The environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) causes a wide range of toxic, teratogenic, and carcinogenic effects. TCDD is a ligand for the aromatic hydrocarbon receptor (AHR), a ligand-activated transcription factor believed to be the primary mediator of these effects. Activation of the AHR by TCDD also elicits a variety of effects on cell cycle progression, ranging from proliferation to arrest. In this report, we have characterized further the role of the activated AHR in cell cycle regulation. In human mammary carcinoma MCF-7 and mouse hepatoma Hepa-1 cells, TCDD treatment decreased the number of cells in S phase and caused the accumulation of cells in G1. In Hepa-1 cells, this effect correlated with the transcriptional repression of several E2F-regulated genes required for S phase progression. AHR-mediated gene repression was dependent on its interaction with retinoblastoma protein but was independent of its transactivation function because AHR mutants lacking DNA binding or transactivation domains repressed E2F-dependent expression as effectively as wild type AHR. Overexpression of p300 suppressed retinoblastoma protein-dependent gene repression, and this effect was reversed by TCDD. Chromatin immunoprecipitation assays showed that TCDD treatment caused the recruitment of AHR to E2F-dependent promoters and the concurrent displacement of p300. These results delineate a novel mechanism whereby the AHR, a known transcriptional activator, also mediates gene repression by pathways involving combinatorial interactions at E2F-responsive promoters, leading to the repression of E2F-dependent, S phase-specific genes. The AHR seems to act as an environmental checkpoint that senses exposure to environmental toxicants and responds by signaling cell cycle inhibition.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the prototypical compound of a class of environmental contaminants that includes many halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons and is a model for the outcome of exposure to these compounds as well as coplanar polychlorinated biphenyls. Exposure to TCDD results in a plethora of toxic and carcinogenic responses in animals, including liver toxicity (1), immunosuppression (2, 3), reproductive and developmental dysfunction (4–6), endometriosis (7, 8), and tumor promotion (9). The most immediate exposure outcome in humans is chloracne (10). Long term effects in humans range from immunological and reproductive perturbations (11) to cardiovascular disease (12, 13) and cancer (14–18). TCDD itself is poorly metabolized, which leads to biological accumulation and production of sustained effects (19).

Although the in vitro effects of TCDD are wide ranging, the in vivo effects are just as varied and often contradictory, affecting cell proliferation, apoptosis, and differentiation, depending on the cell type examined. TCDD acts as an endocrine disruptor in cell cultures, inhibiting several estrogen-induced responses such as growth of human mammary and endometrial cancer cell lines (20). TCDD induces the proliferation (21) and terminal differentiation (22, 23) of human keratinocytes and both enhances (24) and inhibits (25, 26) rat hepatocyte proliferation rates. Induction of apoptosis has been reported for immature thymocytes from rats and mice treated with TCDD in vivo (27–29). In cultures of rat hepatocytes, TCDD also induces apoptosis (24) but inhibits UV light-induced apoptosis (30).

The molecular basis for the biological effects of TCDD is largely speculative. The majority of the effects of TCDD exposure are mediated by the AHR (31, 32), a ligand-activated transcription factor that, upon ligand binding, translocates to the nucleus where it complexes with ARNT (33). The AHR/ARNT heterodimer stimulates the transcription of genes in the cytochrome P450 Cyp1 family as well as several phase II detoxification genes (34, 35), via transactivation through enhancer domains known as AHR-, dioxin-, or xenobiotic-response elements (36). Gene transactivation mediated by the AHR represents an adaptive response required for the detoxification of foreign compounds. However, this effect does not adequately explain the range of toxic outcomes resulting from TCDD exposure in different systems, particularly regarding gene repression effects as seen for transforming growth factor-β2 (37, 38), fibrinogen γ chain and plasmin mRNAs (39), cyclin A (40), and others (41). Global gene expression studies indicate that the AHR participates in the direct and indirect modulation of the transcriptional program (41), at least in part by associating with additional transcription factors (42–44) and coactivators or corepressors (45–47). Such associations may be partially responsible for the myriad effects of the ligand-activated AHR in the regulation of proliferation, apoptosis, differentiation, and signal transduction pathways (41).

Several lines of evidence indicate that AHR activation directly modulates normal cell cycle regulation. Ma and Whitlock...
were also cotransfected with a pCMV vector and by immunoblot analysis. C33A cells harbor a mutation in the RB gene and express an unstable mutant RB protein (55). Hypophosphorylated RB binds E2F to inhibit transcriptional activity and mediate active repression of E2F-responsive genes, thereby inhibiting cell cycle progression from G1 into S phase (51). Inactivation of RB by phosphorylation relieves this repression, allowing cells to enter into S phase. Previous observations from our laboratory have shown that the activated AHR synergizes with RB to repress gene expression (44), which might explain the involvement of the AHR in the G1 phase of the cell cycle. However, little is known regarding the possible role that AHR-mediated transcriptional activity and ARNT interactions might play in the repression and what role, if any, is played by chromatin remodeling factors, known to mediate E2F-dependent transcription, in the overall outcome of AHR-RB interactions. The purpose of the present research was to address these central questions and to test the hypothesis that through its interaction with RB, the AHR functions as a repressor of transcription. Our results help elucidate the complex role played by AHR signaling during the cell cycle and delineate a novel role for the AHR, a known transactivator, in gene repression.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfections, and Growth Conditions—The mouse Hepa-1c1c7 cell line, a subclone of the Hepa-1 hepatoma cell line (52), and two of its mutant derivatives were used in these studies. The C35 derivative line expresses a DNA binding-defective AHR (53), and c4 cells lack ARNT (34). Exponentially growing cells were cultured in minimal essential medium-α (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen), 1.0% 100× antibiotic/antimycotic solution (Sigma), and 38 mM NaHCO3 at 37 °C in a humidified 5% CO2 atmosphere. The human breast carcinoma cell line MCF-7 (54) was cultured similarly. The murine hepatic cell line C3A (55) was cultured in the same medium containing 10% fetal bovine serum. When indicated, cultures were treated with TCDD at a final concentration of 10 nM in Me2SO vehicle, and control cultures were treated with an equivalent volume of Me2SO. C33A cells do not express AHR, as determined from the lack of activity of a transfected AHR/ARNT-responsive luciferase reporter plasmid and by immunoblot analysis. C33A cells harbor a mutation in the RB gene and express an unstable mutant RB protein (55).

Transient transfection experiments were performed using LipofectAMINE Plus (Invitrogen) on cells grown to 50–75% confluence in 24-well plates or 60-mm dishes. All transfection mixtures were brought to the same amount of total DNA by the addition of the appropriate amount of empty vector. To control for variations in transfection efficiency, cells were cotransfected with a pCMVβgal plasmid (Clontech) and transfections were normalized to β-galactosidase expression. Cells were transfected in serum-free medium, and 2 hr later a volume of medium containing twice the final concentration of culture serum was added. After overnight incubation, cells were washed once with phosphate-buffered saline and cultured in complete culture medium with TCDD or with vehicle. Between 24 and 48 h after transfection, cells were lysed in reporter lysis buffer (Promega), and luciferase and β-galactosidase activities were measured. Each transfection was repeated a minimum of two times, and the results shown are the mean ± S.D. of one representative set of results. Where noted, the results shown are the mean ± S.E. of log-transformed results from multiple independent experiments.

Plasmid Constructs—The plasmid pcDNAI/B6AHR, used to express the high affinity variant of murine AHR, has also been described previously (56). Mutant derivatives of the pcDNAI/B6AHR plasmid are schematically shown in Fig. 3A. Plasmid pA495–805 was derived from pcDNAI/B6AHR by deletion of 933 nucleotides encoding the COOH-terminal 311 amino acids of the AHR peptide; this plasmid encodes a dominant-negative variant of the murine AHR, expressing a protein capable of binding ligand and dimerizing with ARNT but lacking transactivation activity (56). The plasmid pΔ323–494 contains a deletion of the LXCXE motif and the PAS B domain, and plasmid pΔ323–867 lacks the LXCXE motif, the PAS B domain, as well as part of the transactivation domain (57). Expression of the peptides encoded by each AHR truncation mutant was confirmed by Western blot.

The reporter p3×E2Fluc, containing three E2F-responsive elements, has been described previously (58). The reporter construct pHRDtkLuc (56) is a luciferase reporter plasmid containing the AHR-responsive domain of the mouse Cyp1a1 gene, has been described previously (58). Expression of the reporter plasmid pAHRDLuc (56) was monitored by measuring luciferase activity (56).

In Vitro Transcription/Translation—1 μM of plasmids encoding the wild-type AHR or truncation mutants was used in 50-μl in vitro transcription/translation reactions with a TNT-coupled reticulocyte lysate system (Promega), following the manufacturer’s instructions and included 30 μCi of [35S]methionine (Amersham Biosciences). Reactions were incubated at 30 °C for 1.5 h. Translated proteins were used immediately for pull-down assays.

Pull-down Assays—Bacteria expressing a human RB protein tagged with Hi-tag at the amino terminus were lysed in 500 μl of 6× guanidine HCl, 0.1 M NaH2PO4, 0.01 M Tris (pH 8.0) and the lysate was incubated with a nickel-agarose slurry (Qiagen) in 2 ml of buffer containing 8 M urea, 0.1 M NaH2PO4, 0.01 M Tris (pH 8.0). The slurry was washed through a column, washed three times with 1 ml of 20 mM Tris (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, and stored in 1 volume of buffer at 4 °C. Control and RB-bound columns were established using 100-μl aliquots of the preadsorbed slurry. Full-length and truncation peptides of the mouse AHR were in vitro translated with [35S]methionine and precleared through nickel-agarose columns. Equal volumes of AHR or truncated peptides were recycled 10 times through control and RB-bound columns. Each column was washed twice with 2 column volumes of 20 mM Tris (pH 7.6), 150 mM NaCl, 0.1% Triton X-100. Bound proteins were eluted with 1.5 column volumes of 8 M urea, 0.1 M NaH2PO4, and 0.01 M Tris (pH 6.3) to disrupt nickel coordination of the Hi-tag. Eluted fractions were denatured by boiling and analyzed by SDS-PAGE in 7.5% acrylamide gels. Detection of [35S]-labeled AHR was by autoradiography. Radioactive quantitation was carried out with a Storm 860 PhosphorImager (Molecular Dynamics, Inc.).

Flow Cytometry—For cell cycle studies, MCF-7 cells were maintained in serum-free minimal essential medium supplemented with 1% antibiotic/antimycotic solution for 72 h. Cells were subsequently fed with medium containing 5% fetal bovine serum and 10 nM TCDD or an equivalent volume of Me2SO vehicle and cultured for intervals ranging from 24 to 30 h. Cells were pulsed with BrdUrd (BD Biosciences) for 1 h at the indicated time point and harvested for cell cycle analysis using a Beckman Coulter Epics XL flow cytometer (Fullerton, CA). The percentage of cells entering S phase was determined by gating BrdUrd-positive cells and subtracting background fluorescence using an unlabeled control. DNA content of the total cell population was determined by 7-aminoactinomycin D staining, and the fraction of cells in G1 was determined from the fraction of cells with 2 × DNA content. Hepa-1 cells were processed similarly for analysis of BrdUrd incorporation. Cells were cultured continuously to ~80–90% confluence under standard conditions and treated with 10 nM TCDD or vehicle control for the time period indicated.

Total RNA Isolation, Reverse Transcription, and Real Time PCR—Hepa-1 cells were cultured in 10-cm plates to 80–90% confluence and treated under standard culture conditions with Me2SO vehicle or with 1 μg/ml actinomycin D for 1 h. Actinomycin-treated cells were treated further with Me2SO vehicle or with 10 nM TCDD for 0, 4, or 8 h prior to harvesting of RNA. Total cellular RNA was isolated with TRI Reagent (Molecular Research Center, Inc.). Immediately after heating at 65 °C.
for 10 min, first strand cDNAs were synthesized from 15 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen) and random primers. Diluted cDNAs were subjected to PCR amplifications with gene-specific primer sets for the various E2F-responsive and control genes (Table I). Reaction volumes of 25 μl contained 12.5 μl of Brilliant SYBR Green QRT-PCR Master Mix (Stratagene), 0.4 μM primers, and 2–4 μl of cDNA template. Real time quantitative PCR was performed using a Smart Cycler rapid thermal cycler (Cepheid), and fluorescence was measured after each of the repetitive cycles. A typical protocol included a 2-min denaturation step at 95 °C followed by 40 cycles of 95 °C denaturation for 30 s, annealing for 30 s at a primer-optimized temperature, and 72 °C extension for 30 s. Detection of the fluorescent product was carried out during the 72 °C extension period, and emission data were quantified using threshold cycle (Ct) values. Ct values for all genes analyzed were determined two to four times, averaged, and normalized to values for β-actin. The fold change from control Ct values (vector-transfected, Me2SO-treated) was determined for each sample, as determined by the following calculation:

\[
\text{fold change} = 2^{-\Delta\Delta C_t} \tag{1}
\]

where ΔΔCt = (Ct(target) - Ct(control) time 1) - (Ct(target) - Ct(control) time 0) - PCR product specificity from each primer pair was confirmed using melting curve analysis and subsequent agarose gel electrophoresis.

To assess whether physiologic levels of activated AHR and RB could cooperate equally well in the inhibition of cell cycle progression, we used the RB- and AHR-positive MCF-7 breast carcinoma cell line for analysis of BrdUrd incorporation. Cells were serum starved for 3 days followed by release into medium containing 5% fetal bovine serum and either Me2SO vehicle or 10 nM TCDD for various time intervals ranging from 10 to 30 h. Cells were pulse labeled with BrdUrd for 1 h at the indicated time points and harvested for flow cytometry analysis. The results indicate that TCDD treatment retards progression of cells into S phase (Fig. 1A). The difference in the number of cells entering S phase between control and TCDD-treated cells increased significantly over the time course examined. As expected, the control group proceeded into a state of exponential growth, whereas S phase progression in the TCDD group lagged behind, with a significantly lower percentage of the total cell population.

Similar experiments were carried out using the Hepa-1c1c7 mouse hepatoma cell line. Hepa-1 cells were cultured to 80–90% confluence and treated with Me2SO vehicle or 10 nM TCDD for 16 or 24 h. Cells were pulse labeled with BrdUrd for 1 h at the indicated time points and harvested for flow cytometry analysis. As in MCF-7 cells, there was a significant 10% decrease in the number of BrdUrd-positive cells in cultures treated with TCDD, suggesting that TCDD retarded DNA synthesis in Hepa-1 cells (Fig. 1B). The decrease in the number of cells incorporating BrdUrd in response to TCDD treatment was accompanied by a corresponding increase in the number of G1 and G2-M cells (Fig. 1C), indicating that TCDD acts primarily by promoting the accumulation of cells in G1 and blocking progression from G1 to S phase. Similar results have been obtained in the rat 5L hepatoma cell line, where the effect of TCDD on the cell cycle has been shown to be dependent on the presence of a functional AHR (64, 65). These results confirm those in both this and other cell lines and indicate that the effect of AHR overexpression observed in transfection studies (44) is relevant to physiological conditions.

**RESULTS**

**TCDD Inhibits S Phase Progression in Multiple Cell Lines**—Results of previous work from our laboratory have shown that in RB- and AHR-negative Saos-2 cells, ectopically overexpressed AHR and RB cooperate to suppress entry of transfected cells into S phase. This effect was attributed to a reduction in E2F-dependent gene expression, as assessed using transfection of reporter constructs, required for DNA synthesis and cell cycle progression (44). Additional observations indicated that AHR activation by TCDD treatment in serum-starved, RB-positive cells resulted in a significant reduction in both RB phosphorylation and cyclin D1 expression (63). To determine whether physiologic levels of activated AHR and RB could cooperate equally well in the inhibition of cell cycle progression, we used the RB- and AHR-positive MCF-7 breast carcinoma cell line for analysis of BrdUrd incorporation. Cells were serum starved for 3 days followed by release into medium containing 5% fetal bovine serum and either Me2SO vehicle or 10 nM TCDD for various time intervals ranging from 10 to 30 h. Cells were pulse labeled with BrdUrd for 1 h at the indicated time points and harvested for flow cytometry analysis. The results indicate that TCDD treatment retards progression of cells into S phase (Fig. 1A). The difference in the number of cells entering S phase between control and TCDD-treated cells increased significantly over the time course examined. As expected, the control group proceeded into a state of exponential growth, whereas S phase progression in the TCDD group lagged behind, with a significantly lower percentage of the total cell population.

**TABLE I**

Gene-specific primer sets for real time PCR analysis of relative mRNA expression levels

| Gene name      | Accession no. | Forward primer (5' → 3') | Reverse primer (5' → 3') | Product size |
|----------------|---------------|--------------------------|--------------------------|--------------|
| β-Actin        | M12481        | CATCCTGTTAGGCCTTTGGTGGCC | ACCGGAGCTGATGACGTTGTC    | 287          |
| Cyp1a1         | NM_009992     | GTGCCGGAGCTTACAAATGAGGG  | ACCATAAATCCCGCGGACC      | 319          |
| Cdk2           | NM_183417     | GCCCATGTGGAAGATGACCC     | GCTGCTGCGCTGTTGCC        | 250          |
| Cyclin E       | NM_076733     | TCTCCTCCCTGAGGACTCC      | ATTCGCCGGATCCAAACAA      | 203          |
| Dblf           | N0_010049     | GCAAGGAAAAAGGCAATCA      | GCCGACCGATCCCAAAAGCA     | 325          |
| DNA pola       | NM_008892     | TCTACGGTGACCTCCATTC      | AACCTGCCTGCTATGGCC       | 240          |
| p27Kip1        | NM_009875     | GCCGCTGTGACCGAGGATTC    | TTCTTTGGGCTGTCTGACCC     | 325          |
real time reverse transcription-PCR analysis. Cells were cultured in the presence of serum to 80–90% confluence, treated with Me2SO vehicle, 10 nM TCDD, 1 μg/ml actinomycin D, or 1 μg/ml actinomycin D for 1 h followed by 10 nM TCDD, and harvested at 0, 4, and 8 h after treatment. Treatment with actinomycin D provides a base line for the level of mRNA remaining at any given time after inhibition of transcription. RNA was isolated from these cells, and the mRNA levels of Cyp1a1, p27Kip1, and the E2F-regulated genes cdk2, Cyclin E, Dhfr, and DNA polymerase (pol) were determined by real time reverse transcription-PCR and normalized to β-actin mRNA levels. We measured p27Kip1 mRNA levels because the effect of TCDD on cell cycle progression in 5L rat hepatoma cells has been attributed to p27 accumulation (66). As expected, after 4 h of TCDD treatment, the level of Cyp1a1 mRNA increased 100-fold over control, and this increase was maintained throughout the 8-h treatment period (Fig. 2). Between 0 and 4 h of TCDD treatment, all four E2F-regulated genes showed a similar pattern of time-dependent decrease in mRNA expression relative to Me2SO-treated controls. At this time, the pattern of inhibition by TCDD mirrors that found for cells treated with actinomycin D or with actinomycin D plus TCDD, suggesting that TCDD treatment shuts off completely the transcription of all four E2F-dependent genes tested. This pattern continued to be maintained for Dhfr and pol α, but cdk2 and Cyclin E mRNAs began to recover toward control levels by 8 h after TCDD treatment. Notwithstanding, both cdk2 and Cyclin E mRNA levels were still decreased signifi-

**Table II**

| Gene name | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) | Product size |
|-----------|---------------------------|---------------------------|--------------|
| Cyp1a1    | CTATCTCTTAAACCCACCCCAACTAAGTATGGTGGAGGAAAGGGTG | CTAAATATGTTGAGAAAGGGTG | 357          |
| Cdk2      | CAGCCCTTGACAATTTGTCCTCCGTAGACCAGAAACACC | TCCGCTGTCTTGCCCAATC | 275          |
| Cyclin E  | CCGGACATTCTGAGCAGGCGTGGTG | TCCGCTGTCTTGCCCAATC | 213          |
| Dhfr      | CAGCCATCATCTAGCCGAGCAGCCTAC | TTGGAAACGGGAGCCGGAAG | 103          |
| DNA polα  | AGCTCCAGACGACGACTCAC | AGCTCCAGACGACGACTCAC | 215          |

**Fig. 1.** TCDD inhibits S phase progression in MCF-7 and Hepa-1 cell lines. A, MCF-7 breast carcinoma cells were serum starved in minimal essential medium−α for 3 days. Cells were released into medium containing 5% fetal bovine serum and either Me2SO vehicle (DMSO) or 10 nm TCDD for 0–30 h. Cells were pulse labeled with BrdUrd for 1 h at the indicated times. Cells were fixed and processed for BrdUrd incorporation to detect DNA synthesis and stained with 7-aminoactinomycin D to determine cell cycle phase by flow cytometry. The percentage of S phase cells was determined by gating for the BrdUrd-positive cell population. Asterisks (⁎) indicate significant differences (p < 0.05) from Me2SO-treated controls using one-way analysis of variance. B, Hepa-1c1c7 hepatoma cells were cultured to 80–90% confluence and treated with Me2SO vehicle or 10 nM TCDD for 16 or 24 h. BrdUrd labeling and flow cytometry analysis were performed as in A. Asterisks (⁎) indicate significant differences (p < 0.05) from Me2SO-treated controls using one-way analysis of variance. C, Hepa-1 cells were cultured and treated as in B. Cell cycle distribution was then determined by flow cytometry. DNA content (7-aminoactinomycin D intensity) is plotted against cell number. The percentage of cells in G0-G1, S, and G2-M phases was determined by ModFit software.
Fig. 2. TCDD inhibits expression of E2F-dependent, S phase-specific genes. Hepa-1c1c7 cells were cultured in the presence of serum to 80–90% confluence and treated with Me2SO vehicle (DMSO), 10 nM TCDD, or actinomycin D, or actinomycin D plus 10 nM TCDD for the time periods indicated. Thereafter, cells were harvested for total RNA isolation, and mRNA of the indicated genes was quantified by real time PCR amplification. Results represent threshold cycle (Ct) values normalized to β-actin. The values shown represent the mean ± S.D. of at least two determinations from one representative experiment. Cyp1a1, cytochrome P450IA1; p27kip1, cyclin-dependent kinase inhibitor 1B; Cdk2, cyclin-dependent kinase 2; Cyclin E, cyclin E1; Dhfr, dihydrofolate reductase; Pol α, DNA polymerase α.

Transactivation Is Not Required for AHR-mediated Repression of Gene Expression—The observation that TCDD inhibits cell cycle progression in a variety of cell lines has been attributed to multiple different mechanisms, including the AHR-mediated up-regulation of genes that inhibit cell cycle progression (66) as well as a direct AHR-RB interaction resulting in enhanced down-regulation of genes required for cell cycle progression (44). The relative contribution of AHR-mediated transactivation to the down-regulation of E2F-regulated genes in cooperation with RB has never been investigated. Previous studies have shown that AHR and RB interact in vitro and that several domains of the AHR, including the glutamine-rich region of the carboxyl-terminal transactivation domain and an RB binding LXXCX motif, contribute to this interaction (43, 44). To analyze specific regions of the AHR for their contribution to the interaction with RB, we tested AHR deletion mutants for their ability to bind RB in an in vitro pull-down assay. Several mutant AHR peptides were constructed containing deletions of the amino-terminal LXXCX motif at amino acid 325 of the PAS domain and the glutamine-rich sequence within the carboxyl-terminal transactivation domain (Fig. 3A). In vitro synthesized full-length or mutant 35S-labeled AHR was passed through nickel-agarose columns saturated with His6-tagged RB prepared in Escherichia coli. Control columns contained a lysate of host E. coli cells. Bound 35S-labeled AHR proteins were analyzed by electrophoresis in 7.5% SDS-polyacrylamide gels. The fraction of labeled AHR bound to control columns was negligible for the full-length protein and all mutant peptides. Quantitation of input and bound 35S-labeled AHR from the RB-containing columns by PhosphorImager analysis showed that 39.5% of labeled full-length AHR protein input was bound by RB. The AHR deletion mutants bound RB to varying degrees, all significantly less than full-length AHR (Fig. 3B). These data suggest that not just two but possibly several domains and perhaps proper folding and three-dimensional confirmation of those domains are responsible for maximal interaction between AHR and RB as observed in these and other in vitro experiments.

The binding data presented above suggest that the relative loss of RB binding by truncated mutants of AHR may impair their ability to cooperate with RB to repress E2F-regulated gene expression. We therefore used AHR truncation mutants (Fig. 3A) in transient transfection assays and compared their ability to transactivate an AHR-dependent reporter gene with their ability to repress an E2F-dependent reporter gene. Each truncated AHR peptide was first tested for its ability to activate AHR-dependent transcription in the human cervical carcinoma C33A cell line, which lacks expression of AHR, ARNT, and a stable RB protein. As expected, full-length AHR induced high levels of luciferase activity from the pAHRtkLuc3 reporter, driven by the AHR-responsive enhancer from the mouse Cyp1a1 gene (Fig. 3C). Transactivation activity was retained by the Δ323–494 mutant, which is constitutively active but unresponsive to TCDD stimulation and was largely decreased for the Δ323–607 mutant.

E2F-dependent transcriptional activity was assayed using the luciferase reporter plasmid p3×E2FLuc that responds to transactivation by E2F. Transfection of this plasmid into C33A cells resulted in high luciferase expression levels (Fig. 3D). Ectopic expression of RB reduced luciferase activity to 60% of
control. As expected, simultaneous expression of RB and the full-length AHR further reduced luciferase expression to 30% of control, indicative of the cooperative relationship between AHR and RB in the transcriptional repression of E2F-dependent promoters. Additional AHR truncation mutants, including the transcriptionally inactive mutants Δ323–494 and Δ323–607, were equally able to repress reporter activity in cooperation with RB. In contrast, the transcriptionally active AHR mutant Δ323–494 was unable to repress E2F-dependent gene expression. These effects were independent of TCDD treatment, as ectopically expressed AHR is activated and localized to the nucleus in the absence of ligand (56). These results suggest that the repressive effect on E2F-dependent gene expression does not require the maximal binding levels observed with full-length AHR in the in vitro binding experiments (see Fig. 3B). All of the truncated AHR peptides which cooperated with RB to repress E2F-dependent transcription showed little or no effect on luciferase expression in the AHR-dependent gene expression assay, suggesting that the effect of AHR on E2F-dependent functions is an independent event unrelated to the ability of the AHR to transactivate gene expression. To ensure that these results were indeed the result of expression of the transfected proteins, whole cell extracts of transfected C33A cells were separated in 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, and protein expression was analyzed for all transfected plasmids by Western immunoblot. AHR, ARNT, and RB proteins corresponding to all transfected plasmids were expressed at comparable levels (data not shown).

**Fig. 3.** AHR-mediated gene repression does not require transactivation. A, AHR deletion mutant series used for subsequent binding and transcription assays. Two domains previously implicated in RB binding, the LXCXE motif and the glutamine rich-domain, are shown. Additional elements include the bHLH domain required for dimerization, DNA binding, and nuclear localization, and the PAS domain implicated in ARNT dimerization and ligand binding. B, full-length AHR is required for maximal binding to RB. Bacterially synthesized vector or RB protein tagged with His6 at the amino terminus was incubated with nickel-agarose slurry, cleared through a column, and stored at 4°C. Control and RB-bound columns were established using 100-μl aliquots of the preadsorbed slurry. Full-length and truncation peptides of the mouse AHR were in vitro translated and labeled with [35S]methionine. Equal volumes of AHR or truncated peptides were recycled 10 times through control and RB-bound columns. Bound proteins were eluted using 8 M urea, and eluted fractions were denatured by boiling and analyzed by SDS-PAGE in 7.5% acrylamide gels. Detection of 35S-labeled AHR was by autoradiography. The graph represents quantitation of bound protein, normalized to control, by PhosphorImager analysis. C, AHR-mediated transactivation is not required for repression of E2F-dependent transcription. C33A cell cultures were transfected with the control plasmid pCMVgal together with the reporter p3E2FLuc. Additional cotransfected plasmids were as follows: —, vector; wtAHR, pcDNAI/B6AHR. AHR mutants are as described under “Experimental Procedures.” In addition, the plasmid pcDNAI/mARNT was cotransfected with all AHR-expressing plasmids. The ordinate represents luciferase activity normalized to β-galactosidase. The values shown represent the mean ± S.E. of at least 12 log-transformed determinations from four independent experiments. Asterisks (*) indicate significant differences (p < 0.05) from RB-transfected cells using one-way analysis of variance. D, AHR-dependent transactivation is impaired with expression of AHR mutant proteins. C33A cell cultures were transfected with the control plasmid pCMVgal together with the reporter pAhRDtkLuc5. Additional cotransfected plasmids were as follows: —, vector; RB, pCMVNeoRB; wtAHR, pcDNAI/B6AHR. AHR mutants are as described under “Experimental Procedures.” In addition, the plasmid pcDNAI/mARNT was cotransfected with all AHR-expressing plasmids. The ordinate represents luciferase activity normalized to β-galactosidase. The values shown represent the mean ± S.D. from one representative experiment of at least two independent trials. The vehicle control was Me2SO (DMSO).
Cells were treated with Me2SO vehicle (DMSO) with the p3E2FLuc plasmid. The gene expression using repression, we tested the effect of ARNT on E2F-dependent transcription by the AHR is unknown. To determine the role played by ARNT in the AHR-dependent effects on gene transcriptional repression by the AHR is not required for the cooperative repression of TCDD-inducible genes (68).

In the nucleus, AHR forms a transcriptionally active heterodimer with ARNT/HIF-1α (67), and the AHR-ARNT interaction is required for maximal transactivation of TCDD-inducible genes (68). Because the transactivation function of AHR is not required for the cooperative repression of E2F-dependent gene expression by AHR-RB complexes, we hypothesized that ARNT might also be dispensable for this effect. It is known that ARNT is not required for the interaction between AHR and RB (43), but its effect in mediating transcriptional repression by the AHR is unknown. To determine the role played by ARNT in the AHR-dependent effects on gene repression, we tested the effect of ARNT on E2F-dependent gene expression using c4 cells in transient transfection assays with the p3×E2Fluc plasmid. The c4 cell line is derived from the mouse Hepa-1 hepatoma cell line (34) and lacks expression of ARNT as well as RB protein. As shown in Fig. 4A, transfection of AHR into c4 cells enhanced RB-mediated repression of E2F-dependent gene expression by about 50%, as reported previously in Hepa-1 cells (44). Coexpression of AHR and wild type ARNT in these cells showed no further reduction in luciferase expression compared with AHR alone, indicating that interaction between AHR and ARNT is not necessary for repression of E2F-dependent gene expression by AHR.

To verify that the endogenous AHR would repress gene expression independently of DNA binding, we used the p3×E2Fluc plasmid in transient transfection assays in Hepa-1 cells, containing a wild type AHR, and in the c35 cell line, a Hepa-1 derivative that contains normal levels of a mutant AHR that is incapable of binding to DNA (53). In Hepa-1 cells, in agreement with previous results (44), AHR activation by TCDD treatment repressed E2F-dependent gene expression and more so in cooperation with overexpressed levels of RB (Fig. 4B, left panel). The same results were observed in the c35 cell line (Fig. 4B, right panel), emphasizing the fact that AHR-mediated DNA binding and transactivation are not requirements for repression of E2F-regulated genes by TCDD.

**Gene Repression by AHR-RB Interactions**

**Fig. 4.** ARNT dimerization is not required for AHR-mediated gene repression. A, formation of an AHR-ARNT complex is not required for repression of E2F-dependent transcription. c4 cell cultures were transfected with the control plasmid pCMVβgal together with the reporter plasmid p3×E2FLuc. Additional cotransfected plasmids were as follows: —, vector; RB, pCMVNeoRB; AHR, pcDNA1/NeoARNT; ARNT, pcDNA1/NeoARNT. The ordinate represents luciferase activity normalized to β-galactosidase. The values shown represent mean ± S.D. from one representative experiment of at least three independent trials. B, Hepa-1 cell cultures (left panel) or the c35 cell line harboring a mutant AHR defective in DNA binding (right panel) were transfected with the control plasmid pCMVβgal together with the reporter p3×E2Fluc. Additional cotransfected plasmids were as follows: —, vector; RB, pCMVNeoRB. The ordinate represents luciferase activity normalized to β-galactosidase. Cells were transfected with Me2SO vehicle (DMSO) or with 10 nM TCDD in Me2SO for 24 h after transfection. The values shown represent the mean ± S.D. from one representative experiment of at least two independent trials.

**A Transcriptionally Competent AHR Complex Is Not Required for Gene Repression**—In the nucleus, AHR forms a transcriptionally active heterodimer with ARNT/HIF-1α (67), and the AHR-ARNT interaction is required for maximal transactivation of TCDD-inducible genes (68). Because the transactivation function of AHR is not required for the cooperative repression of E2F-dependent gene expression by AHR-RB complexes, we hypothesized that ARNT might also be dispensable for this effect. It is known that ARNT is not required for the interaction between AHR and RB (43), but its effect in mediating transcriptional repression by the AHR is unknown. To determine the role played by ARNT in the AHR-dependent effects on gene repression, we tested the effect of ARNT on E2F-dependent gene expression using c4 cells in transient transfection assays with the p3×E2Fluc plasmid. The c4 cell line is derived from the mouse Hepa-1 hepatoma cell line (34) and lacks expression of ARNT as well as RB protein. As shown in Fig. 4A, transfection of AHR into c4 cells enhanced RB-mediated repression of E2F-dependent gene expression by about 50%, as reported previously in Hepa-1 cells (44). Coexpression of AHR and wild type ARNT in these cells showed no further reduction in luciferase expression compared with AHR alone, indicating that interaction between AHR and ARNT is not necessary for repression of E2F-dependent gene expression by AHR.

To verify that the endogenous AHR would repress gene expression independently of DNA binding, we used the p3×E2Fluc plasmid in transient transfection assays in Hepa-1 cells, containing a wild type AHR, and in the c35 cell line, a Hepa-1 derivative that contains normal levels of a mutant AHR that is incapable of binding to DNA (53). In Hepa-1 cells, in agreement with previous results (44), AHR activation by TCDD treatment repressed E2F-dependent gene expression and more so in cooperation with overexpressed levels of RB (Fig. 4B, left panel). The same results were observed in the c35 cell line (Fig. 4B, right panel), emphasizing the fact that AHR-mediated DNA binding and transactivation are not requirements for repression of E2F-regulated genes by TCDD.

**TCDD Reverses p300-mediated Induction of E2F-dependent Gene Expression**—E2F-dependent gene expression is regulated by the recruitment of a variety of corepressors and coactivators to both RB-bound and free forms of E2F. Active repression of certain E2F-responsive genes is mediated by the RB-dependent recruitment of corepressors, including HDAC and chromatin remodeling factors, to RB-E2F-DP complexes (for review, see Refs. 50 and 69). In addition, the ability of E2F to act as an activator of transcription depends in part on an interaction with the HDACs p300/CBP and p/CAF (70, 71). A role for corepressor/coactivator activities is also recognized as important for the regulation of the AHR complex (46, 72). Therefore tested the dependence of AHR transcriptional repression on specific corepressor and coactivator activities required for normal E2F-dependent regulation of transcription. We used the p3×E2Fluc plasmid for transient transfection assays in Hepa-1 cells, containing a wild type AHR. As expected, AHR activation by TCDD treatment repressed E2F-dependent gene expression (Fig. 5). Maximal repression of luciferase activity was obtained by cotransfection of either HDAC-1 or BRG-1 with RB. There was a trend toward further repression in TCDD-treated cells cotransfected with HDAC-1, although the effect was not statistically significant. When p300 was included in the transfection, repression of luciferase activity by RB was abolished, an effect that was blocked by TCDD treatment. In contrast, expression of a dominant-negative p300 did not reverse the repression of luciferase activity by RB. These results suggest that the activated AHR might prevent recruitment of p300 to the E2F complex, effectively enabling continued repression of gene transcription by RB-bound corepressors. Similar results were obtained in c35 cells (data not shown).

**Activation of the AHR by TCDD Displaces p300 from E2F-dependent Promoters**—The transient transfection experiments reported above suggest the possibility that AHR activation resulting from TCDD treatment represses E2F-dependent gene expression by blocking the recruitment of p300 to E2F-dependent promoters. To test this hypothesis, we used ChIP assays to...
analyze protein constituents at native promoters. Hepa-1 cells were treated with Me2SO vehicle or 10 nM TCDD for 1.5 h, and native chromatin was prepared from nuclear lysates. Immunoprecipitations were carried out using antibodies to AHR, E2F1, HDAC-1, p300, and RB. Controls containing no antibody were included to confirm the specificity of the precipitations. Purified genomic DNA was subjected to PCR amplifications with gene-specific primers. Input lane represents promoter-specific amplification of 0.2% of the total chromatin sample. Products were separated and analyzed using 10% PAGE. Cyp1a1, cytochrome P450 1a1; Cdk2, cyclin-dependent kinase 2; Cyclin E, cyclin E1; Dhdr, dihydrofolate reductase; Pol α, DNA polymerase α.

**Fig. 6.** Recruitment of AHR to E2F-regulated promoters results in loss of p300. Hepa-1 cell culture as stated in the legend to Fig. 3 and treated with either Me2SO vehicle (DMSO) or 10 nM TCDD for 1.5 h. Cells were harvested, and native chromatin from nuclear lysates was prepared. Immunoprecipitations were carried out using antibodies to AHR, E2F1, HDAC-1, p300, and RB as indicated. A control (No Ab) using no antibody was included to confirm the specificity of the precipitations. Purified genomic DNA was subjected to PCR amplifications with gene-specific primers. Input lane represents promoter-specific amplification of 0.2% of the total chromatin sample. Products were separated and analyzed using 10% PAGE. Cyp1a1, cytochrome P450 1a1; Cdk2, cyclin-dependent kinase 2; Cyclin E, cyclin E1; Dhdr, dihydrofolate reductase; Pol α, DNA polymerase α.

**Gene Repression by AHR-RB Interactions**

In this paper, we show that AHR activation by TCDD inhibits cell cycle progression, accumulating cells in G₁, and represses the transcription of E2F-dependent genes through interaction with RB. Recruitment of the activated AHR to E2F-regulated promoters blocks the recruitment of p300 and the concomitant up-regulation of E2F-dependent genes. We conclude that the two processes are interrelated and that inhibition of p300 binding is one of the molecular events responsible for the persistence of active gene repression by AHR-RB complexes. We present evidence to show that the repressor activity of the AHR is fully independent of its transcriptional activity because it does not require its transactivation or DNA binding domains or interaction with ARNT. Hence, we propose that repression is the overall effect of ternary or quaternary interactions taking place at E2F-regulated promoters.

Previous work has shown that the molecular interaction between the AHR and the RB protein leads to enhanced repression of E2F-dependent genes and cell cycle inhibition. RB-mediated transcriptional repression is dependent on two distinct protein-binding sites in RB: the large A/B pocket, which binds E2F, and the A/B pocket, which binds LXCXE peptide motifs (58). Deletion analysis of the AHR reveals that neither the LXCXE motif nor the glutamine-rich region of the AHR can mediate the maximal interaction levels between the two proteins observed in vitro with full-length AHR. In experiments using RB deletion mutants,² we find that the large A/B pocket of RB is required for binding with the AHR, confirming that RB sequences other than those that bind the LXCXE motif of AHR are necessary for their interaction. These findings may explain previous observations that T antigen only partially relieves AHR effects on RB-mediated repression (44). However, maximal binding to RB is not critical for enhanced repression of E2F-dependent genes by AHR, as evidenced by transfection assays in which several AHR deletion mutants, none of which is capable of maximal interaction with RB in vitro, can repress gene expression as efficiently as the wild type protein. This result is consistent with data showing that expression of a variant AHR with a mutation in the LXCXE motif can still mediate G₁ arrest (73) and that LXCXE-dependent interactions are not essential for RB to exert growth arrest (74). It is also notable that additional protein contacts may be involved in the interaction. This is underscored by the fact that the AHR can pull down E2F1 and that ectopic expression or activation of the AHR by TCDD can mediate repression of E2F-dependent genes in the absence of RB.³

Activation of the AHR by TCDD blocks cell cycle progression in various cell lines and under a number of different conditions, including in pancreatic cell cultures (75), in specific subclones

² J. L. Marlowe and A. Puga, unpublished data.
³ J. L. Marlowe and A. Puga, manuscript in preparation.
of PLHC-1 cells (76), and in the estrogen-induced proliferation of MCF-7 cells (63), among others. Inhibition of cyclin-dependent kinase activities, inhibition of RB phosphorylation and induction of p27Kip1 and p21WAF1 have been observed and proposed as potential mechanisms responsible for the block. Overexpression of AHR was also found to enhance RB-mediated inhibition of S phase progression in Saos-2 cells when both proteins were expressed ectopically (44). We have determined that this effect is not merely the consequence of protein overexpression, as TCDD significantly retards S phase progression in MCF-7 and in Hepa-1 cells, both of which express endogenous physiologic levels of AHR and RB. This is consistent with recent observations in MCF-7 cells showing that ablation of endogenous AHR expression by use of small interfering RNA results in enhanced progression of cells from G0/G1 to S phase (77). In our experiments with TCDD-treated Hepa-1 cells, the number of cells in G1 increases by the same extent that the number of cells incorporating BrdUrd decreases, suggesting induction of cyclin-dependent kinase activities, inhibition of RB phosphorylation and recruitment of coactivator proteins by E2F (71). p300/CBP-mediated cell cycle progression inhibition (66). Our results show that the inhibition of S phase progression after TCDD treatment coincides with a significant decrease in the expression of at least four genes that are positively regulated by E2F family members and necessary for S phase progression (50). This decrease is accompanied by a doubling of p27 mRNA levels. Accumulation of p27 mRNA and protein in 5L rat hepatoma cells has been proposed as the major cause of the AHR-mediated cell cycle progression inhibition (68). Our results indicate that decreases in expression of E2F-dependent genes occur concomitantly with a moderate increase in p27 expression, suggesting that the two processes are linked and that the ultimate outcome on cell cycle progression might be the result of their cooperation.

The data presented here eliminate several potential mechanisms by which the AHR acts to enhance repression of E2F-dependent genes. Repression mediated by the AHR is independent of its ability to transactivate gene expression, the as yet best characterized of AHR functions. Repression of E2F-dependent genes is also independent of DNA binding by the AHR, emphasizing the requirement for protein interactions in mediating this effect. Although nuclear localization is required in vivo for the AHR-RB interaction, in vitro binding assays show that ARNT is not needed for formation of the AHR-RB complex (44), nor is ARNT required for maximal repression of E2F-dependent gene expression by the AHR. Our studies, however, were not directed at analyzing the role of CYP1A1 expression on the extent of cell cycle inhibition and thus do not address recent work from Elferink and colleagues (78) that suggests that the activity of the AHR-dependent CYP1A1 protein prevents ARNT-mediated G1 arrest by metabolizing AHR ligands and negatively regulating the duration of AHR activation.

Binding of RB to E2F at E2F-responsive promoters recruits corepressor proteins that mediate active transcriptional repression (51). Repression is relieved by RB phosphorylation and recruitment of coactivator proteins by E2F (71). p300/CBP activity is also required for cell cycle progression and E2F activity (79), underscoring its necessity for up-regulation of E2F-dependent transcription. We have shown previously that AHR attenuates both Cdk2 protein levels and Cdk2-associated kinase activity (40) and thus helps maintain RB in a hypophosphorylated state. It may be that in addition, the AHR blocks critical interaction sites required for the recruitment of p300/CBP by E2F and hence helps block transcriptional induction by also preventing the reorganization of chromatin at E2F-responsive promoters. These two effects may in fact be causally related. This concept is supported by our results showing an additive effect of TCDD on HDAC-mediated repression of E2F-dependent gene expression. The ChIP assay with asynchronously growing cells shown here cannot address this issue properly because it represents the combination of results from cells at all stages of the cell cycle, and cells in S and G2-M would mask results specific for cells in G1.

There is no current paradigm that satisfactorily explains more than half of the gene regulatory effects of dioxin exposure, namely, the mechanisms responsible for its large effect as a repressor of gene expression (41). We believe that one of these mechanisms involves the repression of E2F-dependent gene transcription mediated by the activated AHR. As shown herein, repression depends to a large extent on the interactions of AHR with RB and possibly with E2F itself. It appears that one of the critical functions of the activated AHR is to serve as an environmental checkpoint that senses exposure to damaging environmental toxicants and responds by signaling cell cycle inhibition.

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