Interactions between the Aryl Hydrocarbon Receptor and P-TEFb

SEQUENTIAL RECRUITMENT OF TRANSCRIPTION FACTORS AND DIFFERENTIAL PHOSPHORYLATION OF C-TERMINAL DOMAIN OF RNA POLYMERASE II AT cyp1a1 PROMOTER*

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The expression of the cytochrome P450 1A1 gene (cyp1a1) is regulated by the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor that mediates most toxic responses induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In the nucleus, ligand-activated AhR binds to the xenobiotic response elements, initiating chromatin remodeling and recruitment of coregulators, leading to the formation of preinitiation complex followed by elongation. Here, we report that ligand-activated AhR recruits the positive transcription elongation factor (P-TEFb) and RNA polymerase II (RNA PII) to the cyp1a1 promoter with concomitant phosphorylation of the RNA PII carboxyl domain (CTD). Interestingly, the serine 2 and serine 5 of the heptapeptide repeats (YSPTSPS) were sequentially phosphorylated upon TCDD treatment. Inhibition of P-TEFb kinase activity by 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) suppressed CTD phosphorylation (especially serine 2 phosphorylation) and abolished processive elongation without disrupting the assembly of the preinitiation complex at the cyp1a1 promoter. Remarkably, we found that activation of NF-κB by TNF-α selectively inhibited TCDD-induced serine 2 phosphorylation in mouse liver cells, suggesting that residue-specific phosphorylation of RNA PII CTD at the cyp1a1 promoter is an important regulatory point upon which signal “cross-talk” converges. Finally, we show that ligand-activated AhR associated with P-TEFb through the C terminus of cyclin T1, suggesting that AhR recruit the P-TEFb to the cyp1a1 promoter whereupon its kinase subunit phosphorylates the RNA PII CTD.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to the basic helix-loop-helix/

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1 The abbreviations used are: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; cyclin T1, gene of cytochrome P450 1A1; XRE, xenobiotic response element; P-TEFb, positive transcription elongation factor; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; GST, glutathione S-transferase; RNA PII, RNA polymerase II; CTD, C-terminal domain; PIPES, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; CHIP, chromatin immunoprecipitation assay; XRE, xenobiotic response elements.

Per-ARNT-Sim (bHLH-PAS) family and mediates most TCDD-induced toxic responses. The AhR is at the high echelon of transcriptional regulatory circuitry regulating many aspects of physiological processes in addition to the xenobiotic metabolism (1, 2). It has been shown that, in mouse hepatoma cells, the AhR resides in the cytoplasm in association with heat shock protein 90 (Hsp90) (3, 4) and an immunophilin protein (5–7). Upon activation by ligand, the AhR translocates into the nucleus and binds to another bHLH-PAS protein called the AhR nuclear translocator (ARNT) (8). The heterodimeric protein complex then binds to the xenobiotic response elements (XREs) (9), which are enhancer sequences located in the regulatory regions of AhR-controlled genes such as the gene from cytochrome P450 1A1 (cyp1a1), and activates gene expression. Cyp1a1 is a member of the cytochromes P450 monoxygenase superfamily, which plays an important role in xenobiotic metabolism as well as in carcinogenesis. Historically, many important mechanistic aspects of AhR-regulated gene expression have been investigated utilizing transcriptional regulation of mouse cyp1a1 as a model system (1).

The ligand-dependent cyp1a1 transcriptional regulation is a dynamic process involving AhR binding to the XRE, controlled recruitment of coregulators as well as general transcription factors, chromatin remodeling and histone modifications (10–14). These processes lead to the assembly of the preinitiation complex at the cyp1a1 promoter, which is followed by transcription elongation. Actinomycin D treatment, which blocks the nucleosomal changes downstream from the transcription start site has no effect on chromatin remodeling around the promoter region upstream from the transcription start site, suggesting that assembly of the preinitiation complex and elongation are distinct processes (15).

Transcription elongation by RNA PII is a highly regulated process and its complexity has only recently been appreciated. The largest subunit of mammalian RNA PII possesses 52 repeats of heptapeptide with consensus YSPTSPS motif (16). Hyperphosphorylated CTD is associated with active elongating RNA PII, while inactive RNA PII is hypophosphorylated. It is recognized that after formation of the preinitiation complex, RNA PII is subjected to negative regulation by the negative transcription elongation factor (N-TEF) (17, 18). The N-TEF is composed of the DRB sensitivity-inducing factor (DSIF) and the negative elongation factor (NELF). NELF is a transcription factor complex that cooperates with DSIF/hSpt4-hSpt5 to repress elongation by RNA PII (19). In order for RNA PII to overcome the negative regulation and engage in processive transcription, P-TEFb is required. P-TEFb consists of a regulatory subunit (either cyclin T1, T2, or K) and an enzymatic subunit (CDK9) (18). CDK9 phosphorylates the C-terminal
domain (CTD) of the largest subunit of RNA Pol II (20). Hypophosphorylated CTD is associated with RNA Pol II paused at the initiation stage and is inactive in transcript elongation. P-TEFB phosphorylates CTD to release RNA Pol II from the arrested state. Thus, CTD-specific kinases and phosphatases can function as transcriptional activators or repressors regulating the activity of RNA Pol II at different stages of transcription.

Phosphorylation status of specific serine residues, especially serine 2 and serine 5, are associated with different isoforms of RNA Pol II engaging at different stages of transcription cycle (20–22). Serine 2 has been shown to be phosphorylated by P-TEFB (23) and is associated with RNA Pol II engaging in transcript elongation (22), while serine 5 phosphorylation is involved in recruitment and activation of the mammalian capping enzyme (24, 25) and involved in the processing of primary RNA transcript.

In this study, we show for the first time, that activation of AhR leads to recruitment of P-TEFB to the cyp1a1 promoter followed by differential phosphorylation of the RNA Pol II CTD. NF-κB activation antagonizes TCD-induced serine 2 phosphorylation. Furthermore, we demonstrated that cyclinT1 directly interacts with AhR in vitro as well as in vivo. Our results suggest that elongation control is an important point of regulation of cyp1a1 expression.

MATERIALS AND METHODS

Plasmid Constructs and Vectors—pCyclin T1 and pCDK9 were kindly provided by D. Price (University of Iowa). For yeast two hybrid and glutathione S-transferase (GST) pull-down assays, Cyclin T1 expression plasmids were made by inserting the PCR-generated cyclin T1 fragments into the pGAD424 (Clontech) and pGEX-5X-3 (Amersham Biosciences). The PCR primers used are: fragment 1: 5′-726: OLI, GGC-GATCCCCCACTGGAAGGAGAGAGAGGAAG and OL726, ACCGTCGT- AACCATGTTGAAAGGAGACG; for fragment 2, OL1 and OL250, GCACCTGACTAATGTTGGGAGTTTTCTCCAAAA; for fragment 233–726: OL233, GCCGATCCCAACACTTTAGAATCAAGAGATA and AAGTAGGAGGCAGGCACAATGTC. The PCR primers used were: CAGAAACACAGATCCTGG and TATTTTGCACCCACATGG. The PCR products were modified with restriction enzymes BamHI and SalI for insertion into the plasmid vectors. Plasmid pGL3-CYP1A1-Luc was created by inserting the PCR product of the mouse cyp1a1 upstream regulatory region (∼1395 to + 1) into the pG3 basic vector (Promega). Plasmid pCDK9 was made as a fusion protein by subcloning the CTD sequence of CDK9 into the pGEX-4T-1 plasmid (Amersham Biosciences) using BamHI and XhoI sites.

RNAIsolation and Ribonuclease Protection Assay for Determination of cyp1a1 Transcripts—Hepa1c1c7 cells were seeded into 100-mm cell culture plates and cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 mg/ml amphotericin B (Invitrogen). Cells were washed twice with ice-cold phosphate-buffered saline, harvested by scraping, and collected by centrifugation at 600 g. Total cellular RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The ribonuclease protection assays were performed on 20 μg of total RNA and 20,000 cpm of antisense probes using RPA III Ribonuclease Protection Assay Kit (Ambion) according to manufacturer’s recommendations.

Northern Blot—Total RNA from Hepa1c1c7 cells was isolated using TRIzol reagent. Twenty micrograms of total RNA from each sample were separated on a 1% agarose/formaldehyde gel and transferred overnight onto a nylon membrane. After UV-cross-linking, membrane was prehybridized for 4 h at 42 °C in prehybridization buffer (6× SSC, 5× Denhardt’s reagent, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA), and then probed overnight at 42 °C with cyp1a1 cDNA probe labeled with α-32PdCTP using Random primer labeling systems (Invitrogen) at 1× 10^6 cpm/ml hybridization buffer (6× SSC, 0.5% SDS, 100 μg/ml denatured fragmented salmon sperm DNA, 50% formamide). After hybridization, the membrane was washed 3 × 5 min in buffer I (2× SSC, 0.5% SDS), 1 × 15 min in buffer II (2× SSC, 0.1% SDS), and then washed with buffer III (0.1% SSC, 0.1% SDS) at 65 °C until the background became low. The wet membrane was exposed at −70 °C using Kodak film. As a control, the blot was stripped and re-probed with α-32P-labeled cDNA for rat GAPDH. Plasmid that contained rat GAPDH cDNA (pBSKKI1) was obtained from Binas (Texas A&M University). Mouse cyp1a1 cDNA was obtained using RT-PCR using total RNA from Hepa1c1c7 cells. The PCR primers were: CCAACACACACACAGAGATA and AAGTAGGAGGCAGGCACAATGTC. The PCR product was inserted to pGem-T easy vector (Promega). The BamHI-HindIII fragment of GAPDH and Pst fragment of cyp1a1 was used as templates for labeling, respectively.

In Vivo Comminucupression assay—The comminucupression assays were based on a published procedure with modifications (26, 27). Hepa1c1c7 cells were maintained in 100-mm cell culture plates on day 1 and transfection performed on day 2 when growth reached 80% confluence, the cells were treated with TCD (10 μM) or Me2SO (vehicle control) for 60 min. Before harvest, the cells were washed twice with ice-cold phosphate-buffered saline, harvested by scraping, and collected by centrifugation at 600 × g. Nuclear of the cells (two plates from each treatment) were isolated based on a published procedure (26). The isolated nuclei were lysed in buffer (20 mM Hepes, pH 7.4, 125 mM NaCl, 1% Triton X-100, 10 mM EDTA, 2 mM EGTA, 2 mM Na3VO4, 50 mM NaF, 20 mM ZnCl2, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 mm DTT, 5 μg/ml leupeptin) and centrifuged for 15 min at 12,000 × g, and supernatant fractions were collected. For comminucupression assays, the antisera were added to the lysate, and the mixture was incubated for 4 h on a rotatory shaker. 30 μl of GammaBind Plus Sepharose slurry (50 μg beads) (Ambersham Biosciences) were added to precipitate the antibody-antigen complexes. The beads were washed three times in lysis buffer and then boiled in 2× SDS sample buffer. The proteins were separated by 8% SDS–polyacrylamide gel. The proteins on the gel were transferred to nitrocellulose membranes (Biodyl) and the membranes were blocked with 5% bovine serum albumin in TBST buffer (20 mM Tris-HCL, pH 7.6, 137 mM NaCl, 2.68 mM KCl, 0.05% Tween 20), and incubated with appropriate primary antibodies at 37 °C for 60 min. Blots were washed three times with TBST, then incubated with a 1:2000 dilution of immunofinity-purified goat anti-rabbit IgG linked to alkaline phosphate. Blots were reacted with X-phosphate (New England Biolabs), and subsequently developed using NBT/BCIP (Sigma) as the substrate.

YeastTwo-hybrid Interaction Assay—Yeast two-hybrid assays were performed according to the Match Maker Gal4 two-hybrid user manual (Clontech). Human AhR cDNA was obtained by PCR amplification of pSport huAhR and the product was cloned into the trip reporter yeast expression plasmid pGPD424 (Clontech) in-frame with the DNA binding domain of Gal4, resulting in the pGBT9AhR plasmid. Similarly, cyclin T1 cDNA fragments were cloned into the leup yeast vector pGAD424 (Clontech) in-frame with the Gal4 transcription activation domain, resulting in plasmid pGAD424CycT1 (1,726). pGAD424CycT1 (1,726), and pGAD424CycT1 (2,353–2736). As a positive control the human ARNT cDNA without the cDNA fragment was also cloned into pGAD424. The primers used for generation of the cDNA fragment were: GGGGCGGATCCTGGCGGTGTGCAGCATCCAGCAGAGGAAATCTGGGCCAACATC. The assays for the interactions between AhR and cyclin T1 were performed as follows: Saccharomyces

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Interaction between AhR and P-TEFb

Ligand-activated AhR Recruits Cyclin T1, CDK9, and RNA Pol II to the cyp1a1 Promoter with Concomitant Phosphorylation of RNA Pol II CTD—To investigate the elongation control of cyp1a1 transcription, we used in vivo chromatin immunoprecipitation assay to analyze the recruitment of the cyclin T1, CDK9, and RNA Pol II to the cyp1a1 promoter and the residue-specific phosphorylation of the C-terminal domain (CTD) of the RNA Pol II in response to AhR activation. In Hepa1c1c7 cells, 30 min after TCDD treatment, AhR and ARNT began to associate with the cyp1a1 regulatory region followed by the recruitment of RNA Pol II at the promoter region (30–60 min after TCDD treatment) (Fig. 1). P-TEFb complex was recruited to the promoter region at 60 min and coincided with strong phosphorylation of serine 2 of the CTD. Interestingly, although the strongest phosphorylation of serine 2 of RNA Pol II CTD was detectable at 60 min, strongest phosphorylation of serine 5 was detected after serine 2 phosphorylation. In addition, the increases of serine 5 phosphorylation correlated with decreases of serine 2 phosphorylation, suggesting that phosphorylations of serine 2 and serine 5 are controlled by separate mechanisms (Fig. 1).

Inhibition of Kinase Activity of P-TEFb Selectively Blocks cyp1a1 Transcription Elongation by RNA Pol II but Not the Assembly of the Preinitiation Complex—Phosphorylation of the CTD of RNA Pol II is associated with transition from the initiation to elongation stage of transcription (16). To further investigate the role of P-TEFb in cyp1a1 elongation control, we used the specific P-TEFb inhibitor DRB, to test the involvement of P-TEFb in cyp1a1 elongation. DRB treatment (30 μM, 2 h) inhibited phosphorylation of serine 2 and reduced serine 5 phosphorylation of CTD and prevented the RNA Pol II from transcribing the cyp1a1 distal region as determined by ChIP assay (compare lanes 5 and 6 in Fig. 2A). It appeared that serine 2 was more sensitive to inhibition by DRB than serine 5. Interestingly, in contrast to the distal region, the association of the RNA Pol II with the cyp1a1 promoter region was not affected by DRB treatment (compare lanes 3 with 6 in Fig. 2A in Pol II rows). These results suggest that while elongation is sensitive to DRB treatment, assembly of the preinitiation complex is a distinct step and is resistant to CDK9 inhibition. As a confirmation of effects of inhibition of CTD phosphorylation on AhR-regulated gene expression, we transiently transfected pGL3-CYP1A1-Luc in Hepa1c1c7 cells and treated the transfected cells with TCDD and DRB. The pGL3-CYP1A1-Luc is a luciferase reporter gene driven by the upstream sequences (~1395 to ~1) of mouse cyp1a1 and is activated by AhR ligands. Similar reporter gene plasmid has been constructed and used by
Hepa1c1c7 cells were treated with either TCDD (10 nM, 2 h) or CDK9 (phosphoserine 2, 5 of CTD, cyclin T1, and cyclin T2, 5 of CTD, cyclin T1, and CTDK9 (lanes 7–12). The upstream regulatory and promoter regions are schematically illustrated (bottom panel).

**FIG. 1.** Activation of AhR by TCDD leads to sequential recruitment of AhR complex, RNA PII, and P-TEFb to the *cyp1a1* promoter with differential phosphorylation of RNA PII CTD. ChIP assays were performed to determine the time-dependent promoter occupancy following TCDD treatment of Hepa1c1c7 cells. Antibodies (Ab) used for ChIP assay are indicated on the side of the panels. The associations of AhR complex with the *cyp1a1* regulatory region was detected using antibodies against AhR and ARNT (lanes 1–6). The sequential recruitment of RNA PII, P-TEFb, and differential phosphorylations of RNA PII CTD were analyzed using antibodies against RNA PII, phosphoserine 2, 5 of CTD, cyclin T1, and CDK9 (lanes 7–12). These unregulated regulatory and promoter regions are schematically illustrated (bottom panel).

**FIG. 2.** DRB inhibits the phosphorylation of RNA PII CTD and prevents the RNA PII from transcribing *cyp1a1* sequences. A. Hepa1c1c7 cells were treated with either TCDD (10 nM, 2 h) or co-treated with TCDD (10 nM, 2 h) and DRB (30 μM, 2 h). ChIP assays were performed to determine the association of RNA PII with different regions of the *cyp1a1* gene as well as the phosphorylation status of serines 2 and 5 of the RNA PII CTD after the treatments. B, DRB suppresses transcription by AhR as determined by luciferase reporter gene assay. Hepa1c1c7 cells were transfected with pGL3-CYP1A1-Luc. The transfected cells were treated with TCDD and/or DRB as indicated for 18 h. Luciferase reporter gene activity was determined using a luminometer after the treatment.

**FIG. 3.** TCDD (10 nM, 2 h), robust transcription could be detected as the marked increases in the levels of transcripts at distal as well as the proximal promoter regions (Fig. 3A, compare lanes 1 and 2). Critically, DRB treatment reduced levels of total runoff transcripts to the basal level but did not affect the total level of transcription at the proximal promoter region (Fig. 3A, lane 3), suggesting the RNA PII complex was still at the proximal promoter region engaging in abortive transcription, which generates only short transcripts.

The results of the RNase protection assays are in agreement with the promoter occupancy by RNA PII at the *cyp1a1* promoter and association of RNA PII at distal regions in response to TCDD and DRB treatments (Fig. 2). As confirmation for the RNase protection results, we measured the mRNA levels of the treated samples by Northern blot analysis. Inhibition of P-TEFb by DRB completely suppressed the TCDD-induced mRNA of *cyp1a1* in Hepa1c1c7 cells. Curiously, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) appeared to be much less sensitive to DRB treatment, although this could be due to the longer half-life of GAPDH mRNA (Fig. 3B).

**FIG. 4.** Physical and Functional Interactions Between AhR and P-TEFb in Hepa1c1c7 Cells—Recruitment of P-TEFb complex to the *cyp1a1* promoter in response to AhR activation suggests that there may be a direct interaction between AhR and P-TEFb. To test this possibility, we performed communoprecipitation assays to detect potential complex formation between AhR and cyclin T1 in Hepa1c1c7 cells. Cyclin T1 is a regulatory subunit of P-TEFb and has been shown to directly associate with several transactivators (28–31). The results of our experiment showed that AhR formed complex in vivo with cyclin T1 as determined by sequential immunoprecipitation and Western blot analysis, using antibody against cyclin T1 to immunoprecipitate the complex. The presence of AhR in the precipitated complex was detected by Western blot analysis with antibody against AhR (Fig. 4A). To analyze the functional consequences of the P-TEFb/AhR interaction, we transiently coexpressed pGL3-CYP1A1-Luc and cyclin T1 and/or CDK9 in Hepa1c1c7 cells. Transient expression of cyclin T1 markedly enhanced the dioxin-induced luciferase reporter gene activity (Fig. 4B). Without activation by agonist, expression of cyclin T1 or CDK9 alone was ineffective in enhancing the pGL3-CYP1A1-Luc reporter gene activity, which is consistent with the role of P-TEFb in the control of elongation downstream from the agonist-induced initiation. Transient expression of CDK9 in Hepa1c1c7 cells also enhanced the activation of pGL3-CYP1A1-Luc gene expression, although the enhancement by CDK9 was not as strong as that by cyclin T1. No further enhancement of the reporter gene activity was archived by coexpression of CDK9.
with cyclin T1. These results suggest that P-TEFb (particularly cyclin T1) plays a role in regulating the AhR-mediated gene expression through physical and functional interaction with AhR receptor complex.

**Phosphorylation of RNA Pol II CTD at the cyp1a1 Promoter Is Co-regulated by AhR and NF-κB**—In earlier studies, we found mutual repression between AhR and NF-κB signal pathways and this antagonism is reflected in the histone H4 acetylation and deacetylation at the cyp1a1 upstream regulatory region (14, 27). Since the results of our current study suggest that transcription elongation is also a regulatory step for cyp1a1 gene expression, we are interested in analyzing if this step is subject to the positive and negative transcriptional regulation imposed by AhR and NF-κB. We analyzed the serine 2 and serine 5 phosphorylation of RNA PII CTD at the cyp1a1 promoter in response to TCDD and TNF-α treatments using ChIP assay. As expected, we found that both serine 2 and serine 5 were phosphorylated in response to TCDD treatment. However, TNF-α treatment selectively inhibited serine 2 phosphorylation while TCDD-induced serine 5 phosphorylation was not significantly suppressed (Fig. 5). These results are consistent with the reported glucocorticoid receptor (GR)-mediated suppression of NF-κB where GR differentially inhibits RNA PII CTD serine 2 phosphorylation at the IL-8 and ICAM promoters, which are regulated by NF-κB (34).

**AhR and Cyclin T1 Interact Directly as Determined by Yeast Two-hybrid as Well as GST Pull-down Assays**—The results from the coimmunoprecipitation assays (Fig. 4A) suggest that AhR associate with the cyclin T1 in vivo. To further characterize these interactions, we performed yeast two-hybrid assays as well as GST pull-down assays to analyze the interaction domains within cyclin T1 that mediate its interaction with AhR (Fig. 6). Human cyclin T1 is a polypeptide (726 amino acids) consisting of a N-terminal cyclin box (amino acids 1–250), which is conserved among cyclins T1, T2, and K, and the C-terminal domain, which contains a coiled-coil motif (amino acids 379–430), a histidine-rich region (amino acids 506–530), and a PEST sequence (amino acids 709–726) (32). The C-terminal domain is known to interact directly with RNA PII and is essential for elongation activity of P-TEFb (33). To perform yeast two hybrid assay, full-length human AhR cDNA was inserted in-frame into the yeast two hybrid bait vector (pASII, Clontech) and cyclin T1 cDNAs were inserted into pGAD 424 plasmid (Clontech). The chimeras were transfected into S. cerevisiae SFY56 and transfectants were grown in liquid culture and assayed for β-galactosidase activity after treatment with AhR ligand β-naphthoflavone (Fig. 6B). About 2–3-fold induction by β-naphthoflavone was observed when AhR was co-expressed with pGAD424 (empty vector). However, co-expression of AhR with full-length cyclin T1 resulted in significant ligand-induced induction (about 8-fold). The interaction between cyclin T1 and AhR is mainly mediated by the C terminus of the cyclin T1 (CycT1 233–726) and the N terminus of cyclin T1 interacted marginally. As a positive control, coexpression of AhR with ARNT (a known interactive partner of AhR) resulted in high levels of β-galactosidase activity upon induction with BNF. These results suggest that cyclin T1 interacts directly with AhR in vivo in a ligand-dependent manner, and the association is mediated by the C-terminal domains of cyclin T1. Next we performed GST pull-down assays to further analyze the interaction between AhR and cyclin T1. *In vitro* translated and radiolabeled AhR protein was incubated with cyclin

![Figure 3](image-url) **Fig. 3.** DRB inhibits the run-off transcription without affecting the transcription initiation. *A,* ribonuclease protection assay. Arrows to the right of the panel point to the protected transcripts from the proximal and distal regions of cyp1a1. The lower part of the panel shows the schematic illustration of the cyp1a1 promoter and transcribed region. The positions of proximal and distal antisense riboprobes are indicated. *B,* Northern blot analysis. Hepa1c1c7 cells were treated with TCDD and/or DRB as indicated for 2 h, and total RNA was isolated from the treated cells for Northern blot analysis using radiolabeled probes against coding regions of cyp1a1 and GAPDH.

![Figure 4](image-url) **Fig. 4.** *In vivo* association between AhR and cyclin T1, and functional enhancement of AhR-regulated gene expression by P-TEFb. *A,* Hepa1c1c7 cells were treated with TCDD (10 nM, 60 min), and the nuclear proteins were extracted and immunoprecipitated with antibodies against cyclin T1, AhR or a nonspecific control antibody (IgG). The presence of AhR in the precipitated complex was detected by Western blot analysis with antibody against AhR. *B,* Hepa1c1c7 cells were transiently cotransfected with pGL3-CYP1A1-Luc and p-cyclin T1 and p-CDK9 through lipofection. Six hours after transfection, cells were treated with TCDD (10 nM for 18 h), and the luciferase activity was determined using a luminometer. All data are means ± S.D. of triplicate transfections from a single representative experiment.
Interaction between AhR and P-TEFβ

In this study, we have investigated transcription elongation control of cypla1 expression in response to AhR activation. Using the ChIP assay, we found that TCDD treatment in Hepa1c1c7 cells induced binding of the AhR complex to the regulatory region of cypla1 followed by promoter occupancy by RNA PII and recruitment of P-TEFβ to the promoter region. The association of P-TEFβ with the promoter was correlated with elongation, the serine 5-phosphorylated RNA PII and recruitment of P-TEFβ to the promoter region. Using synthetic peptides containing 6 tandem repeats of YSPTSPS,HO and Shuman (25) showed that phosphorylation of serine 5 induced capping of RNA transcript in vitro. Since capping is an early event during transcript elongation, it is somewhat surprising that our results with ChIP assay showed serine 5 was phosphorylated after serine 2 in vivo. Conceivably, because the time required for serine 5 phosphorylation associated with capping is much shorter than serine 2 phosphorylation associated with elongation, the serine 5-phosphorylated RNA PII moved away from the proximal promoter region and was not detected till the surge of serine 5 phosphorylation from second round of transcription. Interestingly, we observed that TCDD-induced serine 2 phosphorylation decreased over time and the decreases correlated with increases of the serine 5 phosphorylation (Figs. 1 and 5), suggesting that phosphorylation of serine 2 and serine 5 is controlled by distinct mechanisms. It has been shown that CDK7 of TFIIH phosphorylates serine 5 while CDK9 of P-TEFβ phosphorylates serine 2, the differential phosphorylation of serines 2 and 5 of CTD at the cypla1 promoter may be due to different kinases with different substrate specificity (20–23). Thus, the CTD appears to function as a molecular switchboard where hyper- and hypo-phosphorylations sig-

**DISCUSSION**

In 1992, Morgan and Whitlock (15) reported that TCDD induced changes in the nucleosomal positions in both the promoter and transcribed regions of mouse cyp1a1 gene with an interesting difference: the nucleosomal changes in the transcribed regions were sensitive to inhibition by actinomycin D, while the TCDD-induced nucleosomal changes in the promoter were insensitive to the same treatments. Actinomycin D binds to DNA and blocks the movement of RNA PII, thus inhibiting transcription. These results suggest that within a single cyp1a1 gene, the transcription is regulated by two inter-connected but distinct mechanisms involving controls of initiation and elongation. In the last two decades, the transcriptional processes leading to initiation have been investigated extensively, but little is known about the process of transcription elongation of cyp1a1.

In this study, we have investigated transcription elongation control of cypla1 expression in response to AhR activation. Using the ChIP assay, we found that TCDD treatment in Hepa1c1c7 cells induced binding of the AhR complex to the regulatory region of cypla1 followed by promoter occupancy by RNA PII and recruitment of P-TEFβ to the promoter region. The association of P-TEFβ with the promoter was correlated with strong phosphorylation of serine 2 of the RNA PII CTD (Figs. 1 and 2). Intriguingly, although both serine 2 and serine 5 are phosphorylated in response to AhR activation, there is an interesting time lag between the serine 2 and serine 5 phosphorylation at the cypla1 promoter with serine 2 being phosphorylated in advance of serine 5 (Figs. 1 and 5). It is not known why strongest phosphorylation of serine 5 was detected after serine 2 phosphorylation at the cypla1 promoter. Using synthetic peptides containing 6 tandem repeats of YSPTSPS,
which contains coiled-coil, His, and PEST motifs. It has been selectively associated with the C-terminal domain of cyclin T1, a direct binding of AhR with cyclin T1. Interestingly, AhR es: by coimmunoprecipitation assay, we found AhR/cyclin T1 interaction between AhR and cyclin T1 using three complementary approaches.

The results of this study suggest a model in which AhR regulates the transcription elongation by recruiting the P-TEFb complex to the promoter region of cyp1a1 gene through direct protein-protein interaction (Fig. 7). XRE-bound AhR recruits P-TEFb to the vicinity of cyp1a1 promoter where the CDK9 of P-TEFb phosphorylates the CTD of RNA PII, thereby releasing it from the arrested state for processive transcription. This model also suggests that transcription elongation is an important point of convergence for the interactions between the AhR and different transcription factors, since the activity of P-TEFb is regulated by many signaling mechanisms. Furthermore, as many therapeutic agents have now been developed targeting P-TEFb, their effects on AhR-regulated xenobiotic detoxification need to be analyzed.

The intricacy of transcriptional regulation of cyp1a1 expression continues to unravel. In addition to the transcriptional processes that lead to assembly of the preinitiation complex at the promoter region, AhR also regulates the transcription elongation by interaction with the general elongation factor P-TEFb, thereby regulating elongation through phosphorylation of the CTD of RNA PII.

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Interaction between AhR and P-TEFb

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