Regulation of Cyp1a1 Induction by Dioxin as a Function of Cell Cycle Phase

RONALD P. SANTINI, SCOTT MYRAND,1 CORNELIS ELFERINK,2 and JOHN J. REINERS JR.
Institute of Environmental Health Sciences, Wayne State University, Detroit, Michigan
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ABSTRACT
Analyses of CYP1A1 mRNA were used to monitor the responsiveness of murine hepatoma 1c1c7 and human monocytic U937 cells in different phases of the cell cycle to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Concentrations of TCDD capable of inducing CYP1A1 were not cytostatic to either cell line. Steady-state CYP1A1 mRNA contents were reduced (45–90%) in TCDD-treated cultures arrested in G2/M as a consequence of exposure to microtubule disrupters (Colcemid, estramustine, vinblastine) or the microtubule stabilizer Taxol, relative to TCDD-treated asynchronous 1c1c7 cultures. The accumulation of mRNAs corresponding to Nmo1, another TCDD-inducible gene of the Ah battery, was also reduced in TCDD-treated G2/M cultures. Quantitative reverse transcription-polymerase chain reaction analyses of CYP1A1 heterogeneous nuclear RNA (hnRNA) revealed that Cyp1a1 transcription was suppressed in G2/M cells. This suppression reflected neither changes in the relative content of the proteins comprising the aryl hydrocarbon receptor (AHR) complex nor a suppression of AHR activation and translocation to the nucleus. Release of 1c1c7 cultures arrested in G2/M restored TCDD responsiveness. Centrifugal elutriation of TCDD-treated asynchronously growing U937 cells was used to prepare populations of cells in specific phases of the cell cycle. Within 3 h of TCDD exposure late G1/early S phase cells had CYP1A1 mRNA contents ~1.4- and 3-fold higher than the contents of asynchronous/early G1, and G2/M cultures, respectively. These studies suggest that the transcriptional activation of members of the Ah battery by TCDD is cell cycle-dependent, and markedly suppressed in G2/M cells.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor. In the absence of ligand it resides in the cytoplasm complexed with two heat shock protein 90 molecules and an immunophilin-like molecule (Schmidt and Bradfield, 1996; Carver et al., 1998). Upon ligand binding the AHR translocates to the nucleus where it complexes with the aryl hydrocarbon nuclear translocator (ARNT) protein. The resulting heterodimer recognizes and binds to dioxin responsive elements in target genes, and in conjunction with a series of coactivating proteins, stimulates target gene transcription (Nebert, 1994; Hankinson, 1995; Schmidt and Bradfield, 1996). To date, a variety of coplanar aromatic molecules have been identified as AHR ligands. Among them are several flavonoids, polycyclic aromatic hydrocarbons, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

Genes of the “Ah battery” encode proteins involved in the phase I and II metabolism of xenobiotics and are transcriptionally activated by TCDD through an AHR-dependent process. For example, TCDD treatment elevates CYP1A1 (nomencature used for CYP genes, mRNAs, and proteins, and is as recommended by Nelson et al., 1996), CYP1A2, and CYP1B1 expression in a variety of tissues (Nebert, 1994). These P450s have been implicated in the bioactivation of numerous procarcinogens. Conversely, TCDD also stimulates the transcription of GSTAI, AHD4, NQO1, NQO2, and UGT1A6 (Nebert, 1994). The proteins corresponding to these latter genes are involved in the detoxification of many carcinogens and cytotoxic agents used in chemotherapy.

In addition to its aforementioned activities, the AHR may also be a cell cycle regulatory protein. For example, the cytostatic activity of TCDD in the rat hepatoma 5L cell line is dependent upon the presence of the AHR (Weiss et al., 1996; Elferink et al., 2001). Similarly, recent studies suggest that a portion of the cytostatic activities of several flavonoids re-
lates to their functioning as AHR ligands (Reiners et al., 1999). The AHR may also influence cell cycle progression via a ligand-independent mechanism. At least three groups have shown that the time it takes to traverse the cell cycle, in the absence of exogenous AHR ligand, is inversely related to the amount of AHR protein expressed (Ma and Whitlock, 1996; Weiss et al., 1996; Reiners and Clift, 1999).

Although several studies suggest that the AHR can function as a modulator of cell cycle progression, very little is known about the regulation of AHR function and expression as a function of the cell cycle. It has been reported that contact arrested murine NMuLi c1 mouse liver cells (Becker and Bartholomew, 1979), and serum/growth factor arrested murine 3T3 fibroblasts fail to respond to AHR ligands (Vaziri et al., 1996). In the case of the fibroblasts this response was shown to reflect AHR turnover and a suppression of AHR expression in the arrested G0 population (Vaziri et al., 1996). We previously reported that the transcriptional activation of Cyp1a1 by AHR ligands was suppressed in 1c1c7 hepatoma cells arrested in G2/M as a consequence of exposure to the microtubule disrupter nocadazole (Schöller et al., 1994). These findings raise the issues of whether the transcriptional activation of Cyp1a1 and AHR function may be cell cycle-dependent. In the current study we investigated these problems by analyzing the expression of two TCDD-inducible genes in 1c1c7 cultures arrested in G2/M by treatment with a series of microtubule disrupters (Colcemid, vinblastine, and estramustine) and a microtubule stabilizer (Taxol). As a complement to these studies we also analyzed the TCDD-dependent induction of CYP1A1 in elutriated subpopulations of myelomonocytic leukemia U937 cells representing distinct phases of the cell cycle. These latter studies circumvented any complications associated with chemical-induced cell cycle blockage. They also provided information on all phases of the cell cycle in cycling cells. Our studies demonstrate that AHR-dependent activation of CYP1A1 is cell cycle phase-dependent and is markedly suppressed in G2/M cells.

Materials and Methods

Chemicals. TCDD was purchased from Chemsyn (Lenexa, KS). Colcemid, vinblastine sulfate, and protein A-Sepharose were obtained from Sigma (St. Louis, MO). Taxol was obtained as a gift from the Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingford, CT). Estramustine was obtained from Kabo Pharmacia (Lund, Sweden). Cyclin B antibody was purchased from Invitrogen (Carlsbad, CA).

Cell Culture and Treatment. Wild-type murine Hepa 1c1c7 cells (obtained from Dr. J. Whitlock, Stanford University, Palo Alto, CA) were cultured in α-minimal essential medium supplemented with 5% fetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin, and grown at 32°C in a humidified atmosphere containing 5% CO2. Cultures were passaged by exposure to trypsin/EDTA.

U937 cells were obtained from the American Type Culture Collection (Manassas, VA), and grown in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained in logarithmic growth at a density between 0.1 and 1 × 106 cells/ml at 37°C in a humidified atmosphere consisting of 5% CO2.

Cells were plated/passed at densities that ensured exponential growth for at least 4 days. Culture treatments were generally initiated 2 days after the passing or plating of cells. All chemicals were dissolved in DMSO. 1c1c7 cells having the flat or rounded morphology as a consequence of exposure to microtubule disrupters/stabilizers were isolated as described by Schöller et al. (1994).

Centrifugal Elutriation of U937 Cells. A Beckman Coulter JE-6B elutriation system (Beckman Coulter, Inc., Fullerton, CA) and rotor were used to enrich for populations of U937 cells in different phases of the cell cycle. In general, 1 × 107 cells (1 × 107 cells/ml suspended in RPMI 1640 + 1% fetal bovine serum) were loaded into the separation chamber (sterilized with 6% H2O2 and maintained at ambient temperature at a rotor speed of 2500 rpm) by using an entry flow rate of 15% of the maximum flow rate capacity. After loading the flow rate was increased by 5% increments until 70% of the maximum flow rate capacity was reached. Flow rates of 25, 32, 38, 44, 51, 57, 63.6, 70, 78.5, and 83 ml/min corresponded to maximum flow rate capacities of 20, 25, 30, 35, 40, 45, 50, 55, 60, and 65%, respectively. Approximately 100 ml of flow through was collected at each step. For simplicity, cells recovered at a specific percentage of maximum flow rate capacity are referred to as that elutriation fraction (i.e., cells eluted at 25% of the maximum flow rate capacity constitute elutriation fraction 25). Debris and dead cells were removed in elutriation fractions 20 and 25. Elutriation fractions were pelleted by centrifugation and subsequently washed twice with PBS. Samples of washed cells were removed for isolation of RNA, analyses of DNA contents by FACs, and cell counting. Samples removed for RNA extraction were frozen in liquid nitrogen and stored at −80°C until the time of RNA isolation.

Flow Cytometry and Chromosome Staining. 1c1c7 cultures were harvested and processed for FACs analyses of DNA content as described by Reiners et al. (1999). Suspensions of U937 cells were pelleted by centrifugation, washed twice with PBS before being fixed, and subsequently processed like 1c1c7 cells. DNA analyses were made with a BD Biosciences FACScalibur instrument (BD Biosciences, San Jose, CA). Percentages of cells in the G1, S, and G2/M stages of the cell cycle were determined with a DNA histogram-fitting program (MODFIT; Verity Software, Topsham, ME). A minimum of 10⁴ events/sample was collected for subsequent analyses. Colcemid-treated cells were fixed and stained with aceto-orcein to detect chromosomes as described by Lopes et al. (1993).

RNA Preparation and CYP1A1 mRNA Detection by Northern Analyses. Total cellular RNA was isolated using either commercially available TRIzol reagent, or the acidic phenol extraction method of Chomczynski and Sacchi (1987). RNA was resolved on 1.2% agarose/formaldehyde gels and transferred to nitrocellulose membranes as described by Reiners et al. (1997). The probes used for the detection of 7S and murine and human CYP1A1 RNAs and the conditions used for hybridization have been described in detail (Schöller et al., 1994; Reiners et al., 1997).

Quantitative RT-PCR of CYP1A1 Heterogeneous Nuclear RNA. Total cellular RNA was isolated from 1c1c7 cultures by the method of Chirgwin et al. (1979). The RT-PCR primer sets and protocol used for the quantitative RT-PCR amplification and detection of murine CYP1A1 hnRNAs have been described in detail by Elferink and Reiners (1996). In brief, the protocol uses a CYP1A1 recombinant RNA (rcRNA) internal standard identical to the target hnRNA except for the addition of an engineered unique NcoI restriction site. This rcRNA standard contains Cyp1a1 genomic sequences from nucleotides −47 to +913 and includes all of Cyp1a1 exon 1 and 826 bp of intron 1. The same primers sets are used for RT and PCR amplification of cellular CYP1A1 hnRNA and the CYP1A1 rcRNA internal standard. Digestion of the internal standard PCR product with NcoI cleaves it into two fragments (318 and 350 bp) that can be resolved from the 658-bp cellular CYP1A1 hnRNA PCR product on nondenaturing 5% polyacrylamide gels.

Preparation of rcRNA Internal Standards for CYP1A1 and NM01 RT-PCR. Total cellular RNA was isolated from 1c1c7 cells cultured in the presence of 1 nM TCDD for 1 h by the method of Chirgwin et al. (1979). Reverse transcription reactions were primed using gene-specific CYP1A1 and NM01 RT primers (Table 1). The resulting cDNAs were subsequently used in PCR reactions to create...
Cypl1a and Nmo1 cDNA deletion constructs having internal deletions of 134 bp (nucleotides 1661–1794 of CYP1A1 mRNA) and 169 bp (nucleotides 469–637 of NMO1 mRNA), respectively. Specifically, forward PCR/reverse deletion PCR primers and RT/forward deletion primers were used in PCR reactions to create the 5' and 3' arms, respectively, of the deletion cDNA constructs (Table 1). The resulting 5' and 3' arms were annealed and cloned first into a PCRII vector (Invitrogen). The PCR product was removed using BamHI and EcoRV and then directionally cloned into the BamHI and Smal sites of pKSt/Ve70, a plasmid containing a T7 RNA polymerase start site and a polyadenylated tail (Elferink and Reiners, 1996). After selection for ampicillin resistance, plasmids were subsequently sequenced. Linearized plasmids were used in vitro transcription assays as described by Elferink and Reiners (1996) to produce rcRNAs. In vitro-generated rcRNAs were purified by oligo(dT)-Sepharose chromatography, quantitated spectrophotometrically, digested, and stored at −80°C as single-use aliquots.

### Competitive RT-PCR Assay

Total cellular RNA was isolated from treated 1c1c7 cultures by the method of Chirgwin et al. (1979). Reverse transcription assays contained 2 μg of total cellular RNA, and varying numbers of molecules of rcRNA, and were primed with a gene-specific RT primer (Table 1). After reverse transcription, reactions were diluted 1/10.5 in water, and used for PCR. Amplification with the forward and reverse PCR primers listed in Table 1 yielded a 799-bp product for wild-type CYP1A1 mRNA (nucleotides 1325–2129), a 671-bp PCR product for the internal standard CYP1A1 rcRNA, a 799-bp product for wild-type NMO1 mRNA (nucleotides 115–913), and a 630-bp PCR product for the internal standard NMO1 rcRNA. For PCR amplification cDNAs were heated to 95°C for 2 min and cycled 17 to 25 times (for Cyp1a1 cDNAs) or 27 to 35 times (for Nmo1 cDNAs) at 95°C for 30 s, 61°C for 1 min, and 72°C for 2 min. After the final cycle an extension at 72°C for 10 min was performed. PCR products were labeled by the inclusion of [α-32P]dCTP in the synthesis reactions, separated on 3.5% acrylamide gels, and visualized/quantitated with a Bio-Rad GS-525 molecular imager (Bio-Rad, Hercules, CA) and Molecular Dynamics (Sunnyvale, CA) software.

### Orotic Acid Incorporation

Two days after plating 1c1c7 cultures were treated with 0.2 μM Colcemid. Approximately 21 h later nontreated and Colcemid-treated cultures were washed twice with PBS and refed with α-minimal essential medium containing 5% fetal bovine serum and 1 μCi/ml [14C]orotic acid. After a 90-min incubation at 32°C the cultures were processed for estimation of the amount of orotic acid incorporated into RNA. The procedure used for culture fixation and extraction has been described in detail by Schöller et al. (1994). A second set of dishes was treated with trypsin/EDTA to estimate cell numbers. [14C]Orotic acid incorporation is expressed as dpm/10^5 cells.

### Immunofluorescent Detection of AHR and ARNT

The procedures used for immunofluorescent detection of AHR and ARNT in 1c1c7 cultures have been described in detail (Jones and Reiners, 1997). The antibodies used in these studies were rabbit polyclonal, affinity-purified antibodies made to amino acids 61 to 419 of the murine AHR, and amino acids 318 to 773 of the human ARNT (both antibodies were the generous gift of Dr. A. Poland, NIOSH, Morgantown, WV). Fluorescence was observed using a Nikon Optiphot microscope (Nikon, Tokyo, Japan) equipped with an oil immersion lens and recorded on Kodak TMAX 100 film (Eastman Kodak, Rochester, NY).

### Western Blot Analyses

Cytosolic extracts were prepared as described by Pollenz et al. (1994) with the exception that the 2× lysis buffer also contained 2 μg/ml leupeptin and 2 μg/ml aprotinin. Cytosolic and nuclear proteins were subjected to 7.5% dodecyl sulfate-polyacrylamide gel electrophoresis and then electrophoretically transferred from the gel to nitrocellulose. The procedure described by Schöller et al. (1994), with the substitution of a 2-h incubation with primary antibody, was used for the immunological detection of AHR and ARNT. The primary antibodies used in these studies are described above. Antigen-immunoglobulin conjugates were detected with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ) and recorded on X-ray film.

### Statistical Analyses

Data were analyzed by the Tukey’s Honestly Significant Difference test. The Statistica 5.0 software package (StatSoft, Tulsa, OK) was used to perform these calculations. Differences were considered statistically significant if P < 0.05.

### Results

**Induction of G2/M Arrest by Microtubule Disrupters and Stabilizers.** 1c1c7 cultures were treated with varying concentrations of microtubule disrupters/stabilizers to identify conditions sufficient to cause a G2/M arrest in the absence of cytotoxicity. The panels in the top two rows of Fig. 1 demonstrate that concentrations of Colcemid, vinblastine, and Taxol existed that were capable of suppressing 1c1c7 proliferation in the absence of overt cytotoxicity. The doubling time of 1c1c7 cells grown at 32°C is ~23 h. Because the cultures were harvested 20 to 23 h after the addition of...
mitotic blocker the 50% value for control cell content noted in the first row of panels represents a total suppression of proliferation.

Most concentrations of estramustine capable of suppressing 1c1c7 proliferation were also cytotoxic (Fig. 1, second column of first and second rows). Optimal suppression of proliferation with only modest cytotoxicity could be achieved with 20 μM estramustine. This was the highest concentration of estramustine used in subsequent studies.

Cytostatic concentrations of mitotic blockers gave rise to cells having either a “flat” or “rounded” morphology (Fig. 1, third row). FACs analyses of the DNA contents of these cells indicated that they were tetraploid (Fig. 1, sixth row). Cdc2/cyclin B activities were highly elevated in these cells, suggesting that they were G2/M cells (J. J. Reiners, unpublished data). This accumulation of G2/M cells was accompanied by coordinate losses of G0/G1 cells (Fig. 1, fifth row). Thus, treatment with any one of the four mitotic blockers for ≥20 h was sufficient to generate 1c1c7 cultures containing >95% G2/M cells.

G2 and M phase cells cannot be distinguished on the basis of their DNA contents. To determine whether Colcemid-derived flat and round tetraploid cells represented enriched G2 or M populations their chromosomes were stained with aceto-orcein. After 20 h of 0.2 μM Colcemid exposure 66, 9, and 25% of the flat cells were in G2, prophase, and premetaphase/metaphase, respectively. Hence, Colcemid-treated cells with the flat morphology represented primarily G2 cells. Conversely, cells having the rounded morphology represented primarily mitotic cells.

**Suppression of Cyp1a1 and Nmo1 Induction in G2/M Arrested Cultures.** Treatment of 1c1c7 cultures with mitotic blockers for ~20 h before the addition of TCDD resulted in concentration-dependent reductions in induced, steady-state CYP1A1 mRNA contents (Fig. 2). In general, reductions were seen at concentrations sufficient to induce G2/M arrest (compare Figs. 2 and 1, bottom row). It should be emphasized that the effects of Colcemid and Taxol on CYP1A1 mRNA contents were only seen when the cells were allowed sufficient time to arrest in G2/M before the addition of TCDD. In Fig. 2, TCDD-induced steady-state CYP1A1 mRNA contents in G2/M arrested 1c1c7 cultures. Cultures were pretreated with DMSO or varied concentrations of the microtubule disrupters/stabilizers for 20 to 21 h prior to the addition of TCDD (1 nM). Total RNA was isolated 5 h after the addition of inducer or solvent from cells having normal, flat (F), or rounded (R) morphology for analyses of CYP1A1 or 7S RNAs.
Specifically, analyses of CYP1A1 mRNA contents in cultures treated with TCDD at various times after the addition of 0.2 μM Colcemid demonstrated an inverse relationship between the percentage of cells in G2/M and induced, steady-state CYP1A1 mRNA content (J. J. Reiners, unpublished data).

The experiments reported in Fig. 2 were repeated minimally twice. In general, CYP1A1 mRNA contents in TCDD-treated cultures arrested in G2/M by treatment with Colcemid, estramustine, and vinblastine were 5 to 40% of the values determined in solvent + TCDD-treated cultures. This range reflected a continued decrease in steady-state CYP1A1 mRNA contents as the concentrations of microtubule disrupters were increased above the minimum concentration necessary to achieve G2/M arrest. For example, although both 0.2 and 10 μM Colcemid induced maximum G2/M arrest, steady-state CYP1A1 mRNA contents at 10 μM were ~50% of what were seen at 0.2 μM (Fig. 2). This should not have occurred if reductions in steady-state CYP1A1 mRNA contents only reflected a loss of responsiveness of G2/M cells. Presumably, the higher concentrations of microtubule disrupters have additional effects on CYP1A1 induction that are cell cycle-independent. In subsequent studies we used the minimum concentration of Colcemid (0.2 μM) sufficient to achieve maximum accumulation of G2/M cells.

Vinblastine exhibited suboptimal cytostatic activity at 1 nM (Fig. 1) but strongly suppressed the induction of Cyp1a1 in the attached cell population (Fig. 2). The attached cells were not arrested in G2/M. Rather, their distribution in the phases of the cell cycle mirrored that of cultures treated with noncytostatic concentrations of vinblastine. Hence, vinblastine could suppress Cyp1a1 induction by a mechanism that was independent of the cells being in G2/M.

Taxol was not as effective as the microtubule disrupters in suppressing CYP1A1 mRNA accumulation. A concentration of Taxol (1 μM) sufficient to arrest most 1c1c7 cells in G2/M suppressed CYP1A1 mRNA accumulation by 46 ± 5% (n = 5 experiments). This value did not change if the concentration was raised to 10 μM (two additional studies; J. J. Reiners, unpublished data).

The Nmo1 gene, like Cyp1a1, is a member of the Ah battery and is transcriptionally activated by TCDD through an AHR-dependent mechanism (Nebert, 1994). As a complement to the study reported in Fig. 2 we also used quantitative RT-PCR assays to measure CYP1A1 and NMO1 mRNAs. These assays use engineered, truncated CYP1A1 and NMO1 rcRNAs as internal standards. Analyses of the abilities of varied amounts of these rcRNAs to compete with a fixed amount of cellular RNA after reverse transcription and PCR facilitate direct comparisons of cellular mRNA contents in control and Colcemid-treated cultures. An example of the data generated in such assays is depicted in Fig. 3A. A summary of three separate experiments is shown in Fig. 3, B and C. Exposure of asynchronous 1c1c7 cultures to TCDD for 5 h elevated CYP1A1 mRNA contents ~9-fold (Fig. 3B). In contrast, CYP1A1 mRNA contents were elevated only ~4-fold in similarly treated, nonfractionated Colcemid-arrested cultures. This Colcemid-dependent effect reflected reductions in both the flat and round populations (Fig. 3B). Although the CYP1A1 mRNA contents of TCDD-treated round cells were consistently less than their flat counterparts, the differences were not statistically significant.

NMO1 mRNA content was elevated ~3-fold in asynchr-
portions of the assay an rcRNA internal standard. This internal standard is identical to the target hnRNA except for the addition of an engineered unique NcoI restriction site. Digestion of the PCR products with NcoI cleaves the internal standard PCR product into two fragments (318 and 350 bp) that can be resolved from the cellular CYP1A1 hnRNA PCR product (658 bp).

CYP1A1 hnRNA was detectable in solvent-treated, asynchronous 1c1c7 cultures (Fig. 4). Within 1 h of TCDD treatment CYP1A1 hnRNA contents were elevated ~8-fold. In contrast, CYP1A1 hnRNA contents were elevated ~2.5-fold in Colcemid-arrested, TCDD-treated cultures (Fig. 4). Analyses of Colcemid-derived flat and round cells confirmed that the transcriptional activation of Cyp1a1 was suppressed in both populations.

Effects of Colcemid on Ligand-Induced AHR Translocation. Transcriptional activation of Cyp1a1 or Nmo1 by TCDD involves its binding to the AHR and the subsequent translocation of the AHR to the nucleus. These two steps can be qualitatively assessed by indirect fluorescent monitoring of AHR translocation after exposure to TCDD (Pollenz et al., 1994; Reiners et al., 1997). The panels in the top and bottom rows of Fig. 5 demonstrate that both ARNT and AHR, respectively, are expressed in asynchronous and Colcemid-arrested 1c1c7 cultures. ARNT was nuclear in both asynchronous and Colcemid-arrested cultures (Fig. 5, A and C, respectively), and its location was not affected by TCDD treatment (Fig. 5, B and D). In contrast, the AHR was predominately cytoplasmic in asynchronous and Colcemid-arrested cultures (Fig. 5, E and G, respectively) but translocated to the nucleus after the addition of TCDD (Fig. 5, F and H). It should be noted that our interpretations are restricted to those Colcemid-treated cells having the flat phenotype (and primarily in G2). The rounded cells (examples are highlighted by arrows) represent M phase cells and lack a nuclear envelope.

Effects of Colcemid on Orotic Acid Incorporation. It is conceivable that the reduced CYP1A1 and NMO1 mRNA contents seen in TCDD-treated Colcemid-arrested 1c1c7 cultures reflect a general suppression of transcription in G2/M phase cells. To test this possibility, control and Colcemid-arrested cultures were pulsed with the RNA precursor [14C]orotic acid and monitored for incorporation into acid-insoluble material. Figure 6 demonstrates that Colcemid-treated cultures incorporated ~50% of the amount measured in solvent-treated control cultures. However, Colcemid-derived flat and round cells differed markedly in their abilities to incorporate [14C]orotic acid. Specifically, the round cells showed very poor [14C]orotic acid incorporation, whereas, the flat G2 phase cells incorporated more [14C]orotic acid than asynchronous control cultures.

Responsiveness to TCDD after Release from G2/M Arrest. The replating of asynchronous control cultures did not affect the relative distribution of cells in the various phases of the cell cycle (Fig. 7A, left). Colcemid-arrested 1c1c7 cultures resumed progression through the cell cycle within 12 h of replating (Fig. 7A, right). This reentry was accompanied by the loss of cyclin B-dependent kinase activity, a marker for G2/M phase cells (monitored by assessing histone H1 phosphorylation in an in situ kinase assay; Fig. 7B). The replating of Colcemid-arrested cultures did not result in tightly synchronized cultures. However, relative to replated asynchronous cultures, Colcemid-derived cultures were enriched in S phase cells 24 h after replating (compare panels in Fig. 7A).

Colcemid-arrested 1c1c7 cultures acquired increased responsiveness to TCDD after being replated (Fig. 7C). Treatment with TCDD 12 or 24 h after replating resulted in steady-state CYP1A1 mRNA contents markedly greater than that detected in G2/M cultures and comparable with that of replated TCDD-treated asynchronous cultures (Fig. 7C). It also appeared that replated Colcemid-arrested cultures had higher steady-state CYP1A1 mRNA contents when treated with TCDD 12 h, as opposed to 24 h, after replating. Two additional studies confirmed that 12-h cultures consistently had greater steady-state CYP1A1 mRNA contents than 24-h S phase-enriched cultures after exposure to TCDD. However, the differences were never greater than 30% and were not statistically significant (J. J. Reiners, unpublished data).

The differential responsiveness of asynchronous and Colcemid-arrested G2/M cultures to TCDD did not reflect marked differences in AHR or ARNT. Cultures arrested in G2/M expressed both proteins in amounts comparable with that detected in asynchronous cultures (Fig. 7B, zero time).

TCDD Induction of CYP1A1 in U937 Cells. As a complement to the chemical synchronization studies, we attempted to use centrifugal elution to enrich for phase-specific populations of 1c1c7 cells. However, we were unable to reproducibly obtain fractions highly enriched in distinct quantities by centrifugal elution.
phases of the cell cycle. For that reason we turned our attention to U937 cells, a monocytoid cell line that expresses the AHR (Hayashi et al., 1995), which can be separated into phase-specific populations by centrifugal elutriation (McCabe et al., 2000). CYP1A1 mRNA was not detected in non-treated or DMSO-treated asynchronous U937 cultures (Fig. 8, first three lanes and last lane). In contrast, CYP1A1 mRNA was detected by Northern blot analysis within 3 h of treatment of cultures with TCDD (Fig. 8). Thereafter, steady-state CYP1A1 mRNA contents continued to increase for minimally an additional 15 h. The lowest and highest concentrations of TCDD tested (2 and 10 nM) were very similar in their abilities to stimulate the accumulation of CYP1A1 mRNA at each of the time points investigated. The 2 nM concentration of TCDD was used in subsequent studies.

Centrifugal Elutriation of U937 Cells. TCDD is cytostatic to some cell lines (Vogel and Abel, 1995; Weiss et al.,...
Exposure to 2 nM TCDD did not affect the proliferation of U937 cells (Fig. 9A). Figure 9B demonstrates that centrifugal elutriation can be used to prepare enriched populations of U937 cells in all phases of the cell cycle after short-term exposure to TCDD. Specifically, highly enriched preparations of G₁ phase (elutriation fractions 30 and 35), S phase (elutriation fraction 40), and G₂/M phase cells (elutriation fractions 50 and 55 + 60) could be reproducibly isolated. In general, the sum of the cells in the various elutriation fractions equaled 95 ± 3% of the initial, preseparated population (R. P. Santini and J. J. Reiners, unpublished data). Furthermore, the sum of the cells in phase-specific fractions, when normalized to the total number of cells recovered after elutriation, yielded cell cycle distributions identical to that measured in the starting population. For example, elutriation fractions 30 and 35 collectively represented ~55% of the elutriated population and were almost exclusively G₁ cells (Fig. 9, B and C). The pre-elutriation population was ~50% G₁ cells. Similarly, elutriation fractions 50 + 55 + 60 represented ~9% of the total elutriated population and were ~70% G₂/M cells. The starting pre-elutriation population was ~10% G₂/M cells.

**CYP1A1 mRNA Contents in TCDD-Treated and Elutriated U937 Cells.** We reasoned that analyses of elutriated cells harvested within 3 h of TCDD treatment could provide a relative estimate of the cell cycle responsiveness of CYP1A1 to TCDD. CYP1A1 mRNAs were detected in all elutriation fractions prepared from cultures harvested 3 h after TCDD treatment (Fig. 10A). However, there were marked differences among the fractions in their CYP1A1 mRNA contents. When normalized to 7S RNA contents, the highest CYP1A1 cellular content was noted in elutriation fraction 35. The lowest contents were noted in elutriation fraction 50 (Fig. 10B, top, and three additional experiments). The maximum difference between elutriation fractions in Fig. 10 was ~3-fold. In a single experiment we obtained sufficient cells in elutriation fractions 55 + 60 to isolate amounts of mRNA sufficient for Northern blot analyses. The CYP1A1 mRNA content in this pooled fraction was ~52% of the CYP1A1 mRNA content of nonseparated TCDD-treated cultures (R. P. Santini and J. J. Reiners, unpublished data).

**Discussion**

The current investigation was designed to determine whether the TCDD-mediated induction of CYP1A1 is a cell cycle-regulated process, and specifically suppressed in G₂/M. As an approach we used four chemicals to arrest 1c₁c7 cul-

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**Fig. 8.** TCDD-mediated CYP1A1 induction in U937 cells. Log-phase cultures of U937 cells were treated for 3, 6, or 18 h with 2, 5, or 10 nM TCDD before being harvested for isolation of RNA and subsequent analyses of CYP1A1 and 7S RNAs. Parallel cultures were treated with DMSO and harvested 3, 6, and 18 h after treatment. Analyses were performed on 10 µg of RNA.

**Fig. 9.** Effects of TCDD on U937 cell cycle progression. Asynchronous cultures of U937 cells were treated with 2 nM TCDD or DMSO. A, cultures were harvested at various times after the addition of TCDD (●) or DMSO (○) for estimation of cell numbers. Data represent means ± S.D. of three culture dishes. B, cultures were harvested ~3 h after DMSO or TCDD treatment for analyses of cell cycle stage by flow cytometry (first two columns). The remaining columns in B represent flow cytometry analyses of elutriated fractions of cells harvested ~3 h after TCDD treatment. C, percentage of the total elutriated population in individual elutriation fractions. The data in B and C represent means ± S.D. of three experiments.
concentrations that caused G2/M arrest. However, CYP1A1 suppressed the TCDD-dependent activation of CYP1A1 at optimal G2/M arrest. All of the microtubule disrupters also the minimum concentration of Taxol required to achieve op- timal arrest. TCDD-treated asynchronous starting population. This value was similar to that measured in 1c1c7 cultures arrested with TCDD-treated asynchronous cultures. G2/M cells prepared by this later procedure were used to separate TCDD-treated asynchronous cultures of U937 cells into highly enriched populations of G1, S, and G2/M phase cells. Analyses of the separated cell types demonstrated that the TCDD-dependent induction of Cyp1a1, as well as Nmo1, was suppressed in both cell types. The Nmo1 data are significant because this gene is also a member of the Ah battery, and transcriptionally activated by TCDD via an AHR-dependent process (Nebert, 1994). To date, we know of only one other example in which the activities of a ligand-activated transcription factor are down-regulated in G2 phase cells. Specifically, several glucocorticoid-responsive genes cannot be induced in the G2 phase of the cell cycle (Martin et al., 1969; Fanger et al., 1986; Hsu et al., 1992). This suppression has been shown to correlate with an inhibition of glucocorticoid receptor nuclear accumulation, and site-specific alterations in glucocorticoid receptor phosphorylation (Hsu et al., 1992; Bodwell et al., 1998).

Fig. 10. CYP1A1 mRNA content of TCDD-treated U937 cells separated by elutriation. A, U937 cells were treated with 2 nM TCDD or DMSO for 3 h before being harvested for subsequent isolation of RNAs for analyses of CYP1A1 and 7S RNAs. RNA was also isolated from the elutriated fractions of TCDD-treated cultures. Northern blots in A represent signals obtained on 10 μg of RNA. B, intensities of CYP1A1 and 7S mRNA signals depicted in A, and three additional independent experiments were measured to establish signal strength ratios. The ratio for nonsepa- rated, TCDD-treated cells was set as 1. Data represent means ± S.D. of four experiments. * significantly greater than all other groups, P < 0.01; ** significantly different from all other groups except elutriation fraction 45, P < 0.05.

A

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B

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<td>Elutriation Fraction</td>
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It has been known for some 40 years that transcription is suppressed in mitotic cells (Taylor, 1960; Prescott and Bender, 1962; Hartl et al., 1993). Analyses of uracil acid incorporation into RNA demonstrated that overall RNA synthesis was strongly suppressed in mitotic 1c1c7 cultures generated by Colcemid exposure. Hence, it is conceivable that the suppressed responses we detected in M phase cultures may reflect a general suppression of transcription and are AHR-independent. However, the same explanation may not account for the suppression seen in the Colcemid-derived G2 population. Orotic acid incorporation in these latter cells was comparable with, if not greater than that measured in asyn- chronic 1c1c7 cultures. Although the identities of the RNAs synthesized in G2 phase cells are not known, we assume that cyclin B and p21Cip1 mRNAs are in this population because their basal transcription is cell cycle-dependent and occurs in G2 (Li et al., 1994; Dulic et al., 1998).

The transcriptional activation of Cyp1a1 and Nmo1 by TCDD is mediated by the AHR, in conjunction with ARNT. The suppressed inductions of both genes in G2/M 1c1c7 cultures did not correlate with reductions in either AHR or ARNT, as monitored by Western blot and indirect immuno- fluorescence analyses. It is also unlikely that ligand binding or AHR transformation is affected in G2 cells. This conclusion is based upon the observed translocation of the AHR to the nucleus after TCDD exposure. AHR translocation requires both ligand binding and receptor transformation. In a previous study we demonstrated by EMSA that AHR/ARNT bind- ing to a radiolabeled oligonucleotide containing a dioxin- responsive element was markedly suppressed in nuclear

patterns in G2/M by two different mechanisms (i.e., microtubule disruption or stabilization). As a complement to these studies we also used a model system that did not use chemical synchronization. Specifically, centrifugal elutriation was used to separate TCDD-treated asynchronous cultures of U937 cells into highly enriched populations of G1, S, and G2/M phase cells. G2/M cells prepared by this later procedure contained 52 ± 6% of the CYP1A1 mRNA content of the TCDD-treated asynchronous starting population. This value was similar to that measured in 1c1c7 cultures arrested with the minimum concentration of Taxol required to achieve optimal G2/M arrest. All of the microtubule disrupters also suppressed the TCDD-dependent activation of CYP1A1 at concentrations that caused G2/M arrest. However, CYP1A1 mRNA contents continued to decrease with increasing concentrations of Colcemid, estramustine, and vinblastine. This continued decrease should not have occurred if the effects of the three microtubule disrupters only reflected their abilities to cause G2/M arrest. Furthermore, a concentration of vin- blastine was identified that suppressed the TCDD-induced accumulation of CYP1A1 mRNA in non-G2/M phase cells. These studies emphasize both the caveats associated with chemical synchronization and the need for complementary procedures in the study of cell cycle-regulated processes. Nevertheless, both approaches demonstrated that G2/M phase cells have lower steady-state CYP1A1 mRNA contents after TCDD treatment than similarly treated asynchronous cultures.

Treatment of 1c1c7 cultures with microtubule disrupters and stabilizers lead to a tetraploid population having either a flat or rounded morphology. Staining with aceto-orcein revealed that the flat and rounded cells represented predominantly G2 and mitotic cells, respectively. Analyses of the separated cell types demonstrated that the TCDD-dependent induction of Cyp1a1, as well as Nmo1, was suppressed in both cell types. The Nmo1 data are significant because this gene is also a member of the Ah battery, and transcriptionally activated by TCDD via an AHR-dependent process (Nebert, 1994). To date, we know of only one other example in which the activities of a ligand-activated transcription factor are down-regulated in G2 phase cells. Specifically, several glucocorticoid-responsive genes cannot be induced in the G2 phase of the cell cycle (Martin et al., 1969; Fanger et al., 1986; Hsu et al., 1992). This suppression has been shown to correlate with an inhibition of glucocorticoid receptor nuclear accumulation, and site-specific alterations in glucocorticoid receptor phosphorylation (Hsu et al., 1992; Bodwell et al., 1998).

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experiments obtained from nocodazole-derived G2/M cultures (Schöller et al., 1994). However, recent studies suggest that the procedure used for the isolation of nuclei in these studies also enriched for particles that looked like nuclei, contained DNA, but lacked a true nuclear envelope and had reduced levels of ARNT (J. J. Reiners, unpublished data). It is likely that the EMSA assay used in these studies underestimated AHR/DNA binding. Hence, it is conceivable that the $G_2$ effects seen in the current study could reflect suppressions of AHR/ARNT dimerization, DNA binding, or the transactivating activity of DNA bound AHR/ARNT complex.

Analyses of elutriated U937 cells and replated Coelimid-arrested 1c1c7 cultures demonstrate that TCDD is capable of inducing CYP1A1 throughout the cell cycle. However, the elutriation studies also show that cells in different phases of the cell cycle do not respond identically to TCDD. Specifically, relative to the CYP1A1/7S mRNA ratio determined for TCDD-treated asynchronous cultures (set as 1), ratios of $1.41 \pm 0.09$ (range $1.31-1.68$) and $0.52 \pm 0.06$ (range $0.46-0.63$) occurred in elutriation fractions 35 and 50, respectively. If one accepts that the elutriation fractions depicted in Fig. 9B represent from left to right the progression of cells from $G_1$ to M, our data demonstrate that late G1 cells accumulate ~3-fold more CYP1A1 mRNA than do G2/M cells. It is intriguing that the extremes in the CYP1A1/7S ratios correspond approximately to the sites of the two major checkpoints of the cell cycle. It is conceivable that agents and conditions that lead to $G_1$ and $G_2$ arrest may influence the biological activities of TCDD and other AHR ligands.

The fluctuating responsiveness of CYP1A1 to TCDD accompanying cell cycle progression may reflect pRB coactivation of the AHR. Recent studies with rat hepatoma 5L/BP8 cells demonstrate that the AHR interacts directly with pRB, but only the hypophosphorylated form (Ge and Elferink, 1998; Elferink et al., 2001). This interaction appears necessary for maximal AHR-mediated CYP1A1 expression, consistent with pRB acting as an AHR coactivator (Elferink et al., 2001). Because hypophosphorylated pRB is restricted to the $G_0$ and $G_1$ phases, AHR-mediated transactivation of the CYP1A1 gene would be predicted to be cell cycle-dependent, with maximal induction detected in $G_0$ and $G_1$ phase cells. Our current data are entirely consistent with this prediction.

TCDD is cytostatic to a variety of cell types. It suppresses the proliferation of several human breast cell lines, including MCF-7, T47D, and MDA-MD-486 (Fanger et al., 1986; Forsythe et al., 1995). Because hypophosphorylated pRB is restricted to the $G_0$ and $G_1$ phases, AHR-mediated transactivation of the CYP1A1 gene would be predicted to be cell cycle-dependent, with maximal induction detected in $G_0$ and $G_1$ phase cells. Our current data are entirely consistent with this prediction.

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In summary, these are the first studies that we know of to demonstrate that a microtubule stabilizer and a series of microtubule disrupters suppress the activation of members of the Ah battery by an AHR ligand. These are the first studies that we know of to suggest that responsiveness to TCDD may differ at the sites of the two major checkpoints of the cell cycle.

Acknowledgments

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References


Jones CL and Reiners JJ Jr (1997) Differentiation status of cultured murine kera-


Address correspondence to: John J. Reiners, Jr., Institute of Environmental Health Sciences, Wayne State University, 2727 Second Ave., Rm 4000, Detroit, MI 48201. E-mail: john.reiners.jr@wayne.edu