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Regulatory Factors Involved in Species-specific Modulation of Arylhydrocarbon Receptor (AhR)-dependent Gene Expression in Humans and Mice

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The arylhydrocarbon receptor (AhR) mediates toxicities of dioxins, including the most potent congener 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), by being translocated to the nucleus upon ligand-binding and inducing expression of target genes. Although the species-specific activity of the AhR is primarily attributable to species-specific AhR-ligand affinity, the precise mechanism has not been clarified. We investigated the modulation mechanisms of AhR in Hepa1c1c7 and HepG2 hepatoma cells, which were derived from high-affinity-AhR-expressing C57BL/6 mice and low-affinity-AhR-expressing humans, respectively. Although, consistent with their AhR affinities, TCDD induced a greater amount of cytochrome P450 1A1 (CYP1A1) mRNA, one of the most sensitive AhR-targets, in Hepa1c1c7 cells than in HepG2 cells immediately after exposure, both cells expressed a similar level of CYP1A1 mRNA from 4 h onward. A rapid decrease in the AhR protein after nuclear translocation in Hepa1c1c7 cells was suggested to contribute to suppression of *CYP1A1* induction to the same level as in HepG2 cells. Different profiles of histone deacetylase 1 (HDAC1)-binding to the *CYP1A1* promoter and histone acetylation between both cell lines and lower degradation rate of CYP1A1 mRNA in HepG2 cells were also implicated in regulating their target gene expression. These factors have been highly suggested to be involved in the species-specific modulation mechanism of AhR function.

Key words: arylhydrocarbon receptor (AhR), CYP1A1, Hepa1c1c7, HepG2, TCDD.

Abbreviations: AhR, arylhydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CYP1A1, cytochrome P4501A1; HDAC, histone deacetylase; Hsp90, heat shock protein 90; XAP2, hepatitis B virus X-associated protein; NLS, nuclear localization signal; ARNT, arylhydrocarbon receptor nuclear translocator; XRE, xenobiotic responsive element; TSA, trichostatin A; polII, RNA polymerase II; HAT, histone acetyltransferase; CBP, CREB binding protein; N-CoR, nuclear receptor corepressor.

The arylhydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to the bHLH-PAS superfamily and mediates the toxicity of dioxins, including the most potent congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (*1*, *2*). In the absence of ligands, the AhR is present in the cytosol in association with a complex of Hsp90, cochaperone p23, and the immunophilin-like protein XAP2 (*3–5*). Ligand binding to the AhR triggers a conformational change that exposes the nuclear localization signal (NLS) domain and causes nuclear translocation of the AhR complex (*6*, *7*). In the nucleus, the AhR dimerizes with another transcription factor, the aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR/ARNT heterodimer binds to DNA sequences called xenobiotic responsive elements (XREs) that are distributed in the enhancer regions of the target genes and induces or modulates the target gene expression. Previous studies using ligation-mediated PCR (*8*, *9*) and recent studies by chromatin immunoprecipitation (ChIP) assay (*10–13*) have indicated the involvement of histone acetylation and remodelling of chromatin structure in the course of AhR-dependent gene expression. The nuclear translocated AhR is reported to be subsequently degraded via the ubiquitin/proteasome pathway (*14–17*), which would attenuate the AhR/ARNT activation reaction (*17*).

While TCDD toxicity is thought to be mediated by the AhR-dependent reactions described earlier in mammalians in common, susceptibility to TCDD toxicity varies greatly among animal species and strains (*1*, *18*, *19*). The major cause of the species- and strain-specific differences in susceptibility has primarily been attributed to differences in the affinity of the AhR for the ligand. Mice of the sensitive strain C57BL/6 express AhRs with high affinity for ligands, and mice of the low-responder strain DBA/2 express low-affinity AhRs. The dissociation constant (K_d) for TCDD of the AhR in these strains has been reported to be 0.27 and 1.66 nM, respectively (*20*), and the intensity of the AhR affinity in these two strains correlates well with the intensity of expression of

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AhR-target genes, such as *CYP1A1* (*21*). Human AhR structurally resembles DBA/2 AhR more closely than it resembles C57BL/6 AhR (*20*, *22*), and it has an affinity for TCDD (K_d, 1.58 nM) similar to that of DBA/2 AhR (*20*). On the other hand, our recent study revealed that TCDD exposure results in stronger CYP1A1 mRNA induction in human blood lymphocytes than in C57BL/6 blood lymphocytes (*23*), suggesting the existence of critical factors besides AhR affinity that modulate the expression of the target genes by AhR activation.

Various factors involved in the AhR pathway, including nuclear translocation, initiation of transcription through chromatin remodelling and degradation as described earlier, could be responsible for the species-specific characteristics of AhR function. Precise studies by Perdew and colleagues have reported distinctive differences primarily in the properties of unliganded human and mouse AhR, such as cellular localization, nuclear translocation, nucleocytoplasmic shuttling, and effects of XAP2 on these processes (*5*, *24*). However, contribution of the differences in these properties to the species-specific gene expression by the AhR in human and mouse cells has not been addressed. It has been also undetermined how significantly other steps in the AhR pathway, such as chromatin remodelling and AhR degradation processes, contribute to species-specific characteristics of AhR-dependent gene expression.

In the present study we investigated the AhR activation pathway upon TCDD treatment in Hepa1c1c7 cells, established from C57BL/6 mice, and HepG2 cells, established from humans, in order to explore the factors involved in the species-specific regulation of AhR-dependent gene expression. We compared time-course profiles of *CYP1A1* induction by real-time PCR, AhR localization by Western blotting, and recruitment of AhR and other factors involved in transcription to the *CYP1A1* promoter region by ChIP assay, and also examined the stability of CYP1A1 mRNA in the two representative human and mouse hepatoma cell lines. The results in the present study indicated characteristic differences in AhR accumulation pattern in the nucleus after TCDD-binding, recruitment profile of factors involved in chromatin remodelling, and target gene mRNA stability in Hepa1c1c7 cells and HepG2 cells. These steps were suggested for the first time to be involved in species-specific modulation of AhR function.

MATERIALS AND METHODS

Reagents and cells—TCDD (purity 98%) was purchased from Cambridge Isotope Laboratory. Trichostatin A (TSA) and Actinomycin D were purchased from Wako and Sigma, respectively. Hepa1c1c7 cells were kindly provided by Dr Y. Fujii-Kuriyama (University of Tsukuba), and HepG2 cells were provided by Cell Resource Center for Biomedical Research (Tohoku University). Both cell lines were maintained at 37°C in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% FBS (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin under a humidified atmosphere composed of 95% air and 5% CO_2.

Real-time PCR—Total RNA was isolated from the cells with an RNeasy Mini kit (QIAGEN). After checking the quality of the RNA by electrophoresis, reverse transcription was performed with an RNA LA PCR kit (AMV) ver3.0 (TaKaRa Bio) using 100 ng of total RNA as described previously (*25*). Quantitative real-time PCR analysis was performed on a LightCycler instrument (Roche Diagnostics) as described previously (*23*). The primer sequences and annealing temperatures for each gene are shown in Table 1. The final concentration of $MgCl_2$ in the PCR reaction mixture was adjusted to 4.5 mM for CYP1A1, 3.5 mM for the mouse *CYP1A1* promoter region and 2.5 mM for the human *CYP1A1* promoter region. The following LightCycler run protocol was used: denaturation program (95°C for 10 min), amplification and quantification program repeated 50 times (95°C for 10 s, 63°C for CYP1A1, 62°C for mouse *CYP1A1* promoter or 66°C for human *CYP1A1* promoter for 10 s and 72°C for 10 s for CYP1A1, for 15 s for mouse *CYP1A1* promoter or 5 s for human *CYP1A1* promoter with a single fluorescence measurement), melting curve program (68–99°C for CYP1A1, 67–99°C for mouse *CYP1A1* promoter or 71–99°C for human *CYP1A1* promoter with a heating rate of 0.1°C/s and continuous fluorescence measurement) and finally a cooling step to 40°C. The control cDNA was prepared from the pooled livers of C57BL mice that had been given 3 μg/kg of TCDD orally and dissected 24 h later. The control genome DNA was prepared from Hepa1c1c7 cells or HepG2 cells with a DNeasy tissue kit (QIAGEN). The relative mRNA level per cell was determined by dividing the amount of amplicons obtained by real-time PCR of 100 ng total RNA by the cell number that represents 100 ng total RNA.

Table 1. **List of primers used for real-time PCR**

Description	Primer sequence	Product size (bp)
CYP1A1	5′-CCTCTTTGGAGCTGGGTTTG-3′ 5′-GCTGTGGGGGATGGTGAA-3′	229
CYP1A1 promoter region of mice (−285 to +66 bp)	5′-TTTCCTCAAACCCCTCCCTC-3′ 5′-GAAGTGAAGAGTGTTCTCTAGGAC-3′	351
CYP1A1 promoter region of humans (−39 to +48 bp)	5′-ACGTACAAGCCCGCCTATAA-3′ 5′-TCACAAGGATCAGGGAAGGT-3′	87

Western blotting—Nuclear and cytoplasmic fractions were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent (PIERCE). Western blotting was carried out as described previously (*26*) with a minor modification. Briefly, cells were lyzed in 0.5% Triton X-100 lysis buffer, and the supernatant was collected after centrifugation. The protein concentrations in the supernatant were determined with a BCA protein assay kit (PIERCE). After boiling in SDS sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 0.001% bromphenol blue), the samples were subjected to SDS–PAGE and then transferred to

PVDF membranes (Hybond-P, Amersham Biosciences). The membranes were blocked, allowed to react with anti-AhR (SA-210, BIOMOL), anti-actin (sc-1615, Santa Cruz) or anti-p300 (05-257, Upstate) for 1 h at room temperature, washed, and then allowed to react with horseradish peroxidase-conjugated second antibody (anti-rabbit IgG, anti-goat IgG, or anti-mouse IgG1) for 1 h at room temperature. After washing, the membranes were developed using the ECL Plus Western Blotting Detection System (Amersham Biosciences).

Chromatin immunoprecipitation (ChIP) assay—ChIP assay was carried out by the method of Shibazaki *et al*. (*12*) with a minor modification. Briefly, Hepa1c1c7 cells and HepG2 cells were seeded in 10 cm dishes and cultured in the presence of TCDD. The cells were washed twice with phosphate-buffered saline (−) (PBS), cross-linked with 1% formaldehyde at room temperature for 10 min, and then collected with a cell scraper. They were rinsed twice with ice-cold PBS, suspended in 200 μl lysis buffer and incubated on ice for 10 min. The cells were then sonicated 10 times, 30 s each time, with a Bioruptor UCD-200TM (Cosmo Bio) to give a final DNA size range of 200 to 600 bp. After precleaning the supernatant with protein A-agarose, immunoprecipitation was carried out by incubating with 1 μg of anti-AhR (SA-210, BIOMOL, for Hepa1c1c7, sc-5579, Santa Cruz, for HepG2), 2 μg of anti-RNA polymerase II (05-623, Upstate), 5 μg of anti-acetylated histone H4 (06-866, Upstate), 2.5 μg of anti-CBP (sc-369, Santa Cruz) or 4 μg of anti-HDAC1 antibody (06-720, Upstate) for 16 h at 4°C, and them incubating with protein A-agarose. The recovered protein–DNA complexes were extracted with elution buffer from the protein A-agarose beads, incubated at 65°C for 16 h to reverse the formaldehyde cross-linking, and then treated with proteinase K at 45°C for 1 h. DNA fragments were purified by phenol-chloroform extraction, precipitated and suspended in 10 μl of TE buffer. A 1 μl sample was subjected to real-time PCR. The PCR primers used to detect the *CYP1A1* promoter regions in Hepa1c1c7 cells and HepG2 cells were designed by using PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primer sequences and annealing temperatures are shown in Table 1.

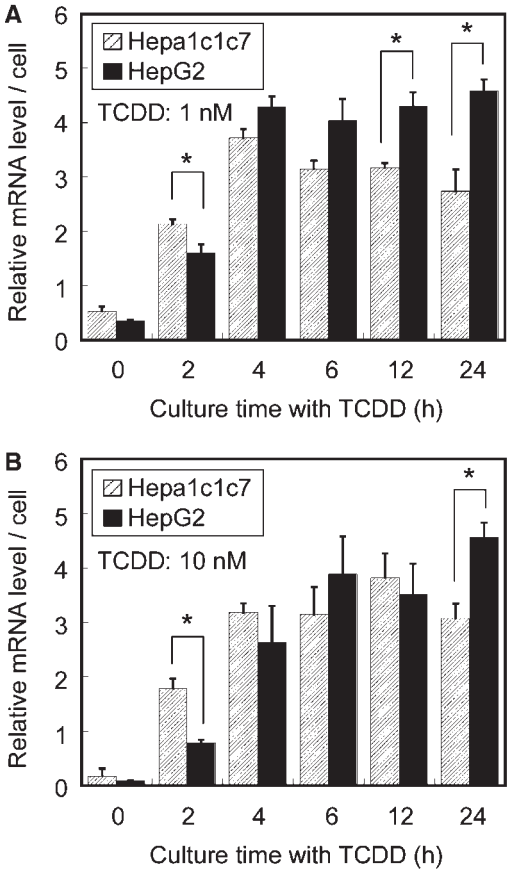

Fig. 1. **Comparison between the time-course of *CYP1A1* induction by TCDD in Hepa1c1c7 cells and HepG2 cells.** Total RNA was prepared from Hepa1c1c7 cells and HepG2 cells cultured in the presence of 1 nM TCDD (A) or 10 nM (B) TCDD for the times indicated and was reverse-transcribed. CYP1A1 mRNA was quantified by real-time PCR as described in 'Materials and methods' section. Total RNA from the liver of a C57BL/6 mouse exposed to TCDD (3 μg/kg) was used as a standard. The data are expressed as means ± SE. *Statistically significant difference between Hepa1c1c7 and HepG2 at $P < 0.05$.

RESULTS

Characteristics of the time course of CYP1A1 induction by TCDD in Hepa1c1c7 cells and HepG2 cells—The time course of *CYP1A1* induction by TCDD was measured in both cell lines by real-time PCR using the primers designed in our previous study (*23*) for regions that were 100% homologous in mice and humans so as to achieve similar PCR efficiency in both species. Upon exposure of Hepa1c1c7 cells to 1 or 10 nM TCDD, CYP1A1 mRNA was greatly and significantly induced at 2 h, further significantly increased at 4 h, and maintained at similar levels thereafter (Fig. 1A and B). The increase in CYP1A1 mRNA from 0 to 2 h was greater than the increase from 2 to 4 h. In HepG2 cells exposed to 1 or 10 nM TCDD, CYP1A1 mRNA was also significantly increased until 4 h, and the increase from 2 to 4 h was greater than the increase from 0 to 2 h (Fig. 1A and B). Thus, more CYP1A1 mRNA was detected in the Hepa1c1c7 cells than in HepG2 cells at 2 h, which coincided with the higher AhR affinity for TCDD in C57BL/6 mice than in humans. From 4 h onward, however, *CYP1A1* induction in HepG2 cells was similar to or rather higher than that in Hepa1c1c7 cells, which did not reflect the difference in affinity of their AhR for TCDD.

Changes in total and nuclear-translocated AhR in Hepa1c1c7 cells and HepG2 cells—The amount of AhR expressed in the cells has been reported to be correlated with the intensity of *CYP1A1* gene expression (*27*). To determine why *CYP1A1* gene expression in Hepa1c1c7 cells and HepG2 cells did not reflect their AhR affinity from 4 h onward, we investigated time-course profiles of the amounts of AhR in the total lysate and nucleus of both cell lines.

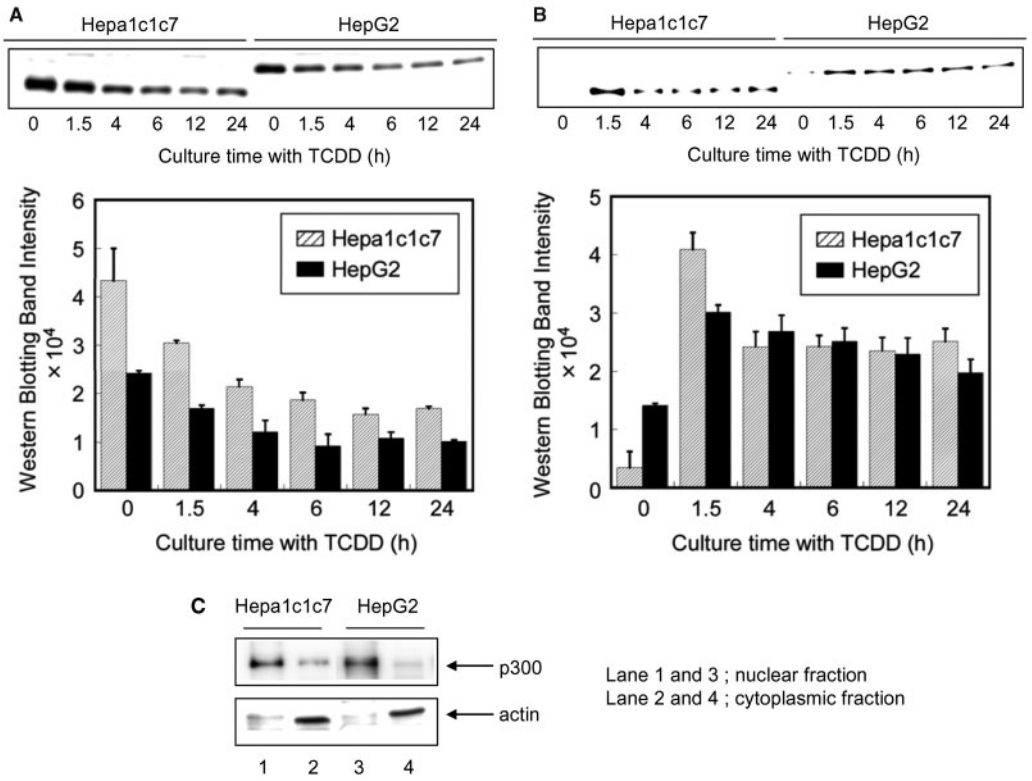

Fig. 2. **Western blotting analysis of total and nuclear-translocated AhR in Hepa1c1c7 cells and HepG2 cells.** Hepa1c1c7 cells and HepG2 cells were cultured in the presence of 10 nM TCDD for 0 to 24 h. Total lysates, cytoplasmic lysates and nuclear lysates were prepared from Hepa1c1c7 cells and HepG2cells. (A) 3 μg of total lysates from Hepa1c1c7 cells or HepG2 cells or (B) 3 μg of nuclear lysates from Hepa1c1c7 cells or 4.5 μg of nuclear lysates from HepG2 cells were resolved by SDS–PAGE and blotted. Blots were stained with 1 μg/ml anti-AhR antibody, followed by anti-rabbit IgG-HRP (1:2000), and visualized by ECL. (C) 4.5 μg of cytoplasmic lysates and 1.5 μg of nuclear lysates of Hepa1c1c7 cells or HepG2 cells were resolved by SDS–PAGE and blotted. Blots were stained with anti-actin antibody (1:800) or 1 μg/ml anti-p300 antibody, followed by anti-goat IgG-HRP (1:2000) for anti-actin antibody or anti-mouse IgG1-HRP (1:2000) for anti-p300 antibody and visualized by ECL. The data are expressed as means ± SE.

The level of AhR protein in the total lysate of both cell lines decreased in a similar manner upon TCDD exposure (Fig. 2A). This finding was consistent with previous studies showing that total and cytoplasmic AhR in various cell types, including Hepa1c1c7 cells and non-hepatic cell lines, was decreased (*14–17*, *28*). The amount of AhR protein in the nucleus of Hepa1c1c7 cells had rapidly increased by about 8-fold at 1.5 h, then immediately decreased to about 60% at 4 h, and remained at the same level until 24 h (Fig. 2B). These results were also consistent with previous studies showing an increase in nuclear AhR in Hepa1c1c7 cells at 1 and 2 h after TCDD exposure and a decrease at 4 h (*14*, *15*). These decreases in cytoplasmic and nuclear AhR were attributed to proteasome-dependent degradation (*14*, *28*). On the other hand, the level of nuclear AhR in HepG2 cells had increased by about 2-fold at 1.5 h, and it did not decrease markedly until 24 h (Fig. 2B). Thus, the greater increase in translocated AhR at 1.5 h in the Hepa1c1c7 cells paralleled the greater affinity of Hepa1c1c7 AhR for the ligand. Then, the amount of translocated AhR was rapidly and characteristically down modulated in Hepa1c1c7 cells, which suggested that proteasome-dependent AhR degradation occurred after nuclear-translocation in Hepa1c1c7 cells played a significant role in suppressing robust expression of the target gene to as low a level as in HepG2 cells.

The purity of the nuclear and cytoplasmic fractions was confirmed by detecting the distribution of p300 and actin, which are specifically present in the nucleus and cytoplasm, respectively, in both the fractions (Fig. 2C).

Time course of AhR and RNA polymerase II binding to the CYP1A1 promoter region in Hepa1c1c7 cells and HepG2 cells—To investigate functional involvement of the nuclear-translocated AhR, we measured binding of the AhR to the *CYP1A1* promoter region in Hepa1c1c7 cells and HepG2 cells by ChIP assay. In Hepa1c1c7 cells, the amounts of AhR binding to the *CYP1A1* promoter region greatly increased, and peaked at 1.5 h after TCDD exposure (Fig. 3), a finding that was highly consistent with the great increase in nuclear AhR at 1.5 h (Fig. 2B). In HepG2 cells, on the other hand, AhR binding to the *CYP1A1* promoter region occurred slowly, and peaked at 4–6 h after TCDD exposure. PolII binding to the *CYP1A1* promoter region in Hepa1c1c7 cells also peaked at 1.5 h, but the peaks were delayed until 4–12 h in HepG2 cells (Fig. 4). Thus, although the time course of AhR/ARNT-binding and PolII recruitment to the *CYP1A1*

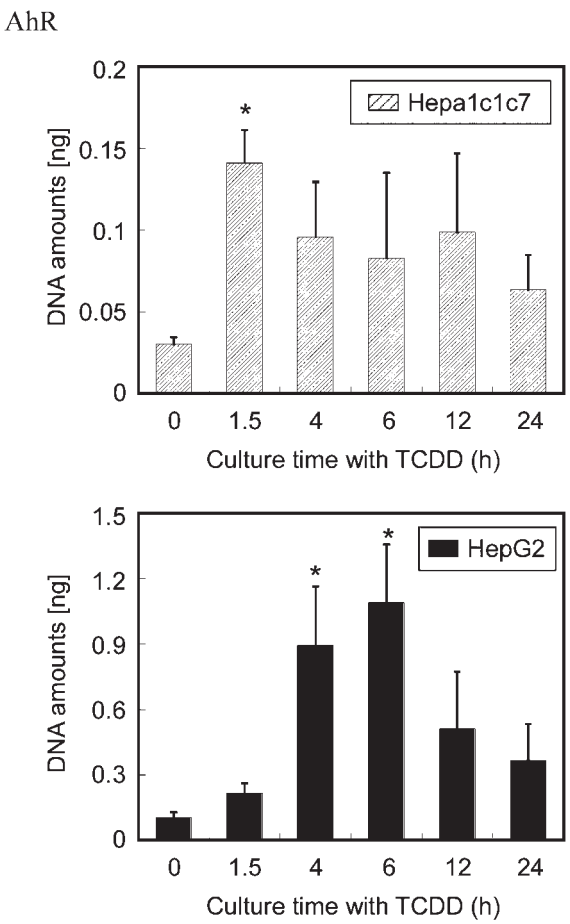

Fig. 3. **Time-course of AhR binding to the *CYP1A1* promoter region in Hepa1c1c7 cells and HepG2 cells.** Hepa1c1c7 cells and HepG2 cells were exposed to 10 nM TCDD for the times indicated. Cells were harvested, and ChIP assays were performed by using anti-AhR antibody as described in 'Materials and methods' section. ChIP assay samples were analysed by real-time PCR using primer pairs that amplify the *CYP1A1* promoter region as indicated in Table 1. Genome DNA from Hepa1c1c7 cells or HepG2 cells was used as a standard. The data are expressed as means ± SE. *Statistically significant difference from 0 h at $P < 0.05$.

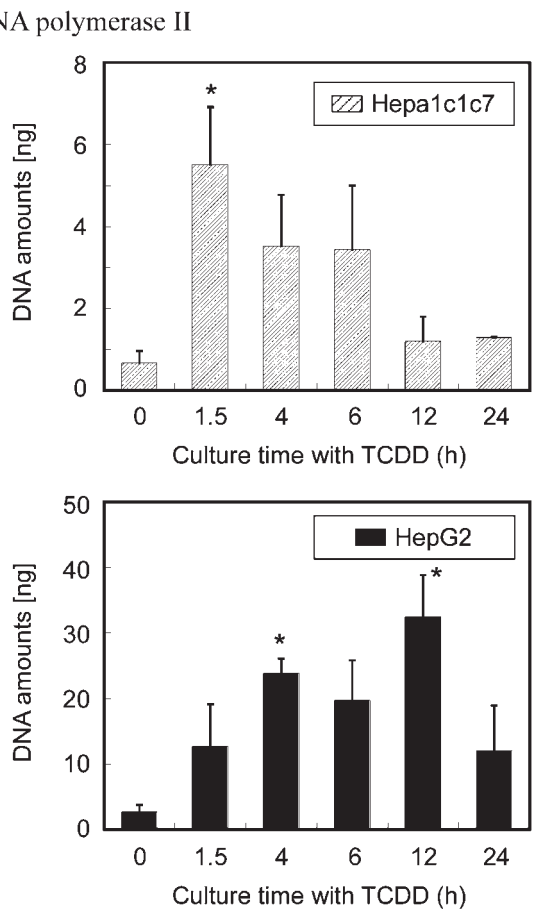

Fig. 4. **Time-course of RNA polymerase II binding to the *CYP1A1* promoter region in Hepa1c1c7 cells and HepG2 cells.** Hepa1c1c7 cells and HepG2 cells were exposed to 10 nM TCDD for the times indicated. Cells were harvested, and ChIP assays were performed by using anti-RNA polymerase II antibody as described in 'Materials and methods' section. ChIP assay samples were analysed by real-time PCR as described in the legend for Fig. 3. The data are expressed as means ± SE. *Statistically significant difference from 0 h at $P < 0.05$.

promoter regions and the time course of changes in the amount of nuclear AhR were similar to each other in Hepa1c1c7 cells, the AhR/ARNT-binding and PolII recruitment to *CYP1A1* promoter did not parallel the amount of nuclear AhR in HepG2 cells.

Histone acetyltransferases and histone deacetylases (HDACs) are involved in the binding of AhR and RNA polymerase II to the CYP1A1 promoter region—To identify the molecules responsible for the distinctive patterns of AhR and PolII binding to the *CYP1A1* promotor region in the individual cell lines, we investigated factors involved in the histone modification, such as histone acetyltransferase (HAT) coactivators CBP and p300. In Hepa1c1c7 cells, CBP recruitment to the *CYP1A1* promoter region was increased at 1.5 h, and the increase paralleled the recruitment of AhR and PolII (Fig. 5). No recruitment of p300 was detected in Hepa1c1c7 cells (data not shown). In HepG2 cells, however, p300 was increased after TCDD exposure (Fig. 5), and there was no change in CBP (data not shown). Involvement of histone acetylation upon TCDD exposure was also confirmed by detection of an increase in acetylated histone H4 bound to the *CYP1A1* promoter region in both cell lines (Fig. 6).

Histone deacetylases (HDACs) play a role in nuclear receptor (NR)-dependent transcription modulation through repressing transcription by deacetylating histones and stabilizing chromatins (*29*, *30*). Since HDAC1 is also implicated in chromatin-remodelling by AhR activation (*31*), we investigated the kinetics of HDAC1 binding to the *CYP1A1* promoter. HDAC1 binding to the *CYP1A1* promoter region decreased upon exposure of HepG2 cells to TCDD (Fig. 7), whereas, HDAC1 recruitment to the *CYP1A1* promoter tended to increase from 1.5 to 6 h by TCDD in Hepa1c1c7 cells (Fig. 7). These results suggest that the sustained HDAC1 binding to the *CYP1A1* promoter region after AhR activation contributes to suppression of the recruitment of AhR and PolII

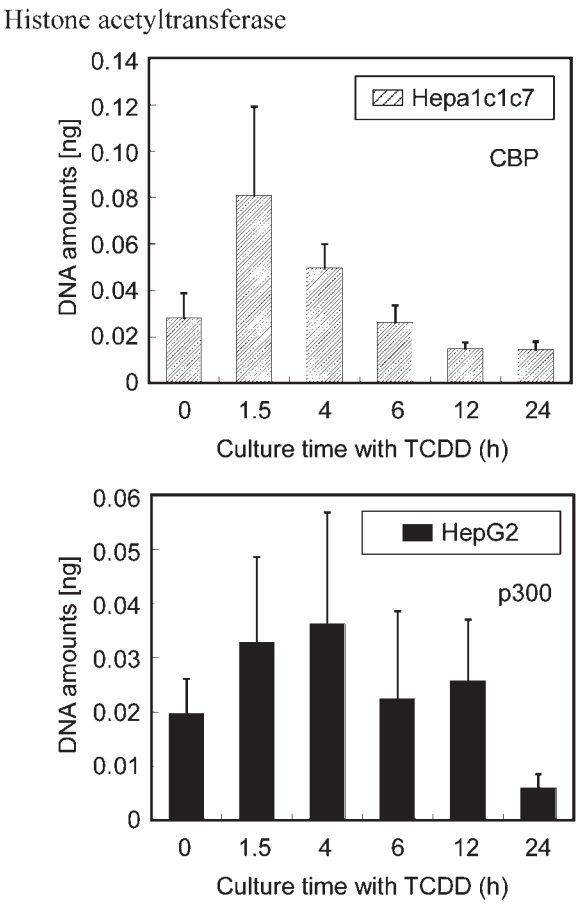

Fig. 5. **Involvement of histone acetyltransferases in the binding of AhR and RNA polymerase II to the *CYP1A1* promoter region.** Hepa1c1c7 cells and HepG2 cells were exposed to 10 nM TCDD for the times indicated. Cells were harvested, and ChIP assays were performed by using anti-CBP antibody for Hepa1c1c7 cells or anti-p300 antibody for HepG2 cells as described in 'Materials and methods' section. ChIP assay samples were analysed by real-time PCR as described in the legend for Fig. 3. The data are expressed as means ± SE. *Statistically significant difference from 0 h at $P < 0.05$.

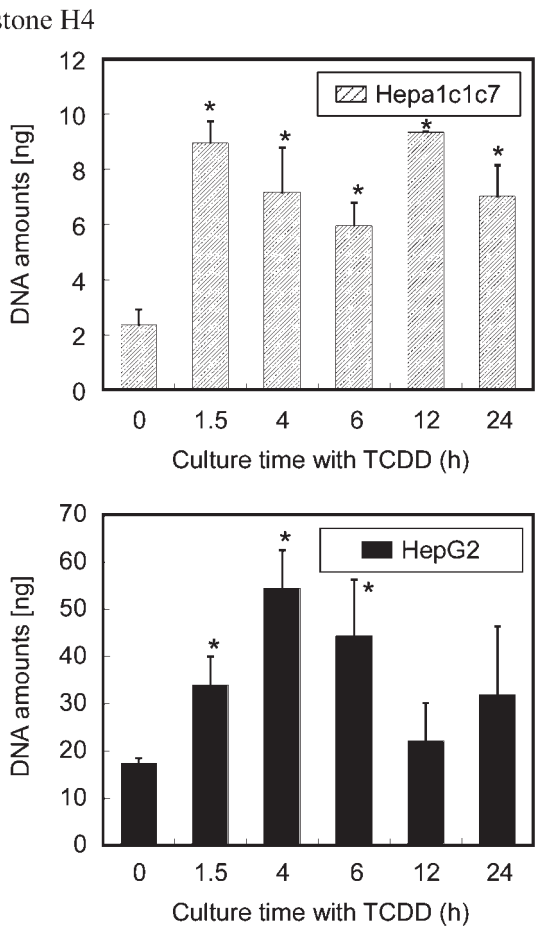

Fig. 6. **Involvement of histone acetylation in the binding of AhR and RNA polymerase II to the *CYP1A1* promoter region.** Hepa1c1c7 cells and HepG2 cells were exposed to 10 nM TCDD for the times indicated. Cells were harvested, and ChIP assays were performed by using anti-acetylated histone H4 antibody as described in 'Materials and methods' section. ChIP assay samples were analysed by real-time PCR as described in the legend for Fig. 3. The data are expressed as means ± SE. *Statistically significant difference from 0 h at $P < 0.05$.

to the *CYP1A1* promoter in Hepa1c1c7 cells, and results in the same levels of CYP1A1 mRNA in Hepa1c1c7 cells as in HepG2 cells. Treatment of cells with the HDAC inhibitor trichostatin A (TSA) resulted in greater *CYP1A1* induction at 4 and 6 h in Hepa1c1c7 cells than in HepG2 cells (Fig. 8), in contrast to the same levels of *CYP1A1* induction in both cells after 4 h in the absence of TSA (Fig. 1). These results also support the notion that different profiles of HDAC1 binding to the *CYP1A1* promoter are also involved in the regulation of *CYP1A1* induction in both cells. Since the cells started to detach from the dishes after 12 h of culture in the presence of TSA, it seemed impossible to accurately compare the *CYP1A1* induction profile in the two cell lines thereafter.

CYP1A1 mRNA stability in Hepa1c1c7 cells and HepG2 cells—We also measured the stability of CYP1A1 mRNA in Hepa1c1c7 and HepG2 cells, since stability affects its steady-state concentration. The half-life of CYP1A1 mRNA was longer in HepG2 cells (4.4 h) than in Hepa1c1c7 cells (3.1 h) (Fig. 9), and the difference may have contributed to the similar levels of CYP1A1 mRNA in both cell lines after exposure to TCDD for 4 h.

DISCUSSION

The major molecular pathway of AhR activation leading to the target gene expression in an XRE-dependent manner has been clearly elucidated (*1*, *2*), but the precise mechanism responsible for species-specific modification of AhR function had remained unclear, except for the discovery that AhR affinity for ligands primarily determines the species-specific activity of the AhR (*1*, *18*, *19*). In the present study we used real-time PCR to compare *CYP1A1* induction by TCDD in C57BL/6 mouse-derived Hepa1c1c7 cells and human-derived HepG2 cells. The results showed greater *CYP1A1* induction in Hepa1c1c7 cells than in HepG2 cells at 2 h, consistent with the higher affinity of Hepa1c1c7 AhR for TCDD.

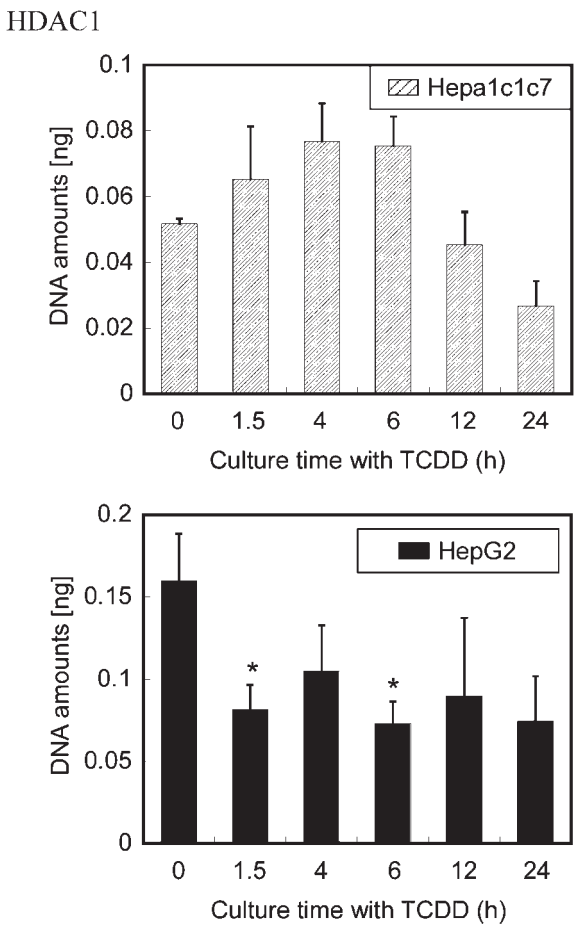

Fig. 7. **Involvement of HDAC1 in the binding of AhR and RNA polymerase II to the *CYP1A1* promoter region.** Hepa1c1c7 cells and HepG2 cells were exposed to 10 nM TCDD for the times indicated. Cells were harvested, and ChIP assays were performed by using anti-HDAC1 antibody as described in 'Materials and methods' section. ChIP assay samples were analysed by real-time PCR as described in the legend for Fig. 3. The data are expressed as means ± SE. *Statistically significant difference from 0 h at $P < 0.05$.

From 4 h onward, however, a similar level of *CYP1A1* expression was observed in both the cell lines, which suggested some mechanisms suppressing AhR function in the mouse cells or enhancing AhR function in the human cells to maintain the AhR-dependent gene expression.

Western-blotting analyses of Hepa1c1c7 AhR demonstrated that nuclear AhR had greatly increased after TCDD exposure, and then had quickly declined at 4 h. These findings were highly consistent with the previous studies showing that nuclear AhR in Hepa1c1c7 cells was increased at 1 and 2 h after TCDD exposure, and greatly reduced at 4 and 6 h (*14*, *28*). On the other hand, the decline in nuclear AhR in HepG2 cells after AhR activation was much less obvious in the present study. Consistent to our findings, many of the previous studies have reported decreased AhR protein in the total cell lysate, cytosolic fraction or nuclear fraction from Hepa1c1c7 cells stimulated with AhR ligand (reviewed in 17). On the other hand, report of changes in nuclear AhR

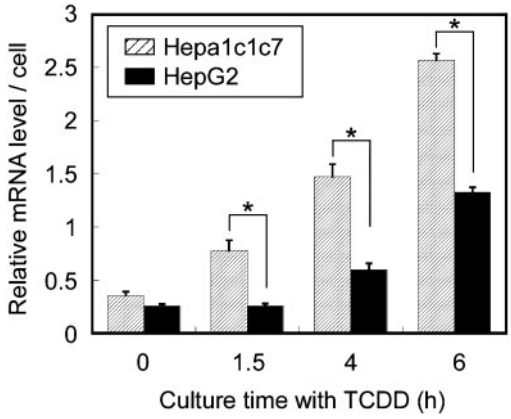

Fig. 8. **Effect of TSA on CYP1A1 mRNA induction in Hepa1c1c7 cells and HepG2 cells.** Total RNA was prepared from Hepa1c1c7 cells and HepG2 cells cultured in the presence of both of 10 nM TCDD and 5 μg/ml TSA for the times indicated, and then reverse-transcribed. CYP1A1 mRNA was quantified by real-time PCR as described in 'Materials and methods' section. Total RNA from the liver of a C57BL mouse exposed to TCDD (3 μg/kg) was used as a standard. The data are expressed as means ± SE. *Statistically significant difference between Hepa1c1c7 and HepG2 at $P < 0.05$.

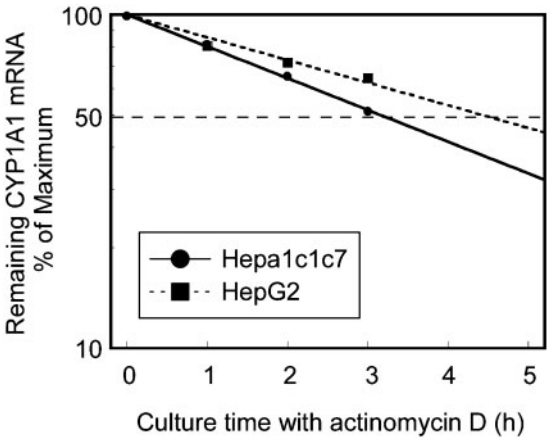

Fig. 9. **Comparison of decay of CYP1A1 mRNA in Hepa1c1c7 cells and HepG2 cells after induction by TCDD.** Hepa1c1c7 cells and HepG2 cells were cultured in the presence of 10 nM TCDD for 4 h, and the medium was replaced with new DMEM containing 5 μg/ml actinomycin D, and the cells were cultured for the times indicated. Total RNA was prepared from Hepa1c1c7 cells and HepG2 cells at the times indicated, and then reverse-transcribed. CYP1A1 mRNA was quantified by real-time PCR as described in 'Materials and methods' section. Total RNA from the liver of a C57BL mouse exposed to TCDD (3 μg/kg) was used as a standard. Data shown are representative of three separate experiments.

protein in HepG2 cells is limited. Although an electrophoretic mobility shift assay in a previous study in HepG2 cells showed that binding of the nuclear AhR/ARNT complex to the XRE sequence in the *CYP1A1* promoter had increased at 1 h after TCDD exposure and gradually declined at 2 and 5 h, the amount of nuclear AhR protein was not measured (*14*). The results of the

present study underscored the difference in the pattern of decrease in Hepa1c1c7 AhR and HepG2 AhR, which closely paralleled the extent of *CYP1A1* induction in each cell line.

The AhR activation-dependent decline in total and nuclear AhR in Hepa1c1c7 cells (*14*, *16*) and various other non-hepatic cells (*14*, *17*) has been shown to be caused by protein degradation via the ubiquitin/proteasome system. Thus, the disparity in nuclear AhR kinetics between the two cell lines found in the present study may be attributable to the difference in sensitivity of the AhR in the two cell lines to ubiquitin/proteasome-dependent degradation upon activation by TCDD. Furthermore, the low-sensitivity of HepG2 AhR to protein-degradation may contribute to maintaining *CYP1A1* induction at the same level as in Hepa1c1c7 cells. The difference in the AhR structure between mouse and human may result in the different susceptibility of the AhR to ubiquitination or proteasome recognition. A previous study has reported the involvement of phosphorylation in AhR degradation (*15*), suggesting the phosphorylation pattern of mouse and human AhR may affect the proteasome recognition of the AhR. In the ubiquitin/proteasome system, the E3 ubiquitin ligases determine target specificity (*32*, *33*). While recent studies have implicated the ubiquitin ligase protein C-terminal hsp70-interacting protein (CHIP) in the degradation of unliganded AhR (*34*, *35*), degradation machinery for ligand-activated AhR has not been clarified. Identification of E3 ligases responsible for ligand-activated AhR degradation would increase understanding of the species-specific differences in the AhR property.

Moreover, the balance between the degradation, nuclear translocation and nuclear export may be important to determine the amount of the nuclear AhR after activation. A previous study by Davarinos and Pollenz showed that the nuclear traslocation of the AhR occurred within 2 h and the proteasome-dependent AhR degradation started at 4 h after TCDD treatment in Hepa1c1c7 cells (*14*). Their data also showed that in the presence of a proteasome inhibitor MG132 in addition to TCDD the nuclear AhR increased at 4 and 6 h comparing to 2 h, showing that translocation of the AhR continued after the degradation started. The characteristics of nucleocytoplasmic AhR shuttling may also affect the amount of nuclear AhR after activation (*24*, *36*). While other studies reported that nucleocytoplasmic shuttling is also required for AhR degradation (*14*, *37*), there exists controversy as to whether the AhR degradation requires nuclear export (*15*, *34*). Thus, the mechanisms responsible for the determination of the steady-state amount of the nuclear AhR in Hepa1c1c7 cells and HepG2 cells after ligand activation will be important to understanding the species-specific mode of AhR function.

Another characteristic difference between the kinetics of AhR property in Hepa1c1c7 cells and HepG2 cells was found in the AhR and PolII recruitment in the target gene promoter region by ChIP assay. PCR primers to detect XRE sequences in the enhancer region of *CYP1A1* in Hepa1c1c7 cells are available, for example the sequence reported by Shibazaki *et al*. (*12*) for the enhancer region from −1141 to −784 bp. Although we tried to design PCR primers that would amplify XRE sequences in the enhancer of the *CYP1A1* gene in HepG2 cells without yielding any extra bands, we did not succeed. A previous study has reported that AhR, PolII and other factors such as CBP, are simultaneously recruited to the enhancer and the promoter (the TATA box) region of *CYP1A1*, where they may form a large multiprotein complex that bridges the two regions (*13*). We therefore used primers to detect the promoter regions of *CYP1A1* for ChIP assay of AhR and all other factors in Hepe1c1c7 cells and HepG2 cells.

The results of the ChIP assay showed that the time course of AhR/ARNT-binding and PolII recruitment to the *CYP1A1* promoter regions were similar to the time course of the changes in nuclear AhR amount in Hepa1c1c7 cells, which peaked at 4 h. On the other hand, the AhR/ARNT-binding and PolII recruitment to the *CYP1A1* promoter in HepG2 cells slowly reached a peak between 4 and 12 h. Previous studies (*10–13*) have reported that chromatin remodelling by histone modification is involved in the modulation of AhR function in a manner similar to the modulation of NR families (*29*, *30*). In the present study, we also detected recruitment of p300/CBP and histone acetylation at the *CYP1A1* promoter region upon TCDD exposure both in Hepa1c1c7 cells and HepG2 cells. The time course of p300/CBP recruitment to the *CYP1A1* promoter region was also similar to the time course of PolII recruitment. Although a previous study reported recruitment of p300 to the *CYP1A1* promoter region in Hepa1c1c7 cells upon exposure to TCDD (*11*), CBP, not p300, was found to be primarily recruited to the *CYP1A1* promoter region in the Hepa1c1c7 cells in the present study. This discrepancy may be due to the difference in antibodies used in the two studies.

HDACs consist of three major groups and play a role in repression of transcription in the transcription regulation by NRs (*29*, *30*, *38*). In the absence of ligand or upon antagonist recruitment, these HDACs are recruited to the target genes of NRs, such as the estrogen receptor (ER) and the thyroid hormone receptor (TR), through binding to corepressors, such as the N-CoR, and they suppress gene expression by stabilizing chromatin. Upon ligand binding to the NRs, coreppressors and HDACs are replaced by coactivators, and chromatin remodelling and gene activation proceed (*29*, *30*). In addition to the steady-state repression in the absence of ligands, recruitment of HDAC1 is reported to be involved in down-regulation of genes activated by NF-κB (*39*) and termination of IL-2 expression (*40*) by a chromatin-dependent mechanism. HDACs have also been implicated in modulation of gene expression by AhR in Hepa1c1c7 cells (*12*, *31*) and rat cells (*41*). In the present study HDAC1 binding was found to decline upon exposure of HepG2 cells to TCDD, in a manner similar to that reported for NRs. In contrast, the results in the present study showed that the HDAC1 binding in Hepa1c1c7 cells tended to increase upon TCDD exposure. The present study also demonstrated higher *CYP1A1* expression in Hepa1c1c7 cells than in HepG2 cells in the presence of the HDAC inhibitor TSA, which also supports that the maintenance of HDAC1 binding to the *CYP1A1* promoter region after AhR activation contributes to

suppress gene expression in Hepa1c1c7 cells. The augmentation of TCDD-induced *CYP1A1* expression by TSA in Hepa1c1c7 cells was consistently reported in the previous study (*12*). On the other hand, a previous study by Wei *et al*. (*31*) reported that HDAC1 binding to the *CYP1A1* promoter region decreased in Hepa1c1c7 cells upon exposure to another AhR ligand, benzo[a]pyrene (B[a]P). As described earlier, in the transcription regulation by NRs, the NRs recruit coactivators or HDACs through recruitment of different factors depending on the ligand (*29*, *30*). Similarly, in the AhR-dependent gene expression the *Bax* promoter is activated by 9,10-dimethylbenzo[a]anthracene-liganded AhR, but not by TCDD-liganded AhR (*42*). Thus, the AhR can recruit different factors depending on the ligand, and that may be the reason for the discrepancy in the HDAC kinetics observed in our study and the study of Wei *et al*. (*31*). Further studies on coactivators/corepressors which regulate chromatin modification by recruiting HAT coactivators and HDACs will improve our understanding of the species-specific modulation mechanism of AhR function.

We also examined the stability of CYP1A1 mRNA in Hepa1c1c7 cells and HepG2 cells in the present study. While previous studies have reported the half life of CYP1A1 mRNA in both cell lines to be 2.4–4.8 h (*43*, *44*), it was measured under different experimental conditions regarding the TCDD concentration and induction time. Our results showed that the CYP1A1 mRNA induced in HepG2 cells by exposure to 10 nM TCDD for 4 h had a longer half life than the CYP1A1 mRNA induced in Hepa1c1c7 cells under the same conditions, and that is also likely to have contributed to the sustained levels of CYP1A1 mRNA in HepG2 cells.

In summary, we have investigated the mechanisms of modulation of AhR function in Hepa1c1c7 cells and HepG2 cells. The results demonstrated differences between the two cell lines in the kinetics of the amount of nuclear-translocated AhR and the AhR and transcription machinery binding to the target gene. These factors have been implicated in determining the species-specific modulation mechanism of AhR function. These may be also involved in the cell-type-specificity of AhR function. The mRNA stability of the target genes would also influence species-specific susceptibility. Further studies focusing on key factors determining the amount of nuclear-translocated AhR, epigenetic factors modulating AhR-target gene transcription through histone modification, and factors modulating mRNA stability will provide further insight into the species-specific function of the AhR.

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