Green Fluorescent Protein (GFP) as a Marker of Aryl Hydrocarbon Receptor (AhR) Function in Developing Zebrafish (*Danio rerio*)

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We developed an inducible *in vivo* reporter system to examine expression of the aryl hydrocarbon receptor (AhR) during development in zebrafish (*Danio rerio*). AhR is a ligand-activated transcription factor that mediates the toxic actions of environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Induction of cytochrome P4501A1 (*CYP1A1*) is an early biomarker of AhR activation. A 1905 base pair region of the human *CYP1A1* promoter/enhancer region was regulated by AhR in zebrafish liver cells after exposure to TCDD (10 nM) in a transient transfection assay. This regulatory region was fused to the cDNA sequence encoding green fluorescent protein (GFP) of jellyfish (*Aequorea victoria*). Transgenic zebrafish were generated to express this AhR-regulated GFP construct. Injected fish exposed to TCDD exhibited induction of GFP in the eye, nose, and vertebrae of zebrafish embryos (48 and 72 hr after fertilization) compared to vehicle controls (DMSO), which did not express GFP. To investigate whether AhR-regulated GFP expression correlated with sites of TCDD toxicity, we exposed wild-type zebrafish to DMSO or TCDD and examined them for morphologic abnormalities. By 5 days after fertilization, TCDD-exposed fish exhibited gross dysmorphogenesis in cranio-facial and vertebral development. *Key words*: aryl hydrocarbon receptor, cytochrome P450, dioxin, green fluorescent protein, polychlorinated compounds. *Environ Health Perspect* 109:845–849 (2001). [Online 14 August 2001] *http://ehpnet1.niehs.nih.gov/docs/2001/109p845-849mattingly/abstract.html*

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that modulates the toxic actions of a class of environmental compounds including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (*1*). Ligand-activated AhR forms a heterodimer with a second protein, aryl hydrocarbon nuclear translocator (Arnt), and binds to Ah response elements (AhREs) in the enhancer regions of AhR-regulated genes such as cytochrome P4501A1 (*CYP1A1*). An endogenous ligand for this receptor has not yet been identified. Several reports in the literature, however, suggest that AhR regulates proliferation and differentiation of many cell types (*2–5*).

That AhR plays an important role during development independent of environmental exposure is supported by AhR knockout mice, which exhibited poor survival, loss of body weight, and impaired liver and immune systems (*6–9*). To date, little information is known about the ontogenetic expression of this receptor. In this study, we examined the temporal expression pattern of AhR and its heterodimer partner, Arnt, during zebrafish development using reverse transcription polymerase chain reaction (RT-PCR). To determine the spatial expression of AhR, we developed an inducible reporter system designed to express green fluorescent protein (GFP) in response to ligand-activated AhR. Several reports in the literature have demonstrated that GFP can be used effectively to describe protein expression in live embryos and juvenile zebrafish (*10–12*). GFP offers several advantages over other reporter systems in that it is nontoxic and can be detected in living animals without the addition of exogenous substrates (*10–12*). We used AhR-regulated GFP expression as an *in vivo* reporter to detect AhR function and to determine whether AhR-regulated GFP expression accurately predicts sites of TCDD-induced dysmorphogenesis during zebrafish development.

Materials and Methods

Chemicals. We obtained TCDD from Cambridge Isotopes Laboratory (Andover, MA). We purchased restriction endonucleases from New England Biolabs (Beverly, MA). Unless specified otherwise, we purchased reagents from commercial sources and used them without further purification.

Zebrafish maintenance. Adult zebrafish were raised and maintained on a 14:10 hr light:dark cycle at 28.5°C and bred in marbled tanks as described by Westerfield (*13*). We fed mature fish three times daily with a combination of Freshwater Aquarium Flakefood (Ocean Star International, Inc., Hayward, CA) and live brine shrimp (Carolina Biological Supply Co., Burlington, NC). Care and treatment of animals were conducted in accordance with guidelines established by the Tulane University Institutional Animal Care and Use Committee.

RNA preparation. We collected RNA from zebrafish embryos using TRI REAGENT RNA Isolation Reagent (Molecular Research Center, Inc., Cincinnati, OH). At targeted stages of development, embryos were frozen, rinsed twice in cold tank water, dissolved in 1 mL of TRI REAGENT, and transferred to a Dounce Homogenizer (Fisher Scientific, Pittsburgh, PA), previously washed with chloroform. Protective chorions surrounding embryos were broken by 10 strokes in the Dounce homogenizer. Embryos were transferred to RNase-free microcentrifuge tubes, and RNA was isolated according to a protocol supplied by the manufacturer.

RT-PCR. We reversed transcribed 10 µg of total RNA according to manufacturer's recommendations (Promega, Madison, WI). Briefly, RNA or water, 20 units of RNasin Ribonuclease Inhibitor, and 1 unit RQ1 DNase (RNase-free) in a total volume of 10 µL were heated to 37°C for 15 min, 70°C for 5 min, and cooled on ice. The reaction mixture was heated to 70°C for 3 min with 200 pmol oligo d $(T)_{16}$ and cooled on ice for 5 min. Reaction volume was increased to 40 µL containing (final concentrations) 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 8 mM $MgCl_2$, 10 mM DTT, 1 mM each of dATP, dCTP, dGTP, and dTTP, 20 units of RNase inhibitor, and 200 units of M-MLV reverse transcriptase. Reaction was incubated at 42°C for 90 min, 70°C for 10 min, placed on ice, and brought to 100 µL in water. We used PCR to amplify zebrafish AhR and Arnt cDNA sequences. We designed AhR and Arnt primers according to published sequences (*14*). Primers used included AhR upper, 5´-CCAAGATTATC TAGGGTTC-CATCAG; AhR lower, 5´-CTCCCAC-AGGCAGAGTATCGCACTGA; β-actin upper, 5´- ATCTGGCACCA CACCTTC-TACAATG; β-actin lower, 5´- GGGGTGT TGAAGGTCTCAAACATGAT. We performed PCR reactions in a final volume of

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50 µL containing (final concentrations) 10 mM Tris-HCl (pH 9.0), 4.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.5 mM each of dATP, dCTP, dGTP, dTTP, 2.5 units of *Taq* DNA Polymerase, 6 µL of the reverse transcription reaction described above, and 20 pmol each of *CYP1A1* primers. All components except enzyme were heated to 95°C for 2 min and cooled on ice for 5 min. *Taq* polymerase was added and samples were cycled 40 times through a 1 min 95°C denaturation step, a 1 min 54°C annealing step, and a 1 min 72°C extension step. Cycling was followed by a 5 min final 72°C extension step. Samples were coamplified with primers for the zebrafish β-actin cDNA sequence for the purpose of a loading control.

Plasmid construction. We constructed two AhR-regulated reporter plasmids, p1A1Luc and p1A1GFP, by fusing a portion of the 5′ regulatory region (–1612/+292) of the AhR-regulated gene *CYP1A1* to the cDNA sequences of firefly luciferase and jellyfish GFP, respectively. This region was previously shown to be regulated by activated AhR (*15–19*). We excised a 1904 base pair fragment from the phMC7.6 plasmid (a generous gift of Y. Fujii-Kuriyama, Sendai, Japan) by PvuII digestion, inserted it into the SmaI site of Bluescript (SK+) vector (Stratagene, La Jolla, CA), and designated it pBU1A1. This portion of the *CYP1A1* 5′ regulatory region is AhR responsive and contains the promoter exon one as well as at least three functional AhREs (*15–19*). We excised this fragment from pBU1A1 by SacI-XhoI digestion and subcloned it into the pGL3-Basic Vector (Promega) upstream of the luciferase cDNA sequence to generate p1A1Luc. We excised the 5′ regulatory region from pBU1A1 by SacI-PstI digestion and subcloned it into the corresponding sites of pEGFP-1 (Clontech, Palo Alto, CA) to generate p1A1GFP.

Zebrafish liver cell culture. P. Collodi (Purdue University, West Lafayette, IN) provided adult zebrafish liver cells (ZFL). We cultivated the cells in ZFL medium consisting of Liebowitz L-15, Dulbecco's Modified Eagle's Medium (DMEM), and Ham's F12 (50:35:15) supplemented with 10 mg/mL insulin, 5% fetal bovine serum (Hyclone, Salt Lake City, UT), and 0.1% trout serum (East Coast Biologics, Inc., New Berwick, ME) in an atmosphere of 5% CO_2/95% air at 28.5°C, as described by Collodi et al. (*20*).

Transient trasfections. We plated ZFL cells in 12-well dishes and transiently transfected them with 1 µg of p1A1Luc using TransFast Reagent (Promega) according to manufacturer's instructions. Following transfection incubation, we replaced ZFL medium with medium containing DMSO (0.1%) or TCDD (1 nM and 10 nM) at different time points. We selected these levels of TCDD because they are equivalent to levels at which humans are thought to be exposed (*21*). After a maximum 48-hr incubation, we rinsed cells twice in phosphate-buffered saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM sodium phosphate, 0.14 mM potassium phosphate) and lysed them on the plate by adding 60 µL reporter lysis buffer 5X (Promega; 25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% Triton X-100). Cell lysates were collected, vortexed, and centrifuged for 20 sec at 13,000 rpm in a microcentrifuge tube (Eppendorf; Brinkman Instruments, Westbury, NY). We added 100 µL luciferase assay reagent (Promega) to supernatant (10 µL) at 25°C, and measured luciferase activity in a luminometer (Monolight 2010; Analytical Luminescence Laboratory, Ann Arbor, MI). We normalized assays to protein concentration estimated using the procedure of Lowry as modified by Peterson (*22*). Bovine serum albumin was the standard. The minimum detectable protein level for this assay was 1 ng.

Microinjection of zebrafish embryos and GFP detection. We micro-injected single-cell zebrafish embryos with p1A1GFP as described (*13*). Briefly, p1A1GFP was linearized with PvuII, purified from an agarose gel, and suspended at 75 µg/mL in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.8) and phenol red (2%) (*23*). We constructed agarose ramps for injections as described (*24*). We transferred eggs to the agarose ramps and injected them with DNA/phenol red solution using a micropipet secured in a micromanipulator (Zeiss, Jena, Germany). We transferred injected eggs to Petri dishes and exposed them to DMSO (0.1%) or TCDD (10 nM) immediately following injection or at 48 hr after fertilization for 24 hr. We monitored embryos for expression of GFP using a Zeiss microscope equipped with a fluorescein isothiocyanate (FITC) filter (λex = 470 nm, λem = 515 nm). We processed images with help from D. Biggs, using the AutoDeblur 2D blind deconvolution algorithm from AutoQuant Imaging, Inc. (Watervliet, NY).

Results and Discussion

Temporal expression of AhR in developing zebrafish. Understanding the role of AhR during development has been limited by the lack of information about the spatio-temporal expression patterns of AhR transcriptional machinery. Difficulties associated with defining AhR and Arnt expression are related partly to limitations posed by mammalian systems, which include the difficulty of reproducible staging and limiting numbers of embryos (*25*). Thus zebrafish provide distinct advantages over other systems in the availability and transparency of eggs.

It was first important to determine whether AhR and Arnt were expressed during development. Because AhR is present in low abundance (5,000–33,000 molecules/cell), RT-PCR was used to amplify AhR mRNA (*26*). Primers were designed based on a portion of published zebrafish AhR cDNA sequences (*14*). We isolated RNA from unfertilized eggs and embryos at several stages during development. We detected AhR mRNA at low levels in the unfertilized egg and in the embryo at 1 and 2 hr after fertilization (data not shown). At 4 hr after fertilization, mRNA levels increased (Figure 1), correlating with the estimated onset of zygotic transcription (*27,28*). We detected AhR transcript at all stages examined (4, 12, 24, 28, 33, 48, and 120 hr after fertilization), although levels appeared to fluctuate, suggesting that this gene is regulated during development.

Design and construction of AhR-responsive constructs. Induction of *CYP1A1* mRNA is an early biochemical marker of ligand-activated AhR. The *CYP1A1* cDNA sequence is highly conserved among vertebrates and invertebrates (*29,30*). The 5′ regulatory region of the human *CYP1A1* gene has been well characterized (*15,17*). Several studies have identified elements of the *CYP1A1* regulatory region that are involved in AhR-mediated transcriptional activation of this gene, including a TATA box, an NF-1 binding site, and eight AhR response elements (AhREs) (*1,15,17*). AhR activates transcription of *CYP1A1* by binding as a heterodimer to one or more of the eight

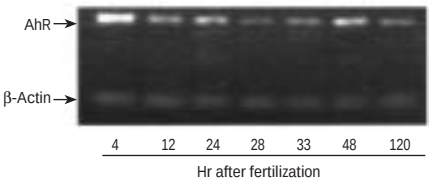

Figure 1. Temporal expression of AhR mRNA during zebrafish development, assessed using RT-PCR. RNA was isolated from embryos at several stages of development, reverse transcribed, and amplified using primers designed using a published sequence for zebrafish AhR cDNA (*14*). Samples were coamplified with primers for zebrafish β-actin, used as a loading control.

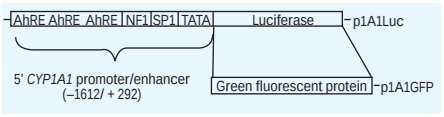

Figure 2. AhR-regulated reporter constructs. Two AhR regulated reporter plasmids, p1A1Luc and p1A1GFP, were constructed by fusing a portion of the 5′ regulatory region (–1612/+292) of the AhR-regulated gene *CYP1A1* to the cDNA sequences of firefly luciferase and jellyfish GFP, respectively. The 5′ *CYP1A1* promoter region includes a TATA box, Sp1, NF1 binding sites, and three AhREs in the upstream enhancer region (*15–19*).

AhREs in combination with other factors of the basal transcriptional machinery (*1,18,19*). Because *CYP1A1* monooxygenase activity depends on AhR activation, we used the *CYP1A1* promoter/enhancer region to design an AhR-responsive reporter construct.

Previous reports have indicated that the first 1905 base pairs of the 5´ regulatory region are sufficient for transcriptional activation of *CYP1A1* by AhR (*1,16,18,19*). This region includes the TATA box, the NF-1 binding site, and three of the eight AhR response elements (*1,16,18,19*). Before injecting zebrafish eggs with a GFP reporter construct under regulatory control of the human *CYP1A1* promoter/enhancer region, we had to determine whether this 5´ regulatory region of *CYP1A1* was responsive to AhR ligands in zebrafish. To test this, we constructed a plasmid by ligating the human *CYP1A1* regulatory region to the cDNA sequence encoding firefly luciferase (Figure 2). This construct was transiently transfected into adult zebrafish liver cells (ZFL)(*20*). Cells were exposed to either DMSO (0.01%) or environmentally relevant concentrations of TCDD (1 nM and 10 nM) for 18, 24, or 48 hr. We determined luciferase activity fluorometrically. Induction of luciferase activity was concentration dependent and reached steady state by 24 hr (Figure 3). These results were consistent with activation observed in human systems using this 5´ regulatory region of *CYP1A1*, and showed that this region was responsive to AhR-mediated induction in the zebrafish system (*2*).

AhR-mediated GFP expression in developing zebrafish. We constructed a second AhR-dependent reporter plasmid for injection into zebrafish embryos. We fused the identical 1905 base pair 5´ regulatory region of the human *CYP1A1* gene to the cDNA sequence encoding GFP (Figure 2). This construct was linearized by digestion with PvuII and microinjected into zebrafish embryos at the one-cell stage as described by Westerfield (*13*). Embryos were immediately exposed to DMSO (0.01%) or TCDD (10 nM) for 24 or 72 hr. Zebrafish were dechorionated and examined by fluorescence microscopy. We observed no GFP expression in DMSO-exposed injected fish, whereas approximately 43% of injected embryos exposed to TCDD exhibited GFP expression. We observed GFP expression most consistently in the eye and nose and along the vertebrae of TCDD-exposed developing zebrafish (24 and 48 hr after fertilization; Figure 4).

GFP expression pattern correlates with sites of TCDD-induced toxicity. Numerous reports (*31–35*) have demonstrated that TCDD exposure causes gross morphologic abnormalities during development. Although the mechanisms underlying TCDD-induced toxicity are not understood, evidence derived from AhR knockout mice has confirmed that toxicity is mediated via AhR (*6–8*). We were interested to determine whether patterns of AhR-dependent GFP expression in transgenic zebrafish correlated with sites of toxicity in developing zebrafish. We exposed wild-type zebrafish to DMSO (0.01%) or TCDD (10 nM) for 5 days after fertilization and examined them for gross morphologic differences. We observed diverse toxicity, including a lack of eyes and a range of vertebral abnormalities (Figure 5). These data suggest that AhR-regulated GFP expression accurately predicts sites of TCDD-induced toxicity and may provide a novel method for examining the physiologic function of AhR. A recent study (*36*) has demonstrated that AhR plays an important role in regulating development of vascular structures in murine embryos through transcriptional regulation of as yet unidentified genes by the AhR. The AhR-regulated GFP expression system described here could be a useful tool to elaborate the molecular mechanism by which AhR regulates important developmental genes in vascular tissues.

Conclusions

In this report, we used zebrafish as a model to investigate the spatio-temporal expression pattern of AhR during development. AhR mRNA was detected by RT-PCR in unfertilized eggs and in embryos at 1 and 2 hr after fertilization, suggesting the presence of maternal transcript. AhR mRNA levels increased dramatically at 4 hr after fertilization and appeared to be regulated throughout development, suggesting that this gene has an important and specific functions during development. We used the 5´ regulatory region of the human *CYP1A1* gene, an

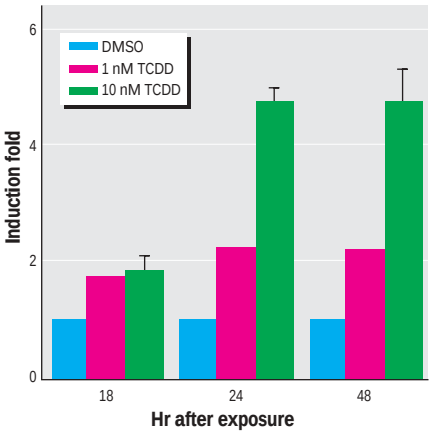

Figure 3. Human *CYP1A1* 5´ regulatory region is responsive in zebrafish liver cells (ZFL). An AhR-responsive reporter plasmid, p1A1Luc, was transiently transfected into zebrafish liver. Cells were exposed to DMSO (0.01%) or TCDD (1 nM and 10 nM) and luciferase activity was assayed after 18, 24, and 48 hr. Results are presented in relative light units normalized to protein content. Values shown represent the mean from two separate experiments performed in triplicate ± SE.

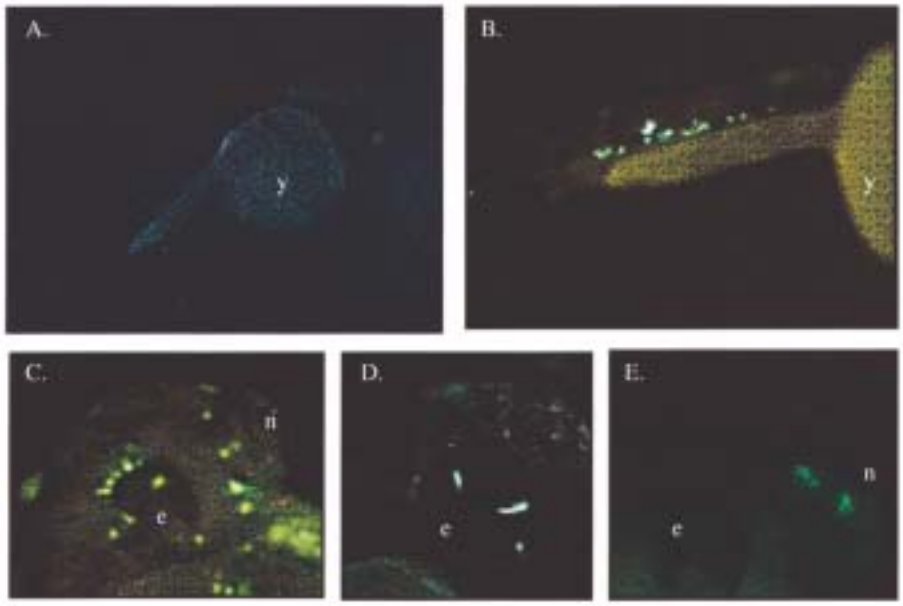

Figure 4. AhR-regulated GFP expression in developing zebrafish exposed to TCDD. Abbreviations: e, eye; n, nose; y, yolk sac. Microinjected zebrafish were exposed to DMSO (0.01%) or TCDD (10 nM) for 48 hr, dechorionated, and analyzed for GFP expression by fluorescent microscopy. Transgenic zebrafish exposed to DMSO did not express GFP (*A*); TCDD-exposed transgenic zebrafish expressed GFP in vertebrae (*B*), face (*C*), eye (*D*) and nose (*E*).

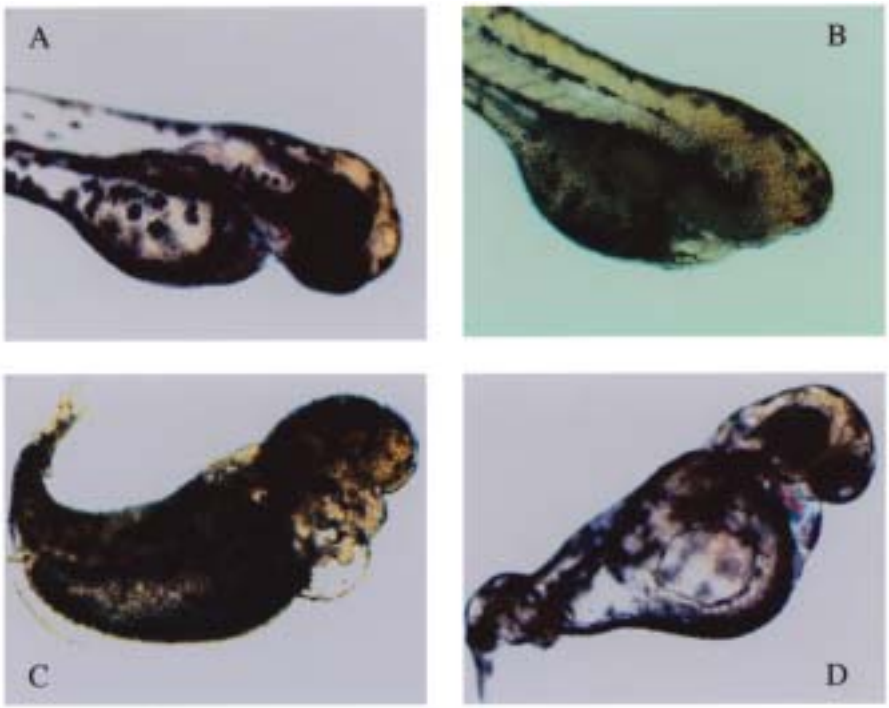

Figure 5. TCDD-induced dysmorphogenesis in developing zebrafish. Wild-type zebrafish embryos were exposed to DMSO (0.01%) or TCDD (10 nM) for the first 24 hr of development. Following incubation for 24 hr, embryos were rinsed and reared in fresh water. Embryos were observed daily for morphologic aberrations. Fish shown are 5 days after fertilization. (*A*) Control zebrafish. (*B*) TCDD-exposed zebrafish lacking eyes. (*C*) TCDD exposed zebrafish exhibiting contorted tail and swollen yolk sac. (*D*) TCDD-exposed zebrafish lacking eyes and exhibiting contorted tail and swollen yolk sac.

AhR-regulated gene, to construct two reporter plasmids, p1A1Luc and p1A1GFP, that express luciferase and GFP, respectively. By transiently transfecting the luciferase construct into zebrafish liver cells, we showed that this regulatory region was functional in the zebrafish system and was induced in response to TCDD, a potent AhR ligand. Microinjection of p1A1GFP into single-cell zebrafish embryos produced GFP expression after exposure to TCDD but not the vehicle control (DMSO). We observed GFP expression up to at least 48 hr, after which the onset of pigmentation limited observation of GFP expression. We detected GFP expression in the eye, nose, and vertebrae, suggesting that AhR may play an important physiologic role in the development of these structures. These data are in agreement with those of other laboratories (*36*), which showed a role for AhR as a regulator of vascular structures in mice. Activation of AhR indicated that TCDD-induced toxicity targeted morphologic development of the vertebrae and cranio-facial structures including the eye. AhR-regulated GFP expression, therefore, appears to be an accurate predictor of TCDD-induced toxicity and may provide important information about the normal physiologic function of this receptor. Expression of AhR-regulated reported groups could be used as an assay system to elucidate functions of potential natural ligands for AhR and may be a powerful tool to elaborate physiologic as well as toxicologic functions of AhR during development. Further, it is possible that specific receptor-regulated GFP expression systems could be useful transgenic biosensor systems to detect xenobiotic toxicants in the environment.

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