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The Aromatic Hydrocarbon Receptor Modulates the Hepa 1c1c7 Cell Cycle and Differentiated State Independently of Dioxin

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The aromatic hydrocarbon receptor (AhR) has been defined and characterized according to its ability to mediate biological responses to exogenous ligands, such as the synthetic environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The natural ligand(s) for AhR is unknown, and we know relatively little about AhR function in the absence of TCDD. Here, we have exploited the availability of AhR-defective (AhR-D) mouse hepatoma (Hepa 1c1c7) cells to analyze AhR's effects under conditions in which TCDD is not present. Our results reveal that AhR-D cells exhibit a different morphology, decreased albumin synthesis, and a prolonged doubling time compared with wild-type cells. Introduction of AhR cDNA into AhR-D cells by stable transfection alters these characteristics such that the cells resemble wild-type cells. Conversely, introduction of antisense AhR cDNA into wild-type cells changes their phenotype such that they resemble AhR-D cells. Fluorescence microscopy reveals that AhR-D cells do not exhibit an increased rate of death. Flow cytometric and biochemical analyses imply that the slowed growth rate of AhR-D cells reflects prolongation of G_1. Our findings reveal a potential link between AhR and the G_1 phase of the Hepa 1c1c7 cell cycle. These effects of AhR occur in the absence of TCDD. We speculate that they represent responses to an endogenous AhR ligand in Hepa 1c1c7 cells.

The aromatic hydrocarbon receptor (AhR) is an intracellular protein that binds exogenous ligands, such as the halogenated aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and mediates their biological effects (16, 28, 31, 34, 39). Much of our understanding of AhR's function stems from analyses of the mechanism by which TCDD induces the transcription of *Cyp1A1*, a gene that encodes the microsomal enzyme cytochrome P4501A1, which oxygenates various xenobiotics as a step in their detoxification (3). AhR is a basic helix-loop-helix protein that dimerizes with a second basic helix-loop-helix protein, the AhR nuclear translocator (Arnt), to form a heteromeric transcription factor (2, 7, 19, 32). The liganded AhR-Arnt heteromer binds to specific DNA sites in an enhancer upstream of the *Cyp1A1* gene, an event which leads to alterations in chromatin structure and the binding of general transcription factors to the *Cyp1A1* promoter (6, 23, 27, 29, 43). Such studies reveal that AhR is part of a novel, ligand-activated transcriptional control system (10, 16, 42).

AhR's prototypical ligand, TCDD, is widespread, potent, and resistant to metabolic breakdown; the latter property allows it to accumulate in the cell and to produce sustained effects. Some effects, such as the induction of xenobiotic-metabolizing enzymes, are adaptive; they enhance the cell's ability to detoxify lipophilic foreign compounds. Other responses to TCDD, such as neoplasia and reproductive toxicity, are adverse and are associated with alterations in endocrine homeostasis, cellular proliferation, and tissue differentiation (20, 30, 31, 34). We do not understand why TCDD, an environmental contaminant of relatively recent origin (4), should produce changes in cell growth and differentiation, which involve fundamental cellular processes that evolved long ago. Presumably, in eliciting such changes, TCDD mimics a naturally occurring ligand for AhR. For example, some edible plants contain AhR ligands (1, 11, 12). Like TCDD, such ligands induce enzymes that metabolize lipophilic compounds; however, unlike TCDD, they are also substrates for the induced enzymes, and therefore their effects are transient. If TCDD acts by mimicking such ligands, changes in cell growth and differentiation might reflect persistent alterations in hormonal and metabolic status that occur secondary to sustained enzyme induction. A second possibility is that TCDD mimics an endogenous AhR ligand, which has not yet been identified. This idea predicts that AhR's effects on growth and differentiation do not require the induction of xenobiotic-metabolizing enzymes and that AhR can modulate growth and differentiation in the absence of TCDD.

Mouse hepatoma (Hepa 1c1c7) cells in culture constitute a powerful experimental system for analyzing the mechanism by which AhR induces transcription in response to exogenous ligands like TCDD (16, 42). One attractive aspect of this system is the availability of AhR-defective (AhR-D) mutants; such cells enable genetic analyses of AhR function (14, 15, 23, 26). Furthermore, the culture system facilitates experimental control of the extracellular milieu and eliminates secondary effects due to hormonal and metabolic changes, which occur in intact animals. Here, we have taken advantage of this cell system to analyze the phenotype of AhR-D cells in greater detail. We find that AhR content influences both the growth and the differentiated state of Hepa 1c1c7 cells and that AhR exerts its effect on growth during the G_1 phase of the cell cycle. These AhR-dependent effects do not require TCDD; therefore, we envision that they reflect the existence of an endogenous AhR ligand in Hepa 1c1c7 cells.

MATERIALS AND METHODS

Materials. Plasmids pRc/CMV and pCH110 were from Invitrogen Corporation (San Diego, Calif.) and Pharmacia LKB Biotechnology Inc. (Piscataway, N.J.), respectively. [α-^{32}P]dATP (3,000 Ci/mmol), [^{14}C]chloramphenicol (57 mCi/mmol), and [^{3}H]thymidine (25 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, Ill.). Monensin was from Sigma (St. Louis, Mo.).

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TCDD was from the National Cancer Institute Chemical Carcinogen Reference Standard Repository.

Cell culture. Wild-type Hepa 1c1c7 and AhR-D variant cells were grown as monolayers in alpha minimal essential medium containing 10% fetal bovine serum as previously described (26). To analyze doubling time, cells were trypsinized and seeded in 60-mm-diameter plates (5×10^4 cells per ml). Cells from triplicate plates were counted at 24-h intervals with a hemocytometer. Cell number (log scale) was plotted versus time (linear scale), and doubling time was calculated from the linear region of the plots.

Stable transfection. For stable expression of AhR, we subcloned full-length AhR cDNA (24) into the mammalian expression vector pRc/CMV, which contains a selectable marker, the neomycin resistance gene. The resulting construct was designated pAhR/CMV. The cells were transfected by using Polybrene (9) and were allowed to grow in nonselective medium for 48 h. The cells were trypsinized and cultured in medium containing G418 (500 μg/ml). After 14 to 16 days, clones were isolated and expanded individually. Cells from each clone were transiently cotransfected with both a reporter plasmid, pMcat5.9, which contains the AhR-dependent transcriptional regulatory region of the *Cyp1A1* gene linked to chloramphenicol acetyltransferase (CAT) cDNA (22), and a eukaryotic β-galactosidase expression vector, pCH110. The transient transfectants were screened for gain of TCDD-inducible CAT activity, as an indicator that AhR function had been restored. Transfectants with high responsiveness to TCDD were studied further as described below.

To express antisense AhR mRNA, AhR cDNA was amplified by PCR using the following primers: primer A, TGTCGTCTAGAGATGAGCAGCGGCGCCAACATCACC, containing an *Xba*I site and nucleotides 1 to 24 of the AhR coding strand (underlined); and primer B, CTGCCAAGCTTACTCTGCACCTTGCTTAGGAATGC, containing a *Hin*dIII site and nucleotides 2392 to 2415 of the AhR noncoding strand (underlined). Plasmid pAhR/CMV was used as the template, and PCR was performed as described previously (24). The PCR fragment was inserted into pRc/CMV at the *Hin*dIII and *Xba*I restriction sites in the reverse orientation with respect to the cytomegalovirus promoter, generating pAhRr/CMV. Wild-type cells were transfected with pAhRr/CMV and were grown in medium containing G418 as described above. After 2 weeks, clones were isolated and expanded individually. Clones were then transiently transfected with plasmids pMcat5.9 and pCH110 and were screened for loss of TCDD-inducible CAT activity, as an indicator that AhR function had diminished. Transfectants with low responsiveness to TCDD were studied further as described below.

Analysis of cell death and apoptosis. Cells in mid-log growth were treated with Hoechst 33242 (10 μg/ml; Sigma) for 20 min. Following Hoechst staining, the medium containing floating cells was collected and saved at 4°C. Attached cells were harvested by mild trypsinization, combined with the floating cells, collected by centrifugation, and resuspended in phosphate-buffered saline (PBS) containing 5% fetal bovine serum. Propidium iodine (PI) was added to a final concentration of 10 μg/ml. Thereafter, stained cells were maintained at 4°C and were analyzed within 30 min by fluorescence microscopy. Under these conditions, both living and dead cells stain with Hoechst 33242 (green nuclear fluorescence), whereas only dead cells stain with PI (red nuclear fluorescence). The difference between the two represents viable cells. Apoptosis was assessed by using a combination of nuclear morphology and fluorescence (37, 38).

Flow cytometry. Unsynchronized cells in mid-log phase were collected by mild trypsinization and gentle centrifugation and were fixed in 70% ethanol at 4°C. The fixed cells were pelleted, washed with distilled water, repelleted, and resuspended for 1 h at 37°C in a PI staining solution (PBS [pH 7.0], 100 μg of DNase-free RNase A per ml, 10 μg of PI per ml). After staining, the cells were pelleted and resuspended in PBS. The PI-stained cells were analyzed by flow cytometry at 488-nm excitation. The PI signal was filtered through a 635-nm bandpass filter to remove scattered light. Linear amplification of fluorescence signals was utilized. Pulse processing electronics (peak integral versus peak height) were used to analyze the DNA signals and to eliminate cell doublets from the analysis (37, 38).

Synchronization and thymidine incorporation. Cells in early log phase were treated with nocodazole (0.4 μg/ml; Sigma) for 8 h. Mitotic cells were detached by gentle agitation and were collected by low-speed centrifugation ($1,000 \times g$, 4 min) at 4°C. The cells were washed twice with ice-cold culture medium and were reseeded in plates at 5×10^4 cells per 35-mm-diameter plate. The cells were pulsed with [^{3}H]thymidine (1 μCi/ml) for 45 min at different times after seeding. Cells were washed once with ice-cold PBS (pH 7.0) and were lysed with 0.075% sodium dodecyl sulfate (SDS). Trichloroacetic acid was added to a final concentration of 10%, and the resulting precipitate was collected by filtration on Whatman glass fiber filters, washed with ice-cold 5% trichloroacetic acid, and analyzed for radioactivity by liquid scintillation counting.

Measurement of CAT activity. Cells transfected with pMcat5.9 and pCH110 were treated with 1 nM TCDD in dimethyl sulfoxide for 16 h prior to harvest. Control cells were treated with dimethyl sulfoxide only. Cell lysates were prepared in Reporter lysis buffer (Promega). CAT activity was measured by using a differential extraction-liquid scintillation assay (Promega). β-Galactosidase activity was measured by using a colorimetric assay (Promega) as instructed by the manufacturer. CAT activity was normalized to β-galactosidase activity to correct for differences in transfection efficiency.

Expression of AhR cDNA in *Escherichia coli* and preparation of polyclonal antibodies. To generate polyclonal antibodies, we expressed a carboxy-terminal fragment of AhR in *E. coli*. The plasmid designated pAhR-BH/ET21b contains a 1,164-bp fragment of the 3′ end of AhR cDNA inserted into the prokaryotic expression vector pET-21b (Novagen). *E. coli* BL21(DE3) cells were transformed by electroporation (Bio-Rad) and grown for plasmid expression as recommended by the manufacturer. Expression was monitored by immunoblotting of cell lysates with monoclonal antibody T7.Tag (Novagen), which is directed against the T7.Tag epitope (the 12 amino acids at the amino terminus of the T7 major capsid protein) linked to the amino terminus of the expressed protein. Because most of the fusion protein was in the form of inclusion bodies, it was purified in the presence of 6 M urea by using the pET His.Tag system (Novagen). The purified protein was renatured by dialysis against PBS containing decreasing concentrations of urea.

To generate polyclonal antiserum, 1 ml of protein suspended in Freund's complete adjuvant at 0.5 mg/ml was injected into rabbits (0.5 ml subcutaneously and 0.5 ml intradermally). The injections were repeated three times, and a test bleed was made after 6 weeks. The antiserum was tested against the antigen by enzyme-linked immunosorbent assay. To purify polyclonal antibodies specific for the receptor, an immunoaffinity column was made by coupling the purified C-terminal antigen (~2 mg) to Affi-Gel 10 (2 ml; Bio-Rad) as instructed by the manufacturer. The rabbit antiserum (~40 ml) was heated at 56°C for 30 min prior to loading onto the affinity column. The column was washed with PBS (pH 7.5) until the A_{280} of the eluent was approximately zero. The antibodies were eluted from the column with (0.2 M glycine HCl [pH 2.5]). The collecting tubes contained 0.15 ml of 1 M Tris Hcl (pH >8.0) to neutralize the eluent. Fractions of high A_{280} were collected and pooled. Immunoblot analyses reveal that the affinity-purified antibody recognizes a single band whose mobility is consistent with the size of the mouse AhR (~95 kDa).

Analysis of albumin expression by immunofluorescence. Immunofluorescent staining of cells was performed according to established procedures (18). Briefly, cells were grown to near confluency on coverglass slips (12-mm circle; Applied Scientific) contained in wells of a 24-well dish. Cells were incubated for 2 h with fresh medium containing 5×10^{-6} M monensin (2,000-fold dilution of a 10^{-2} M stock in ethanol), which blocks albumin secretion (40). Cells were washed twice with 1× PBS, fixed in 3.7% formaldehyde at room temperature for 10 min, and permeabilized in methanol at −20°C for 6 min. Cells were rinsed with PBS and were incubated in PBS containing 1% bovine albumin (Sigma) for 30 min with shaking at room temperature. Cells were then incubated with a 1:200 dilution of rabbit anti-rat albumin immunoglobulin G (IgG) (Organon Teknika, Durham, N.C.) in PBS plus 1% bovine albumin for 1 h with shaking, washed twice with PBS plus 1% albumin for 5 min, and incubated with affinity-purified fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, Pa.) for 1 h in the dark. Cells were then washed three times for 5 min in PBS. The coverglass slips were mounted onto Superfrost micro slides (VWR Scientific, Media, Pa.). Cells were visualized and photographed by using a Zeiss Axiophot fluorescence microscope with a 40× oil immersion objective.

Immunoblot analyses of albumin and AhR proteins. For immunoblotting of albumin, cells were grown to near confluency in 60-mm-diameter plates and were treated with monensin (5×10^{-6} M) for 2 h. The plates were washed twice with PBS, and the cells were lysed in Reporter lysis buffer (Promega); lysates were then sonicated for 5 s. Cell extracts were prepared by centrifugation of the lysates at $14,000 \times g$ for 10 min. Cell extracts for immunoblotting of AhR were prepared in a similar fashion but without monensin treatment. The cell extracts were fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes according to established procedures (35). The blots were blocked in PBS with 5% dry milk and 0.1% Tween 20 for 1 h with shaking and then incubated with either rabbit anti-mouse albumin IgG (Organon Teknika) or rabbit anti-mouse AhR antibody for 1 h. An alkaline phosphatase-conjugated goat anti-rabbit IgG preparation and a nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium substrate solution were used for color visualization (Promega).

RESULTS

Phenotypes of wild-type and AhR-D cells. The wild-type mouse hepatoma cells studied here are derived from a minimal deviation tumor, and the cells express a (partially) differentiated liver phenotype (5). Phase-contrast microscopy reveals that wild-type cells are epithelioid and polygonal, contain multiple nucleoli, have a granular cytoplasm, and form an orderly monolayer at confluence; these features are typical of hepatocytes (Fig. 1). The AhR-D cells were selected by fluorescence-activated cell sorting followed by growth in the presence of benzo[*a*]pyrene; they contain about 90% less AhR than wild-type cells, as measured by TCDD binding (26) and by immunoblotting with anti-AhR antibodies (Fig. 2A). In contrast, Arnt-defective (Arnt-D) cells, which were selected by the pro-

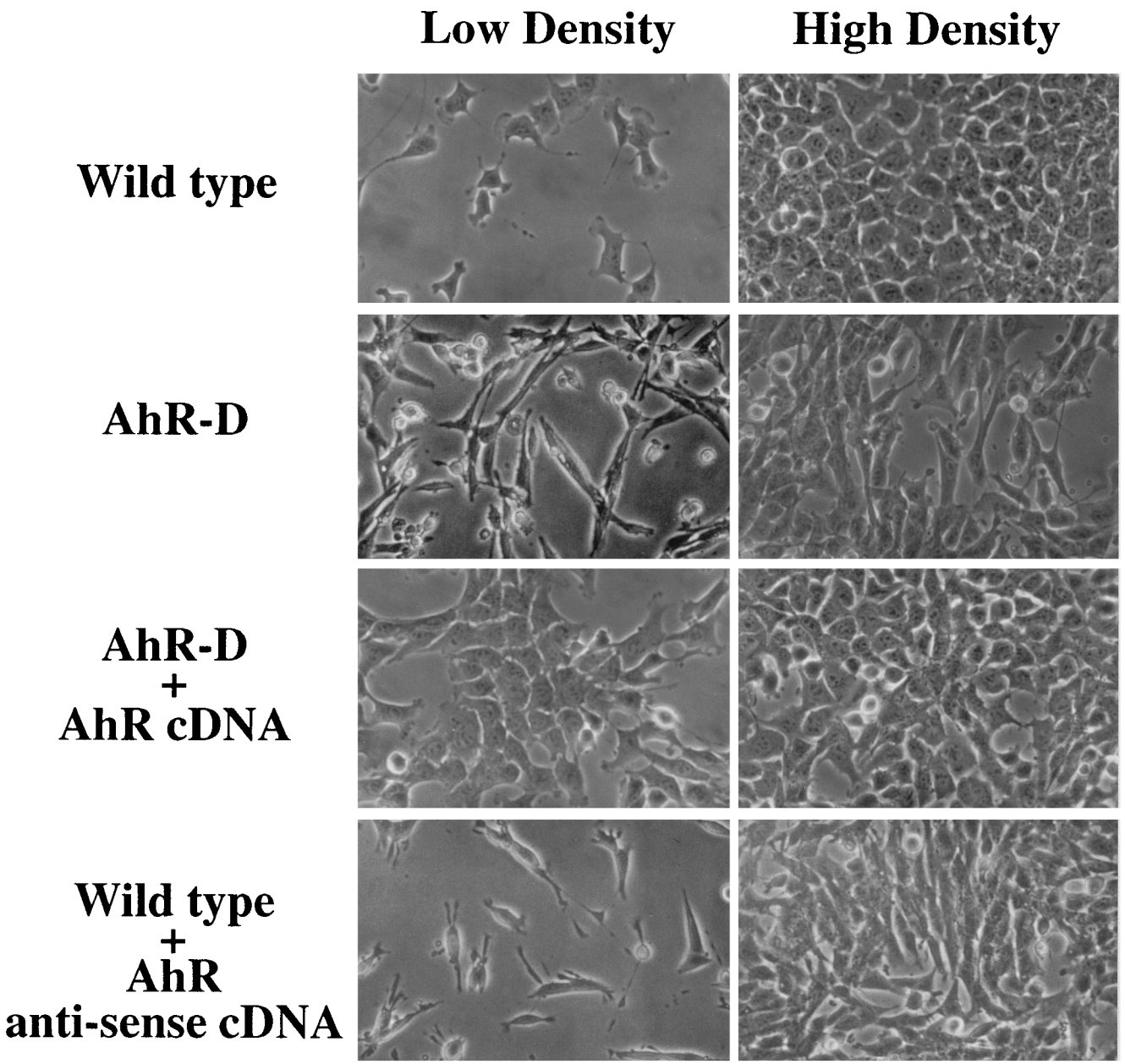

FIG. 1. Morphology of wild-type, AhR-D, and stably transfected cells. Untransfected cells and cells stably transfected with the indicated plasmids were cultured under routine conditions, visualized by phase microscopy, and photographed at low and high density.

cedure used for AhR-D cells, express a normal amount of AhR (26) (Fig. 2A). Decreased AhR is associated with morphological changes; for example, the AhR-D cells appear less well differentiated and more spindle shaped, and they fail to form well-organized monolayers at high density (Fig. 1). Immunofluorescent staining for α-tubulin and actin confirms the differences in shape between wild-type and AhR-D cells (data not shown). Immunofluorescence measurements performed with antialbumin antibody reveal that wild-type cells contain substantial amounts of albumin; in contrast, albumin is barely detectable in AhR-D cells (Fig. 3). Immunoblotting studies indicate that wild-type cells express at least 10-fold more albumin than AhR-D cells (Fig. 4A). The data also reveal that albumin expression in Arnt-D cells is similar to that in wild-type cells (Fig. 4A). Together, these data reveal that decreased AhR content is associated with changes in the differentiated state of Hepa 1c1c7 cells.

Measurements of cell growth reveal that the doubling time for wild-type cells during exponential growth is about 19 h, whereas that for AhR-D cells is about 25 h (Table 1). These findings reveal that decreased AhR content is also associated with decreased proliferation of Hepa 1c1c7 cells in culture.

We observe analogous alterations in morphology and growth in other, independently isolated AhR-D cell strains (data not shown). Thus, this particular phenotype appears typical of AhR deficiency in this cell system. Notably, the mutant phenotype is apparent in the absence of any known AhR ligand.

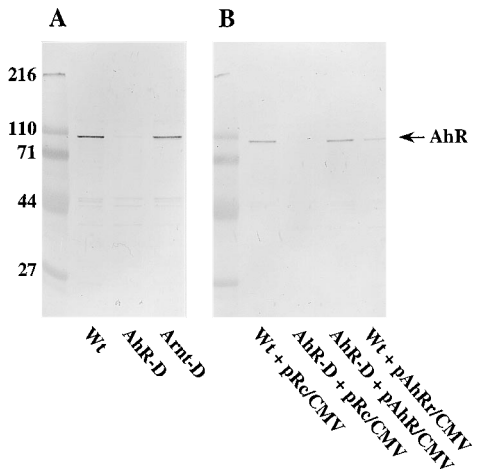

FIG. 2. Immunoblot analysis of AhR in wild-type, AhR-D, Arnt-D, and stably transfected cells. Cell extracts were electrophoresed on an SDS–10% polyacrylamide gel (20 μg of protein per lane), transferred to a nitrocellulose membrane, and immunoblotted with immunoaffinity-purified AhR antibody and alkaline phosphatase-conjugated goat anti-rabbit antibody. (A) Wild type (Wt), AhR-D, and Arnt-D cells; (B) stably transfected cells. Sizes are indicated in kilodaltons.

Thus, these AhR-D cells constitute an interesting experimental system for studying TCDD-independent functions of AhR.

Genetic manipulation of AhR levels in wild-type and AhR-D cells. We performed gene transfer experiments in order to analyze more definitively the role of AhR in the observed phenotypic differences between wild-type and AhR-D cells. First, we stably transfected AhR cDNA into AhR-D cells. To assess AhR function in the recipient cells, we transfected each G418-resistant (i.e., stably transformed) clone with a plasmid (pMcat 5.9) that contains an AhR-dependent CAT gene and measured TCDD-inducible CAT activity in cell extracts. We chose for further study a clone in which TCDD-inducible CAT expression was similar to the wild-type level, implying that substantial restoration of AhR function had occurred (Fig. 5). Consistent with the CAT data, immunoblotting revealed an increased content of AhR protein in the stable transfectants (Fig. 2B). Phase-contrast microscopy revealed that the morphology of the transfected cells had changed such that they now resembled wild-type cells (Fig. 1). Immunoblot analyses indicate that albumin expression had increased in these cells to approximately wild-type levels (Fig. 4B). Furthermore, the doubling time of the transfected cells had decreased and was the same as that of wild-type cells (Table 1). Other, independently isolated stable transfectants exhibited analogous changes in growth and morphology; however, we have not analyzed these clones in detail (data not shown).

To corroborate these findings, we performed the converse experiment, in which we stably transfected wild-type cells with antisense receptor cDNA to decrease their AhR content; we screened the G418-resistant clones for TCDD responsiveness as outlined above. We chose for further study a clone in which TCDD-inducible CAT activity was less than 30% of the wild-

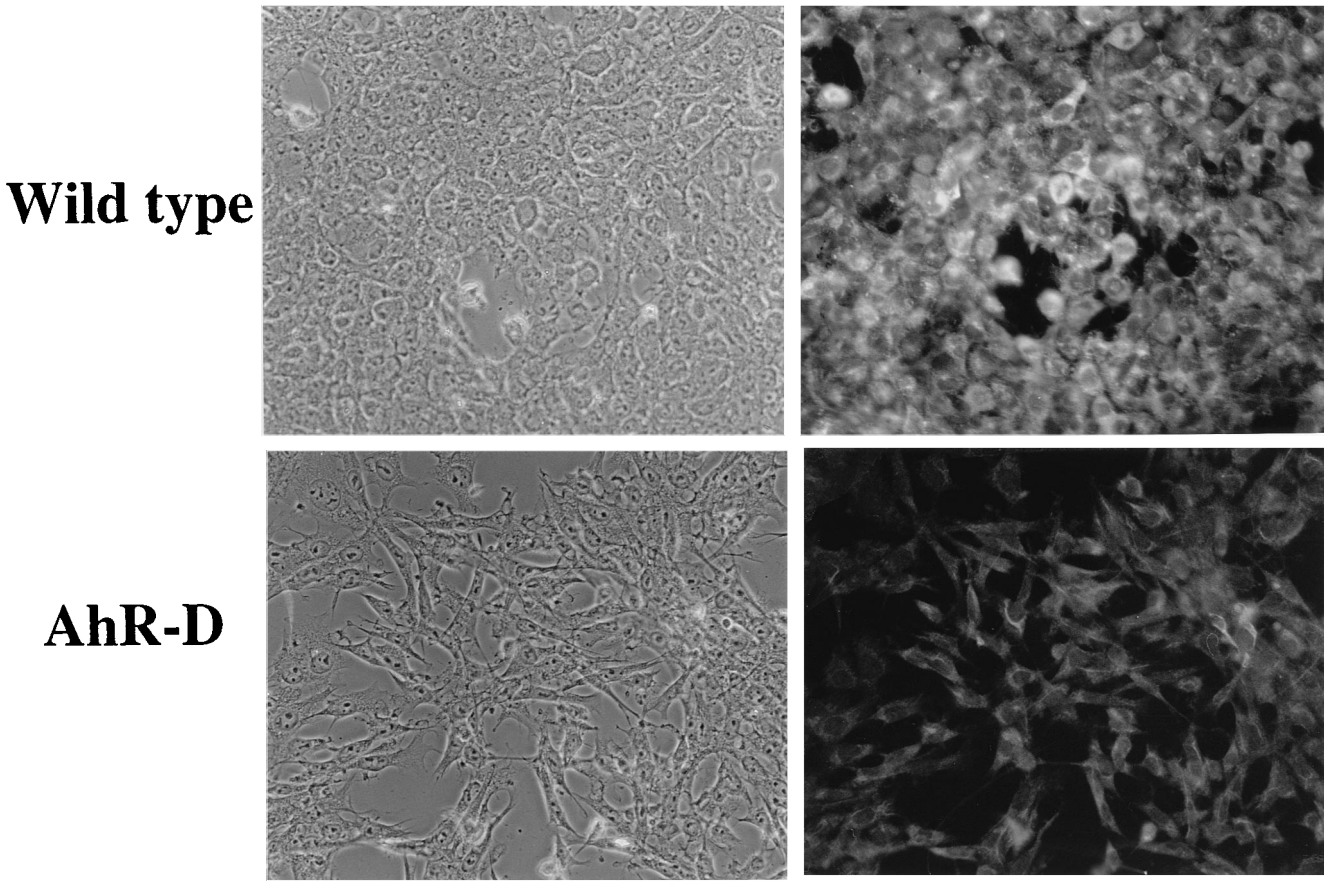

FIG. 3. Analysis of albumin in wild-type and AhR-D cells by immunofluorescence. Cells near confluency were treated with monensin (5×10^{-6} M) for 2 h, fixed, permeabilized, and stained. The cells were visualized by phase-contrast microscopy (left panels) and by fluorescence microscopy (right panels). Magnification, ×368.

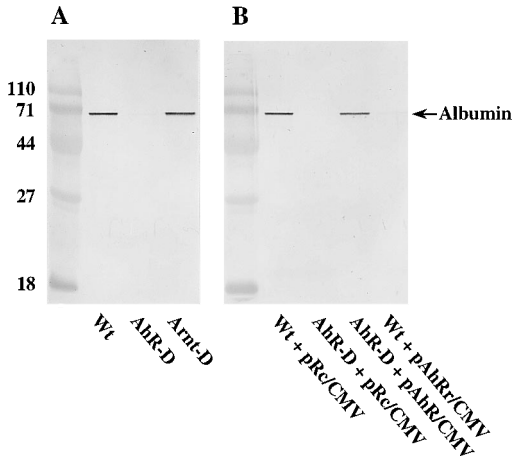

FIG. 4. Immunoblot analysis of albumin in wild-type, AhR-D, Arnt-D, and stably transfected cells. Cells near confluency were treated with monensin (5×10^{-6} M) for 2 h. Cell extracts were fractionated on an SDS–12% polyacrylamide gel (20 μg of protein per lane), transferred to a nitrocellulose membrane, and immunoblotted with rabbit anti-mouse albumin IgG and alkaline phosphatase-conjugated goat anti-rabbit antibody. (A) Albumin expression in wild-type (Wt), AhR-D, and Arnt-D cells; (B) albumin expression in stably transfected cells. Sizes are indicated in kilodaltons.

type level, implying that a substantial loss of AhR function had occurred (Fig. 5). Immunoblotting studies revealed that the level of AhR was decreased in the stably transfected cells (Fig. 2B). Morphologically, the transfectants resembled AhR-D cells (Fig. 1); in addition, they grew more slowly (Table 1) and expressed less albumin (Fig. 4B) than wild-type cells. Thus, the results of the transfection experiments are internally consistent; taken together, the findings implicate AhR in the differentiation and proliferation of Hepa 1c1c7 cells. Furthermore, AhR influences these phenotypes in the absence of TCDD.

AhR's effects on the Hepa 1c1c7 cell cycle. We used fluorescence staining and flow cytometry to analyze in greater detail the mechanism by which AhR affects Hepa 1c1c7 cell proliferation. Two general mechanisms could account for the longer doubling time of AhR-D cells: (i) AhR-D cells die at a faster rate than wild-type cells; and (ii) AhR-D cells proliferate more slowly than wild-type cells. To distinguish between these possibilities, we used a staining procedure to estimate cell death and apoptosis for wild-type and AhR-D cells under normal growth conditions. The results indicate that the proportion of dead AhR-D cells in mid-log phase (13.5%) is similar to that of wild-type cells (11.4%). Similarly, the fraction of cells that undergo apoptosis does not differ substantially between wild-type and AhR-D cells (1.4 and 1.8%, respectively). These findings imply that the increased doubling time of AhR-D cells does not reflect increased cell death; therefore, we infer that AhR-D cells proliferate more slowly than wild-type cells.

To analyze cell proliferation in greater detail, we used flow cytometry to determine whether AhR exerts its effect at a particular phase of the cell cycle. Analyses of unsynchronized populations during logarithmic growth reveal no significant difference between wild-type and AhR-D cells in the fraction traversing S phase or the G_2+M phase; however, the fraction of AhR-D cells in the G_0+G_1 phase is significantly higher than the fraction of wild-type cells in G_0+G_1 (data not shown). Combining these flow cytometric data with the observed doubling times for wild-type and AhR-D cells allows us to estimate the time that each cell type spends in each phase of the cell cycle (Table 2). Our data indicate that there is no significant

TABLE 1. Doubling times of wild-type and AhR-D cells[a]

Cell type	Transfected plasmid	Doubling time (h)
Wild type	pRc/CMV	19, 18
	pAhRr/CMV	23, 24
AhR-D	pRc/CMV	26, 24
	pAhR/CMV	20, 18

[a] Cells were stably transfected with the indicated plasmid, and doubling times were measured during exponential growth as described in Materials and Methods. The data are the results from two experiments.

difference between wild-type and AhR-D cells in the time that they spend in S phase (~7 h) and in the G_2+M phase (~6 h). In contrast, AhR-D cells spend about twice as much time (~12 h) as wild-type cells (~6 h) in the G_0+G_1 phase. These observations imply that AhR selectively influences the G_0+G_1 phase of the Hepa 1c1c7 cell cycle. To confirm this observation, we used flow cytometry to analyze cells stably transfected with either sense or antisense AhR cDNA. The data reveal that stable transfection of AhR cDNA into AhR-D cells selectively shortens the G_0+G_1 phase (from ~12 to ~7 h); in addition, introduction of antisense AhR cDNA into wild-type cells selectively lengthens G_0+G_1 (from ~6 to ~11 h) (Table 2). Again, our data are internally consistent; they reveal that reducing AhR levels in Hepa 1c1c7 cells prolongs the G_0+G_1 phase of the cell cycle. This prolongation can account for the increased doubling time of AhR-D cells.

Two different mechanisms could explain both the increased fraction of AhR-D cells in G_0+G_1 and their increased doubling time: (i) AhR-D cells take longer than wild-type cells to transverse G_1; and (ii) AhR-D cells transverse G_1 at the same rate as wild-type cells, but a substantial number of AhR-D cells exit the cell cycle and enter G_0. To distinguish between these possibilities, we carried out pulse-labeling experiments with synchronized populations, in which we directly measured the time interval between late M phase and S phase. Our results (Fig. 6) reveal that AhR-D cells require more time than wild-type cells to enter S phase following the release of a mitotic block. In analogous experiments, we find that transfection of AhR cDNA into AhR-D cells shortens the late M- to S-phase interval, whereas transfection of antisense AhR cDNA into wild-type cells lengthens it; the change is 2 to 3 h in each case (data not shown). These findings are internally consistent both with the data on growth of unsynchronized cells and with the

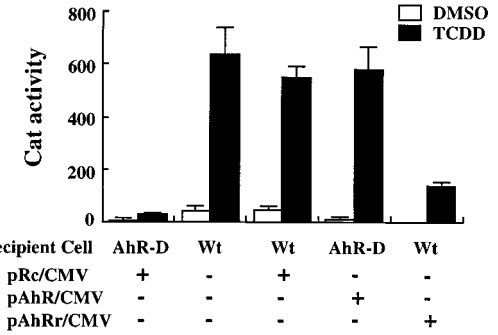

FIG. 5. TCDD responsiveness of stably transfected cells. The indicated stable transfectants were transfected with the reporter plasmid pMcat5.9. CAT specific activity was measured in extracts from uninduced and TCDD (1 nM, 16 h)-induced cells and corrected for transfection efficiency. The data represent average values from three experiments. Brackets indicate standard deviations. DMSO, dimethyl sulfoxide; Wt, wild type.

TABLE 2. Cell cycle analysis of wild-type and AhR-D cells[a]

Cell type	Stably transfected with:	Mean length (h) ± SD of cell cycle phase		
		G_0+G_1[b]	S	G_2+M
Wild type	pRc/CMV	5.8 ± 1.0†,§	6.9 ± 1.3	5.8 ± 1.3
	pAhRr/CMV	11.1 ± 2.1§,¶	6.0 ± 2.7	6.4 ± 1.7
AhR-D	pRc/CMV	11.9 ± 1.7†,‡	7.3 ± 1.3	5.8 ± 1.8
	pAhR/CMV	6.6 ± 1.8‡,¶	8.6 ± 3.1	3.9 ± 1.6

[a] Cells in mid-log phase were stained with PI, and the percentage of cells in each phase was determined by flow cytometry. The means and standard deviations were determined from four experiments and were multiplied by the doubling times of each cell type (Table 1) to calculate the length of each cell cycle phase.

[b] Statistical analysis was performed by using analysis of variance. Only the length of G_0+G_1 is significantly different among the four cell types as a group ($P < 0.05$). Further analyses by analysis of variance identified additional pairs with significant differences (‡, ¶, $P < 0.05$; †, §, $P < 0.01$).

flow cytometric data; they imply that the G_1 phase is longer in AhR-D cells than in wild-type cells. Taken together, our studies suggest that AhR influences Hepa 1c1c7 cell growth (as least in part) by altering the length of the G_1 phase of the cell cycle; this action is independent of TCDD.

DISCUSSION

The AhR-D cells studied here exhibit decreased (but not absent) TCDD binding and decreased (but not absent) responsiveness to TCDD, and we estimate that their AhR content is about 10% of that of wild-type cells (26). We have partially characterized 20 additional AhR-D Hepa 1c1c7 cell strains. These strains appear to be independent clones because they express different levels of AhR (ranging from 5 to 40% of the wild-type level), as measured by TCDD binding (21, 25) and by immunoblotting (data not shown), and because they exhibit different levels of cytochrome P4501A1 induction in response to TCDD (21, 25). The procedure used to isolate AhR-D cells selects against cells that contain AhR (14), yet we have found no strains in which AhR is completely absent. This finding suggests that a finite level of AhR function is required in order for Hepa 1c1c7 cells to survive in culture. This inference is consistent with our observation that decreased AhR content is associated with prolongation of G_1 and a decreased rate of cell proliferation.

Arnt-D cells represent a second type of benzo [*a*] pyrene-resistant variant derived from Hepa 1c1c7 cells (15, 26). These cells also grow more slowly and exhibit altered morphology compared with wild-type cells. However, unlike AhR-D cells, Arnt-D cells are larger and form sinus-like foci in culture (33). In addition, we show here that Arnt-D cells express albumin at a level comparable to the wild-type level. Thus, the phenotype of Arnt-D cells differs from that of AhR-D cells, suggesting that the two defects produce different (but possibly overlapping) alterations in cell function.

It is possible that the association between AhR content and growth rate is peculiar to Hepa 1c1c7 cells. We note that transgenic mice which are AhR deficient exhibit a 50% decrease in liver size; this phenomenon might indicate that AhR is required for hepatocyte proliferation in vivo. However, many other tissues in the transgenic mice appear unaffected by the AhR deficiency; this observation argues against a general requirement for AhR function in cellular growth and differentiation (8). Thus, our findings may be restricted to the particular cell strains studied here and may not be generalizable. This issue may be addressed in the future by detailed analyses either of additional clones and/or of cell populations into which AhR, anti-AhR cDNA, or a dominant-negative mutant has been introduced with high efficiency (e.g., by retroviral infection). The method that we used to screen stably transfected clones for gain or loss of AhR function (measurement of TCDD-inducible CAT activity) does not involve the assessment of their differentiated state or proliferation rate; this approach minimizes investigator bias in identifying particular clones to study. Thus, it is unlikely that selection bias has influenced our findings. The internal consistency of the data also argues against the existence of substantial bias in our experiments.

The observed AhR-dependent changes in cell differentiation and proliferation do not require exposure to TCDD. This result is consistent with observations in AhR-deficient transgenic mice, which imply that AhR also regulates basal transcription of the hepatic *Cyp1A2* and *Ugt1*06* genes in the absence of exogenous ligand (8). The mechanism by which AhR functions independently of TCDD is unknown. We cannot rule out the possibility that AhR is able to function without a ligand (in which case, the protein would not be acting as a receptor at all); however, we view this scenario as unlikely, because it supposes that AhR can act by two fundamentally different mechanisms. Therefore, we envision that AhR requires a ligand for function. Cell culture medium may contain exogenous ligands for AhR, as demonstrated by the transient and weak induction of *Cyp1A1* immediately following a medium change (25). We routinely take steps to minimize the introduction of such ligands into our experiments; furthermore, we are not aware of any such ligands that produce sustained effects (i.e., lasting for more than several hours). Therefore, we doubt that exogenous ligands in the medium can account for our findings. Thus, we are led to conclude that Hepa 1c1c7 cells contain an endogenous ligand that elicits AhR-dependent functions in the absence of TCDD. The observed dependence of cell proliferation on AhR content is consistent with the existence of an endogenous ligand. Other observations that support the existence of an endogenous ligand in Hepa 1c1c7 cells are (i) the increased level of cytochrome P4501A1 mRNA in cells containing a mutant cyto-

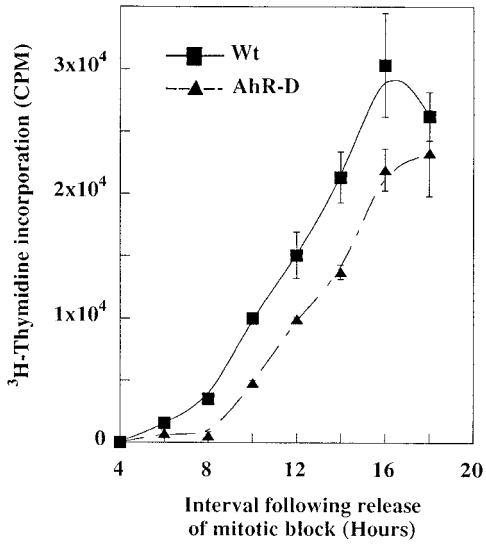

FIG. 6. Interval between late M phase and S phase in wild-type (Wt) and AhR-D cells. Cells were synchronized in M phase with nocodazole, released from the mitotic block, and pulse-labeled with [^{3}H]thymidine as described in Materials and Methods. Data represent means and standard deviations from three samples for each time point.

chrome P4501A1 enzyme (17) and (ii) the induction of cytochrome P4501A1 mRNA in cells grown in suspension in the absence of exogenous ligands (33). We speculate that TCDD mimics the effect of the hypothetical endogenous ligand.

Göttlicher et al. reported that TCDD inhibits the growth of 5L rat hepatoma cells in AhR-dependent fashion, because AhR-defective variants were insensitive to growth inhibition by TCDD (13). Furthermore, growth inhibition by TCDD occurred at the G_1 phase of the cell cycle (41). These observations substantiate our conclusion that AhR influences G_1 in rodent hepatoma cells. The mechanism that links AhR to G_1 progression remains to be determined. One possibility is that AhR participates directly in the transcriptional control of a G_1 cyclin(s) and/or cyclin-dependent kinase(s) and/or phosphatase(s), in response to an endogenous ligand that acts as a growth signal for Hepa 1c1c7 cells. A second possibility is that AhR acts more indirectly, via transcriptional effects on growth factors and/or their cognate receptors. Hepa 1c1c7 cells constitute a potentially useful system for testing these hypotheses.

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