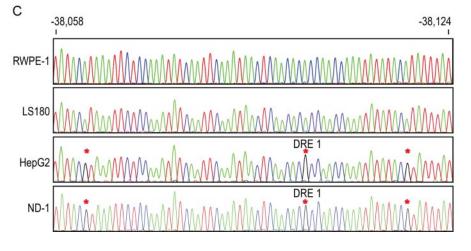


Fig. 5. Analysis of a several human cell lines. A RNA isolated from untreated or TCDD treated (10 nM, 18 h) cells was analyzed by RT-PCR to assess the level of CYP1A2, CYP1A1, and GAPDH RNA. CYP1A2 RNA was amplified for 35 PCR cycles, CYP1A1 RNA was amplified for 30 PCR cycles, and GAPDH RNA was amplified for 28 PCR cycles. B, AhR binding and histone H4 acetylation on the CYP1A locus and GAPDH promoter was assessed in untreated and TCDDtreated (10 nM, 1 h) cells by ChIP. The no-antibody and input controls for these reactions are presented in Supplementary Fig. S1. C, DNA methylation on the CYP1A2 3' enhancer was assessed by bisulfite DNA sequencing. The black lines indicate 5-methylcytosine; the red lines indicate adenine; the green lines indicate thymine and unmethylated cytosine; the blue lines indicate guanine; red asterisks indicates sites containing significant levels of 5-methylcytosine. The location of DRE 1 and the distance from the CYP1A1 transcriptional start site is indicated. Note that the sequencing reaction reads the complimentary strand of the sequence presented in Fig. 3A.

CYP1A1 exhibits increased histone H4 acetylation. These results reveal a strong association between induced histone acetylation and induced gene activity.

We suspected that the inability of AhR to interact with the CYP1A2 3' region in HepG2 and ND-1 cells might be associated with DNA methylation. Previously, we demonstrated that DNA methylation on the CYP1A1 enhancer prevents AhR binding and inhibits CYP1A1 induction in human prostate cancer (Okino et al., 2006); others have shown that the CYP1A2 promoter is methylated in mouse tissues (Jin et al., 2004). Our analysis of DNA methylation (Fig. 5C and data not shown) reveals that all CpG sites within the region analyzed (between -37,900 and -38,125 relative to the CYP1A1 transcription start site) are completely methylated in HepG2 and ND-1 cells. In contrast, no methylation is detected in RWPE-1 and LS180 cells. It is noteworthy that in HepG2 and ND-1 cells, the CpG site within DRE-1 is completely methylated; such methylation was shown previously to inhibit AhR interaction in vitro and in vivo (Shen and Whitlock, 1989). We infer that DNA methylation inactivates the TCDDresponsive enhancer 3' of CYP1A2 in HepG2 and ND-1 cells.

# **Discussion**

The members of the *CYP1* gene family, *CYP1A1*, *CYP1A2*, and *CYP1B1*, are transcriptionally induced by the AhR. Although the TCDD-responsive, AhR-dependent enhancers for *CYP1A1* and *CYP1B1* are well characterized, details regarding a similar *CYP1A2* enhancer remained elusive (Hankinson, 1995; Whitlock et al., 1997; Nebert and Dalton, 2006). Here, we demonstrate that DRE sites 3' of the native *CYP1A2* gene bind AhR in TCDD-treated human prostate and colon cells and function as a TCDD-responsive enhancer in a reporter gene assay. We infer that this region participates in TCDD induction of *CYP1A2* in humans and is thus a bona fide *CYP1A2* enhancer.

Unlike the dioxin-responsive enhancers for *CYP1A1* and *CYP1B1*, which are positioned approximately 1 kb upstream of their respective genes, the *CYP1A2* enhancer is downstream of *CYP1A2* and further away, approximately 15 kb away from the the *CYP1A2* transcriptional start site. There is precedence for an enhancer being located downstream of its target gene; there is also precedence for an enhancer being located at a distance, up to 100 kb away from the promoter

TABLE 2 Quantitation of CYP1A RNA levels

CYP1A1, CYP1A2, and GAPDH RNA levels were estimated by real-time PCR. The -fold TCDD induction was calculated as the ratio of induced CYP1A RNA to basal CYP1A RNA. The induced RNA level was calculated as (induced CYP1A RNA level/corresponding GAPDH RNA level) × 100. The liver and prostate RNA data were from an analysis of a single sample. The other results represent an average of two independent samples. No information regarding the TCDD/xenobiotic exposure of the liver and prostate RNA donors is known; thus, only the level of CYP1A RNA relative to GAPDH is reported.

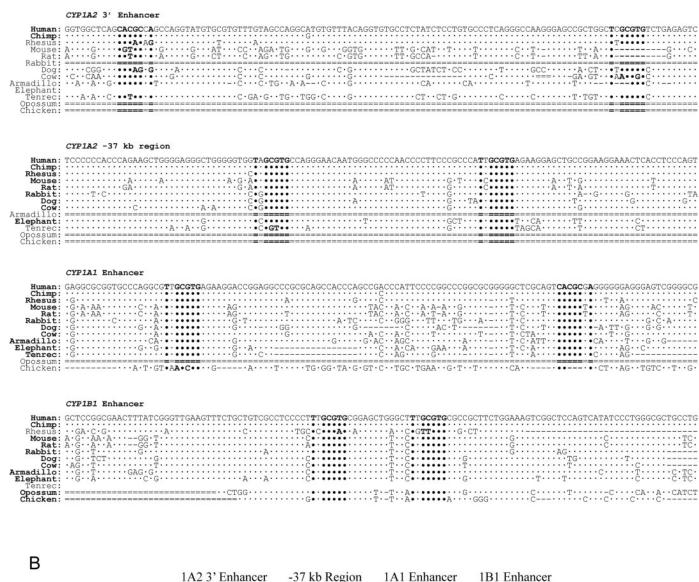
	Fold TCDD Induction		Induced RNA Level (Percentage of GAPDH RNA Level)	
	CYP1A1	CYP1A2	CYP1A1	CYP1A2
RWPE-1	310	45	60	0.020
LS180	6700	3300	65	0.700
HepG2	440	210	73	0.132
ND-1	8	2	0.1	0.002
Liver			4.6	49
Prostate			2	0.053

that it controls (Blackwood and Kadonaga, 1998; Carroll et al., 2005; Maston et al., 2006). Therefore, although the placement and distance of the *CYP1A2* enhancer is unique among characterized AhR-regulated genes, it is not unusual in the context of general enhancer action.

It is significant that HepG2 cells induce CYP1A2 in the absence of AhR binding to its 3' enhancer (Fig. 5). This implies that binding to this enhancer is not required for CYP1A2 induction. Consistent with this, a cross-species DNA sequence homology comparison shows that the DRE sites within this region are not well conserved in other species (Fig. 6). Again, this implies that the CYP1A2 enhancer identified in this study is not required for TCDD-induction of CYP1A2. Therefore, another enhancer probably controls CYP1A2. It is interesting that we find that two closely spaced DRE sites located just 1 kb away from the identified CYP1A2 enhancer exhibit cross-species conservation at a level similar to that found in the *CYP1A1* and *CYP1B1* enhancers (Fig. 6). This suggests that this region may participate in TCDD regulation. However, our ChIP data clearly show that these DREs, located at -37 kb relative to the CYP1A1 transcriptional start site, do not bind AhR in any cell line tested (Fig. 5b). Not surprisingly, analysis of DNA methylation shows that both DRE sites are completely methylated in RWPE-1, LS180, HepG2, and ND-1 cells (data not shown). Thus, DNA methylation probably suppresses the TCDD-responsiveness of this region. We suspect that the growth of cells as a monolayer culture inactivates this region. Indeed, the expression of CYP1A2 in primary hepatocytes is known to dramatically decrease over time, whereas the extent of CYP1A2 promoter methylation increases (Nemoto and Sakurai, 1993; Jin et al., 2004). Future studies that determine the significance of this putative control region may provide novel insights into CYP1A2 regulation.

We have identified a TCDD-responsive CYP1A2 enhancer that can be regulated by DNA methylation and is primarily human-specific. We believe that expression of CYP1A2 in humans involves coordination between this enhancer, the CYP1A1 enhancer, the CYP1A2 promoter, and the putative CYP1A2 regulatory region described above. Obviously, control of CYP1A2 is quite complex and involves multiple regulatory regions and a layer of epigenetic control. In addition, based on the species-specificity of this enhancer, TCDD-regulation of CYP1A2 is likely to differ between humans and most other animal species.

PAHs are toxic and carcinogenic compounds that are ubiquitous in the environment and prevalent in cigarette smoke, automobile exhaust, and charcoal-cooked meats. PAH exposure activates AhR and induces the expression of the CYP1 genes. The CYP1 gene products initiate PAH metabolism through oxidation (Poland and Knutson, 1982; Whitlock et al., 1997). After oxidation, the PAHs are further modified by conjugation with glutathione, ultimately leading to their detoxification and elimination from the body. Studies in knockout mice reveal that induction of CYP1A1 and CYP1A2 is advantageous because animals that lack them are acutely sensitive to chemical toxicity. In contrast, CYP1B1 induction has an adverse effect because Cyp1b1<sup>-/-</sup> animals are protected against PAH toxicity (Nebert et al., 2004; Nebert and Dalton, 2006). Our previous work revealed that some prostate tumors are probably unable to induce CYP1A1 because its enhancer is silenced by DNA hypermethylation (Okino et Α



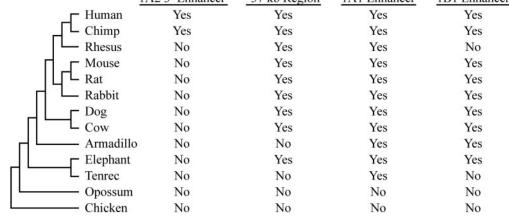


Fig. 6. Cross-species comparison of DNA homology. A, the cross-species homology of specific DNA regions was compared using the vertebrate multiz alignment and conservation track on the University of California at Santa Cruz Genome browser (http://genome.ucsc.edu/) (Kent et al., 2002). DRE sites are in boldface type. Dots represent bases identical with the corresponding human base. Single line, no bases in the aligned region. Double line, aligning species has one or more unalignable bases in the gap region. Species name in boldface type indicates that both AhR binding sites are conserved. B, cross-species conservation of AhR binding sites. Yes indicates that both AhR binding sites are conserved in a particular species. No indicates that the core AhR binding motif of at least one site is not conserved in a particular species.

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al., 2006). In contrast, CYP1B1 is overexpressed in prostate tumors due to gene hypomethylation (Tokizane et al., 2005). Here we show that induction of CYP1A2, like CYP1A1, may be silenced by enhancer hypermethylation in human prostate cancer. Thus, in prostate tumors, two genes that protect against chemical toxicity are suppressed, and a gene that mediates PAH toxicity is overexpressed. The combined effect probably results in increased sensitivity to PAH toxicity. To compound this, two glutathione S-transferases that detoxify PAHs are not expressed in most prostate cancers because their genes, GSTP1 and GSTM1, are inactivated by DNA hypermethylation (Harden et al., 2003; Nakayama et al., 2004; Lodygin et al., 2005). Thus, some prostate tumors are likely to be acutely sensitive to adverse PAH effects. Indeed, several large epidemiological studies demonstrate that smokers, a group that has high PAH exposure, have higher prostate cancer-associated mortality (Rodriguez et al., 1997; Giovannucci et al., 1999; Doll et al., 2005). Future epidemiological studies that assess DNA methylation on genes involved in xenobiotic metabolism may provide insights into this intriguing observation.

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