Aromatic Hydrocarbon Receptor Interaction with the Retinoblastoma Protein Potentiates Repression of E2F-dependent Transcription and Cell Cycle Arrest*

(Received for publication, July 30, 1999, and in revised form, October 28, 1999)

Alvaro Puga‡§, Sonya J. Barnes‡¶, Timothy P. Dalton‡, Ching-yi Chang‡, Erik S. Knudsen**‡‡, and Michael A. Maier§§

From the ‡Center for Environmental Genetics and Department of Environmental Health, **Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati, Ohio 45267-0056

Polyhalogenated aromatic hydrocarbons, of which 2,3,7,8-tetrachloro-p-dioxin (TCDD) is the prototype compound, elicit a variety of toxic, teratogenic, and carcinogenic responses in exposed animals and in humans. In cultured cells, TCDD shows marked effects on the regulation of cell cycle progression, including thymocyte apoptosis, induction of keratinoyte proliferation and terminal differentiation, and inhibition of estrogen-dependent proliferation in breast cancer cells. The presence of an LXCXE domain in the dioxin aromatic hydrocarbon receptor (AHR), suggested that the effects of TCDD on cell cycle regulation might be mediated by protein-protein interactions between AHR and the retinoblastoma protein (RB). Using the yeast two-hybrid system, AHR and RB were in fact shown to bind to each other. In vitro pull-down experiments with truncated AHR peptides indicated that at least two separate AHR domains form independent complexes with hypophosphorylated RB. Immunoprecipitation of whole cell lysates from human breast carcinoma MCF-7 cells, which express both proteins endogenously, revealed that AHR associates with RB in vivo only after receptor transformation and nuclear translocation. However, the AHR nuclear translocator and transcriptional heterodimerization partner, is not required for (nor is it a part of) the AHR-RB complexes detected in vitro. Ectopic expression of AHR and RB in human osteosarcoma SAOS-2 cells, which lack endogenous expression of both proteins, showed that AHR synergizes with RB to repress E2F-dependent transcription and to induce cell cycle arrest. Furthermore, AHR partly blocked T-antigen-mediated reversal of RB-dependent transcriptional repression. These results uncover a potential function for the AHR in cell cycle regulation and suggest that this function may be that of serving as an environmental sensor that signals cell cycle arrest when cells are exposed to certain environmental toxicants.

One of the most puzzling aspects of the biological impact of the dioxins, a group of polyhalogenated aromatic hydrocarbons of which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the prototype compound, is that they cause a profusion of apparently unrelated biological effects. In humans, exposure to TCDD and to many other chlorinated phenolic agents causes choline, a long lasting skin disease characterized by the hyperkeratinization of follicular sebocytes (1). Long term epidemiologic studies have also established a strong link between exposure to high doses of TCDD and certain types of cancers and cardiovascular disease (2–5). TCDD exposure during mouse embryogenesis causes craniofacial anomalies, such as cleft palate, and hydronephrosis (6), whereas in adult rodents, TCDD causes an elevated incidence of hepatic carcinoma and pulmonary and skin tumors (7–9). Other consequences of TCDD exposure include disturbances of lipid metabolism, cardiovascular and craniofacial abnormalities during fish development (10, 11), and immunotoxic (12), reproductive, and endocrine effects (13–16), some of which appear to be present also in exposed humans (17–19).

The effects of TCDD at the cellular level are just as diverse and often contradictory. In human keratinocytes, dioxin has been shown to induce proliferation (20) as well as terminal differentiation (21–23). Immature thymocytes from rats and mice treated with TCDD in vivo, but not in vitro, show increased apoptosis (24–26). In rat hepatocytes, TCDD has also been reported to induce apoptosis (27) as well as to inhibit uv-induced apoptosis (28) and to increase (27) and to decrease (29, 30) proliferation rates. TCDD abrogates the estrogen-dependent proliferation of human breast cancer cells (31).

It is widely accepted that most, if not all, effects of dioxins are mediated by a cytosolic receptor known as the aromatic hydrocarbon (Ah) receptor (AHR) (32). AHR is an 805-amino acid-long ligand-activated transcription factor that forms a transcriptionally active heterodimer with the aromatic hydrocarbon nuclear translocator (ARNT/HIF-1β) (33, 34). In the cytosol, the unliganded AHR is found in a complex with two HSP90 molecules and at least one p45 protein, recently identified as an immunophilin homolog (35, 36). Ligand binding disrupts this complex and causes the nuclear translocation of the AHR. The nuclear AHR/ARNT heterodimer binds to CACGC DNA motifs (Ah receptor elements; also termed DREs and XREs) in the regulatory regions of the CYP1A1, CYP1B1, and CYP1A2 cytochrome P450 genes and of several genes cod-

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This work was supported by NIEHS, National Institutes of Health (NIH), Grants ES06273 and P30 ES06096.

¶ To whom correspondence should be addressed. Tel.: 513-558-0916; Fax: 513-558-0925; E-mail: Alvaro.Puga@UC.EDU.

† Supported by a NIEHS, NIH, mutagenesis and carcinogenesis training grant.

‡ Recipient of a predoctoral fellowship from the Pharmaceutical Research and Manufacturers of America Foundation. Present address: Dept. of Pharmacology, Duke University Medical Center, Durham, NC.

‡‡ A Kimmel Scholar.

§§ Supported by a United States Environmental Protection Agency STAR predoctoral fellowship grant.

---

1 The abbreviations used are: TCDD, 2,3,7,8-tetrachloro-p-dioxin; Ah, aromatic hydrocarbon; AHR, dioxin Ah receptor; ARNT, aromatic hydrocarbon nuclear translocator; RB, retinoblastoma protein; GFP, green fluorescent protein; DHFR, dihydrofolate reductase; PAGE, polyacrylamide gel electrophoresis.
ing for phase II detoxification enzymes, such as glutathione S-transferase Ya, NAD(P)H-dependent quinone oxidoreductase 1, aldehyde dehydrogenase-3, and others and activates their transcription (37).

In the process of studying exogenous ligand-independent mechanisms of AHR activation, we noticed that ectopic expression of AHR in several cell lines lacking endogenous AHR expression caused alterations in cell cycle progression in these cells. These observations, together with reports of cell cycle-dependent TCDD effects on gene expression (38) and of TCDD-dependent (39, 40) and -independent (41) Ah receptor effects on cell cycle progression, prompted us to examine possible mechanisms that would explain the apparent association of the Ah receptor with the cell cycle. Amino acid sequence comparisons revealed that all Ah receptor proteins sequenced to date have a minimal essential medium-dependent transcription and cell cycle progression. The human osteosarcoma (RB/RB) SAOS-2 (45), were cultured in minimal essential medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum and 1% antimycotic/antibiotics in a humidified 5% CO2 atmosphere. SAOS-2 cells do not express AHR, as determined from the lack of expression of a transfected AHR/ARNT-responsive luciferase reporter plasmid. Duplicate or triplicate transient transfections were prepared according to the manufacturer’s directions and were transfected in serum-free medium, and 3 h later one volume of a mixture of essential amino acids minus leucine and tryptophan was inoculated with individual yeast colonies and grown overnight at 30 °C to saturation. To activate the Ah receptor, β-naphthoflavone was brought up to the same amount of total DNA by the addition of the glutamine-rich transactivation domain (53); the L motif is at position 320 in this peptide. Plasmid pG1–425 encodes the carboxyl-terminal 379 amino acids and includes the glutamine-rich transactivation domain (53). A 50-μg aliquot of each bacterially made peptide was adsorbed to nickel-agarose as described above and allowed to interact with 500–800 μg of a whole cell extract of CV-1 cells prepared by sonication in NET-N buffer. Approximately 1 mg of whole cell extract was allowed to interact with 5–8 μg of bacterially made peptide tagged with [35S]methionine, which was precleared through a control nickel-agarose column to which a lysate of Escherichia coli had been added and washed. The effluent from the control column was applied to the RB column by gravity flow and recycled five times. Binding buffer contained 20 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Triton X-100. After washing with 10 column volumes of the same buffer, bound proteins were eluted with 8 M urea, 0.1 M NaH2PO4, 0.01 M Tris, pH 5.9, to disrupt nickel coordination of the His tag. For the converse experiments, two truncation peptides of the mouse AHR cDNA were cloned into the pQE30 vector (Qiagen) for expression of His6-tagged proteins. The plasmid p3576–805 encoded the mouse AHR amino-terminal 375 amino acids, to the terminus of the PAS region, including the basic helix-loop-helix and ligand-binding domains (39) that were subcloned in frame with the glutamine-rich transactivation domain (53). A 50-μg aliquot of each bacterially made peptide was adsorbed to nickel-agarose as described above and allowed to interact with 600–800 μg of a whole cell extract of CV-1 cells prepared by sonication in NET-N buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mM sodium pyrophosphate). Wash and elution protocols were identical as indicated above. All eluted fractions were denatured by boiling and analyzed by SDS-PAGE in 7.5% acrylamide gels. Detection of [35S]AHR was by autoradiography; detection of RB was by Western blot analysis, using rabbit anti-human RB antibodies as described previously (48). Immunoreactive quantitation was carried out with a Storm 860 PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA).

For coimmunoprecipitation experiments, MCF-7 cells were treated with 5 ng TCDD or with Me6SO vehicle for 2 h and lysed by sonication in NET-N buffer. Approximately 1 mg of whole cell extract was allowed to interact with 5–8 μg of anti-RB polyclonal serum (48) or with affinity-purified rabbit anti-mouse AHR antibodies prepared in our laboratory. As control, we used prebleed rabbit serum from the same rabbits used to prepare the anti-AHR antibodies. Binding reactions were carried out for 4 h at 4 °C, and complexes were recovered on mixed protein A plus protein G-Sepharose, washed five times with NET-N, denatured by boiling, and analyzed in 7.5% SDS-PAGE. AHR and RB were detected by Western blot after semidyelotransfer of the proteins to polyvinylidene difluoride membranes, as described previously (54).

**Bacterial Expression and Pull-down Assays—** For cell cycle studies, SAOS-2 cells were transfected with pCMVNeoRB in the presence of varying amounts of pCMV6AHR, as indicated in the corresponding figure legends. To identify transfected cells, pHB2GFP, a plasmid encoding a nuclear-targeted GFP (54), was included in all transfections. After allowing for a 24-h period of cell recovery, cells were labeled with BrdUrd and cultured for an additional 16–24 h. After fixing the cells for
15 min at room temperature in 3.7% formaldehyde, BrdUrd incorporation was detected using rat anti-BrdUrd antibodies (Accurate Scientific) and a rhodamine-conjugated donkey anti-rat secondary antibody as described previously (55). Transfected cells were scored by visualization of the expressed GFP with incident fluorescence light. Approximately 20–40% of the cells were transfected under our experimental conditions. Incorporation of BrdUrd was determined by quantitation of the numbers of GFP-positive and GFP-negative cells that showed rhodamine fluorescence as visualized using a Zeiss epifluorescence microscope. Total cell numbers were determined by Hoescht 3325 staining. Two independent operators, one of which was blind to the experimental protocol, made the quantitation, and their results agreed to within 3%.

RESULTS

AHR and RB Interact in Yeast Two-hybrid Assays—The presence of an LXXCXE motif in the AHR moiety suggested the possibility that AHR and RB could form protein complexes. To test this hypothesis, we cloned cDNAs coding for these proteins in yeast expression vectors and analyzed them in yeast two-hybrid assays. AHR was inserted in frame with the sequences coding for the DNA binding domain (DBD) of Gal4, and RB was cloned in frame with sequences coding for the Gal4 transactivating domain. The two resulting plasmids, pGBT9AHR and pGAD424RB, were transfected into S. cerevisiae yeast cells, and positive transfectants were selected by the methods described under “Experimental Procedures.” The plasmids transfected were as follows. GBT, trp; yeast expression vector pGBT9; GAD, leu; yeast vector pGAD424; AHR, pGBT9AHR; ARNT, pGADARNT; RB, pGAD424RB. The values shown are the mean ± S.D. of three determinations.

FIG. 1. AHR and RB proteins interact in yeast. Yeast expression plasmids were introduced into yeast cells, and positive transfectants were selected by the methods described under “Experimental Procedures.” The plasmids transfected were as follows. GBT, trp; yeast expression vector pGBT9; GAD, leu; yeast vector pGAD424; AHR, pGBT9AHR; ARNT, pGADARNT; RB, pGAD424RB. The values shown are the mean ± S.D. of three determinations. DMSO, Me2SO.

can bind to RB. Complexes formed in the presence of an AHR ligand generate higher β-galactosidase levels, suggesting that a specific AHR/RB interaction takes place that is enhanced by ligand-induced conformational changes in the Ah receptor.
M15 cells had been adsorbed. The effluents from the control columns were cycled five times over the columns containing His$_6$-tagged RB. Proteins bound to control and to RB columns were analyzed by electrophoresis in 7.5% SDS-polyacrylamide gels (Fig. 2). Control columns did not retain any detectable amount of labeled AHR, but a significant fraction of the input $[^{35}S]$AHR was bound to the RB-bearing columns (Fig. 2A). Quantitation of input and bound AHR by PhosphorImager analysis indicated that 2.5% of the labeled AHR protein translated in the absence of TCDD was bound to RB. However, when translation took place in the presence of TCDD ligand, the fraction of $[^{35}S]$AHR that bound to RB nearly doubled, to 4.7% of the total synthesized protein, suggesting that receptor activation and release from HSP90 has a significant effect on the ability of the two proteins to interact.

For the converse experiment, we tagged with a His$_6$ peptide two truncated forms of the AHR, encoding the amino-terminal 375 amino acids and the carboxyl-terminal 379 amino acids, respectively. The $\Delta 376–805$ peptide contains the LHCXE motif LHCAE at position 320 near the end of the PAS domain, whereas the $\Delta 1–423$ peptide harbors the entire glutamine-rich AHR transactivating domain. A whole cell lysate of CV-1 cells, which express high levels of RB, was first precleared through a control nickel-agarose/E. coli M15 lysate column, and the flow-through from this control column was applied to the AHR-nickel-agarose columns. Bound CV-1 proteins were separated in 7.5% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed by Western immunoblotting (Fig. 2B). Both AHR peptides were found to interact with RB, although, as judged from immunoblot intensities, the LHCAE-containing amino-terminal peptide binds to RB to a lesser extent than the carboxyl-terminal peptide, in good agreement with recently published evidence of AHR/RB interactions in vitro (57). It appears also that AHR binds preferentially to the faster migrating, less phosphorylated forms of RB.

To test for AHR/RB interactions in vivo, we used coimmunoprecipitation experiments with anti-AHR and anti-RB specific antibodies. For these experiments, we used whole cell lysates of AHR-positive/RB-positive human breast carcinoma MCF-7 cells treated with Me$_2$SO vehicle or with 5 nM TCDD for 2 h. Lysate aliquots containing 1 mg of protein were incubated with monoclonal rabbit anti-RB antiserum, anti-AHR antiserum, or control rabbit polyclonal serum. Immune complexes were recovered with protein A plus protein G-Sepharose, and bound proteins were eluted by boiling, analyzed by 7.5% SDS-PAGE, blotted, and probed with anti-RB and anti-AHR antibodies (Fig. 2C). Probing with anti-RB showed that anti-AHR antibodies precipitated RB from whole cell extracts of TCDD-treated cells but not from cells mock-treated with Me$_2$SO. Conversely, probing with anti-AHR showed that anti-RB antibodies immunoprecipitated AHR from TCDD-treated cells and to a much lesser extent, if at all, from Me$_2$SO-treated cells. Control serum did not immunoprecipitate any of these proteins. These data
suggest that AHR and RB associate in vivo and that the association requires ligand-dependent nuclear translocation of the Ah receptor, since RB is always localized to the nucleus. In addition, the observation that lysates of MeSO-treated cells do not show the presence of AHR/RB complexes indicates that the observed interaction occurs in vivo and is not merely a consequence of protein association following lysis.

**AHR Cooperates with RB to Repress E2F-dependent Transcription**—To determine whether interaction with AHR affects RB function, we used the luciferase reporter plasmid pX/E2FLUC that responds to E2F-dependent transactivation. Transfection of this plasmid into Hepa-1 cells, which have nearly undetectable levels of RB expression, resulted in high levels of luciferase expression that were reduced to 50% by activation of the endogenous AHR with TCDD. As expected, expression of RB in these cells reduced luciferase expression even further, to levels approximately 15% of control. Like TCDD treatment, overexpression of AHR also reduced luciferase expression to 50% of control, possibly because AHR overexpression leads to its constitutive activation and nuclear localization (46). The combined effects of co-expressing RB and AHR resulted in nearly undetectable luciferase expression, with levels comparable with those directed by pX/mE2FLUC, a control luciferase reporter bearing mutated E2F binding sites (Fig. 3A).

Qualitatively similar results were obtained in SAOS-2 cells, which are RB-negative and do not express AHR (Fig. 3B). In these cells, however, TCDD treatment had no effect on E2F-dependent luciferase expression, indicating that dioxin treatment is of no consequence to E2F-dependent expression in the absence of the Ah receptor. In addition, the effect of AHR expression on luciferase activity was independent of TCDD treatment, as anticipated from the observation that nuclear localization of ectopically expressed AHR is independent of ligand (46).

To analyze in more detail the dose-response relationships between AHR and RB, we cotransfected both expression plasmids at different relative ratios in SAOS-2 cells and measured the levels of residual E2F-dependent luciferase activity. Luciferase activity steadily decreased with increasing AHR/RB ratio, leveling off at a 3:1 ratio, reaching values of 50–60% of those found in the absence of AHR (Fig. 4A). This result was not due to interference by one plasmid on expression of the other, since transfection of increasing amounts of pCMVNeoRB in the presence of a constant amount of pCMVB6AHR resulted in proportionately increasing amounts of RB protein in the transfected cells, without any apparent decrease in the amount of AHR protein (Fig. 4B).

We used the reporter plasmids pDHFRLuc and pmutDHFRLuc to test whether the effect of AHR on E2F-dependent expression resulted from AHR/RB/E2F interactions at the E2F binding site. The DHFR promoter is a natural E2F-responsive promoter that responds to active repression by RB; the mutant DHFR promoter contains a mutation at the E2F binding site that renders it constitutively active and not actively repressible by RB. In both Hepa-1 and Saos-2 cells, luciferase expression directed by the wild type promoter resembled closely that directed by pX/E2FLuc, with RB and RB plus AHR actively repressing luciferase expression (Fig. 5). In contrast, the mutant promoter showed a significant level of activity in the absence of RB, and neither RB nor RB plus AHR had a significant effect on luciferase levels (Fig. 5). This data argues in favor of the view that the effect of AHR on RB-dependent active repression of E2F function is mediated by AHR/RB/E2F ternary interactions at the E2F binding site.

**AHR Partly Blocks T-antigen Sequestration of RB**—The viral oncoproteins adenovirus E1A, SV40 T-antigen, and human papilloma virus E7 bind and displace hypophosphorylated RB from its interaction with E2F, thus allowing for expression of E2F-dependent genes and cell cycle progression (reviewed in Ref. 58). Predictably, co-expression of RB together with SV40 T-antigen in both Hepa-1 and SAOS-2 cells showed reversal of the inhibitory effect of RB on E2F-dependent luciferase expression. Co-expression of AHR and T-antigen had, if anything, a slight stimulatory effect. In contrast, co-expression of RB, AHR, and T-antigen greatly diminished luciferase activity levels relative to those observed in cells expressing RB and T-antigen, but without reaching the low levels resulting from AHR/RB coexpression (Fig. 6). These data indicate that AHR binding to RB can partly block T-antigen from interacting with RB and restore inhibition of E2F-dependent transcription and, conversely, that T-antigen can reverse to some extent the effect of AHR on RB-mediated repression.

**AHR Cooperates with RB to Suppress Entry into S Phase**—One of the main roles of RB during cell cycle progression is to regulate S phase entry; hypophosphorylated RB remains bound to E2F and represses transcription of S phase-specific genes until phosphorylation by cyclin D-activated Cdk4 releases RB from E2F and allows the latter to function as a transactivator (reviewed in Ref. 59). To determine whether AHR binding had an effect on cell cycle regulation by RB, we expressed both proteins in SAOS-2 cells and quantitated S phase entry by BrdUrd incorporation during a 24-h post-transfection period. Plasmid pH2BGFP, expressing GFP fused to histone H2B, was included in all transfections to distinguish between transfected and untransfected cells. Approximately 80% of untransfected (GFP-negative) cells were positive for BrdUrd, indicating that, during the 24-h labeling period, they had entered S phase and incorporated BrdUrd. In the transfected (GFP-positive) cell population, inhibition of S phase entry by RB showed a clear dependence on the dose of the transfected RB expression plasmid. In addition, at all nonsaturating doses of RB, AHR coexpression blocked significantly more cells from entering S than observed in the presence of RB alone (Fig. 7), suggesting that AHR/RB complexes have a stronger effect on suppression of S phase entry than RB alone.

The magnitude of the AHR effect on RB function may be more readily evaluated from the data in Fig. 8, which summarize S phase entry and E2F-dependent activity results as a function of RB plasmid dose. Both the number of cells entering S phase (Fig. 8A) and the residual E2F-dependent activity (Fig. 8B) appear to be significantly diminished by the presence of AHR. Analysis of the slopes of the curves indicates that, for a given effect, 10 times less RB is required in the presence of AHR than in its absence, suggesting that the role of AHR is to synergize with RB and potentiate its function.

**DISCUSSION**

The data presented in this study indicate that the Ah receptor and the retinoblastoma protein can bind and form a stable protein complex. Evidence for the interaction stems from yeast two-hybrid assays, in vitro pull-down experiments, and coimmunoprecipitation analyses with specific antibodies and confirms the observations of Ge and Elferink (57), published while this work was in progress. The present results extend the work of these authors beyond the biochemical analysis into the biological consequences of the interaction and provide the framework for an understanding of its physiologic significance. We show that AHR binding reinforces the ability of RB to repress E2F-dependent transcription and potentiates RB-mediated induction of cell cycle arrest. AHR by itself possesses none of these functions; its role appears to be to cooperate with RB by lowering the amount of RB required to achieve a certain effect.

**AHR/ RB Interactions**

2947
In this context, AHR appears to have a cooperative function with RB similar to that of the BRG1/BRM family of transcriptional activators that cooperate with RB to induce cell cycle arrest (60, 61). As is the case with BRG1, whose functional effects can be blocked by sequestration of RB by the adenovirus E1A protein (60, 61), T-antigen partly relieves AHR effects on RB-dependent repression at the same time that AHR blocks to a large extent the functional effects of T-antigen on RB. Based on this observation, we would predict that the AHR and T-antigen might compete for binding sites in the RB A/B pocket, comprising amino acids 379–572 (A domain) and 646–772 (B domain). However, AHR behaves in a diametrically opposite fashion to the viral oncoproteins; rather than sequestering RB and allowing the cell cycle to proceed, AHR cooperates with RB to prevent entry into S.

In agreement with the data of Ge and Elferink (57), we find that at least two domains of the AHR can interact with RB independently. One domain includes the amino-terminal 375 amino acids and contains the LXXC motif. The second domain is present in the carboxyl-terminal 379 amino acids and con-
in the unliganded cytosolic receptor, is masked by binding of HSP90 (62–64). HSP90 is also found in large concentrations in reticulocyte lysates masking the ligand binding domain of in vitro translated AHR unless translation products are exposed to ligand (65). Overlap between ligand-binding and HSP90-binding domains provides a likely explanation for the observation that twice as much [35S]AHR bound to RB when the receptor was translated in vitro in the presence of TCDD; removal of HSP90 by ligand is likely to unmask the LXCXE motif, thus allowing for the AHR/RB interaction to take place. In vivo, both yeast two-hybrid assays and commounprecipitation analyses show that AHR binds to RB only after receptor activation and nuclear localization. This is not indicative that the LXCXE motif is the only AHR domain that binds to RB, but rather that nuclear translocation of AHR is required for the two proteins to interact. This is not surprising, given the exclusive nuclear localization of RB. Although nuclear localization is required in vivo for the AHR/RB interaction, in vitro binding experiments indicate that ARNT is not needed for, nor is it a part of the AHR/RB complex.

A critical question is whether the activated Ah receptor forms tripartite complexes with RB and E2F at E2F binding sites of natural promoters. We have approached this question by measuring the activity of wild type and mutant DHFR promoter directing luciferase expression. Our data show that the mutation at the E2F binding site in this promoter abolishes a part of that complex. Formal proof of this conclusion must await results from experiments designed to analyze protein interactions at E2F-responsive promoters.

Activation of the Ah receptor by TCDD has recently been shown to block estrogen-induced proliferation of MCF-7 cells by inhibiting Cdk2-, Cdk4-, and Cdk7-dependent kinase activities and estradiol-induced hyperphosphorylation of RB (67). The Ah receptor has also been shown to induce p27kip1 transcription and to inhibit proliferation of rat hepatoma 5L cells and of fetal thymocytes (68). It has also been observed that p21WAF1 levels increase in mouse liver after TCDD treatment (69) and that AHR content, independently of ARNT, modulates aspects of ceramide-induced apoptosis in mouse hepatoma 1c17 cells (70). The work reported here is consistent with these findings and points at yet another mechanism whereby the Ah receptor cooperates in bringing about cell cycle arrest. It may be the failure to respond to one such “AHR checkpoint,” perhaps in combination with the specific genetic make-up of exposed cells or animals, that causes TCDD to be a powerful tumor promoter in mice (71, 72) and a rodent carcinogen (8).

Both AHR and RB are responsive to environmental signals. There are more than 400 environmental toxicants and endogenous compounds that are known AHR ligands (73). Many of these ligands are oxygenated by the cytochrome P450 monoxygenases induced by AHR/ARNT-dependent transactivation and converted into highly oxidized metabolites, many of which, such as the benzo[a]pyrene diol-epoxides, are known DNA-damaging agents that elicit cell cycle checkpoints (74, 75). Consistent with a checkpoint role for AHR, homozygous deletion of the mouse Ah receptor does not lead to uncontrolled cell proliferation, but changes the cellular response to environmental insult (76, 77), suggesting that the role of AHR in the regulation of cell cycle progression is subtle and not critical for survival. A similar phenotype is observed in fibroblasts from RB-deficient mice, in which cell cycle progression is not inherently misregulated (78), but responsiveness to environmental signals is compromised, with failure to arrest in response to DNA damage or TGF-β (78–80). Like AHR, RB is also responsive to a plethora of environmental signals (58) that inhibit cell cycle progression by bringing about the dephosphorylation of RB, thereby activating RB as a transcriptional repressor and a cell cycle-inhibitory molecule (58, 81). The finding that AHR cooperates with RB via a direct interaction suggests an additional mechanism through which environmental signals can function to activate RB. One of the critical functions of the liganded Ah receptor appears to be to act as an environmental sensor that, in the presence of environmental toxicants, binds to RB and signals cell cycle arrest.

**Acknowledgments**—Many colleagues provided stimulating discussions in the initial stages of the development of the idea that the Ah receptor could interact with elements of the cell cycle regulatory machinery. We particularly thank J. Babish, C. J. Ellerink, W. F. Greenlee, M. E. Hahn, and J. J. Stegeman for sharing thoughts. We also thank M. E. Hahn for communicating the fish Ah receptor sequence prior to publication and B. L. Schumann for expert technical assistance.

**REFERENCES**

1. Suskind, R. R. (1985) Scand. J. Work Environ. Health 11, 165–171
2. Bertazzi, P. A. (1991) Sci. Total Environ. 106, 5–20
3. Fingerhut, M. A., Halperin, W. E., Marlow, D. A., Paciotti, L. A., Honchar, P. A., Sweeney, M. H., Greif, A. L., Dill, P. A., Steenland, K., and Suruda, A. J. (1991) N. Engl. J. Med. 324, 212–218
4. Flesch-Janys, D., Berger, J., Gurn, P., Manz, A., Nagel, S., Waltsgott, H., and Dwyer, J. H. (1995) Am. J. Epidemiol. 142, 1165–1175
5. Manz, A., Berger, J., Dwyer, J. H., Flesch-Janys, D., Nagel, S., and Waltsgott, H. (1991) Lancet 338, 959–964
6. Abbott, B. D. (1995) Toxicology 105, 365–373
7. Florstrom, S., Busk, L., Kronave, T., and Ahlborg, U. G. (1991) Fundam. Appl. Toxicol. 16, 375–391
