Expression of Zebrafish Aromatase cyp19a and cyp19b Genes in Response to the Ligands of Estrogen Receptor and Aryl Hydrocarbon Receptor

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Many endocrine-disrupting chemicals act via estrogen receptor (ER) or aryl hydrocarbon receptor (AhR). To investigate the interference between ER and AhR, we studied the effects of 17β-estradiol (E2) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the expression of zebrafish cyp19a (zcyp19a) and cyp19b (zcyp19b) genes, encoding aromatase P450, an important steroidogenic enzyme. In vivo (mRNA quantification in exposed zebrafish larvae) and in vitro (activity of zcyp19-luciferase reporter genes in cell cultures in response to chemicals and zebrafish transcription factors) assays were used. None of the treatments affected zcyp19a, excluding the slight upregulation by E2 observed in vitro. Strong upregulation of zcyp19b by E2 in both assays was downregulated by TCDD. This effect could be rescued by the addition of an AhR antagonist. Antiestrogenic effect of TCDD on the zcyp19b expression in the brain was also observed on the protein level, assessed by immunohistochemistry. TCDD alone did not affect zcyp19b expression in vivo or promoter activity in the presence of zebrafish AhR2 and AhR nuclear translocator 2b (ARNT2b) in vitro. However, in the presence of zebrafish ERα, AhR2, and ARNT2b, TCDD led to a slight upregulation of promoter activity, which was eliminated by either an ER or AhR antagonist. Studies with mutated reporter gene constructs indicated that both mechanisms of TCDD action in vitro were independent of dioxin-responsive elements (DREs) predicted in the promoter. This study shows the usefulness of in vivo zebrafish larval and in vitro zcyp19b reporter gene assays for evaluation of estrogenic chemical actions, provides data on the functionality of DREs predicted in zcyp19 promoters and shows the effects of cross talk between ER and AhR on zcyp19b expression. The antiestrogenic effect of TCDD demonstrated raises further concerns about the neuroendocrine effects of AhR ligands.

Key Words: endocrine disruption; aromatase CYP19; zebrafish estrogen receptor; aryl hydrocarbon receptor; gene expression/ regulation.

In recent years, diverse cases of disturbed sexual differentiation and reproductive abnormalities have been reported in fish (Eggen et al., 2003; Jobling et al., 1998; Segner et al., 2003). These findings can be linked, at least partially, to exposure to so-called endocrine-disrupting chemicals (EDCs) natural or synthetic compounds widely present in the environment that can disrupt hormone action. Aromatase P450, encoded by cyp19 gene(s), is considered to be a potential EDC target because it catalyzes the final step of biosynthesis of estrogens (Simpson et al., 2002), important hormones involved in the control of many physiological processes, including those related to reproduction. Several indications exist that interference with the aromatase CYP19 system in fish might lead to malfunctioning of the reproductive system. For instance, treatment with aromatase inhibitors disrupts gonadal sex differentiation in fish (Fenske and Segner, 2004).

Two structurally distinct cyp19 genes, cyp19a1 (cyp19a) and cyp19a2 (cyp19b), are found in most teleosts, including model species zebrafish (Danio rerio). Zebrafish cyp19a (zcyp19a) is predominantly expressed in gonads, and zebrafish cyp19b (zcyp19b) is mainly found in neuronal tissues, while lower levels of the other isoform are found in both sites and in some other tissues (Chiang et al., 2001; Sawyer et al., 2006). Characterization of zcyp19 promoters in zebrafish led to the assumption that some EDCs, for example, estrogen- and dioxin-like compounds, might interfere with the expression of these genes due to the presence of predicted responsive elements (Kazeto et al., 2001; Tong and Chung, 2003). Estrogen-responsive element (ERE) is found in the zcyp19 promoter, half-EREs, and aryl hydrocarbon responsive elements (also called dioxin-responsive elements, DREs) are predicted in...
zfcyp19a and zfcyp19b promoters (Kazeto et al., 2001; Tong and Chung, 2003). Functionality of ERE in the zfcyp19b promoter is confirmed by in vivo and in vitro studies (Hinfray et al., 2006; Kazeto et al., 2004; Kishida et al., 2001; Menuet et al., 2005), but the functionality of DREs in zfcyp19 promoters has not been unambiguously shown to date (Kazeto et al., 2004). Exposure to both estrogen- (Brion et al., 2004; Fenske et al., 2005; Nash et al., 2004) and dioxin-like (King Heiden et al., 2006) compounds negatively affects fish development and reproduction.

The presence of ERE and DRE sites in the promoter points to potential regulation by respective receptors. Both estrogen receptor (ER) and aryl hydrocarbon receptor (AhR) function as ligand-dependent sequence-specific transcription regulators. Ligand-activated ER homodimer is able to initiate transcription from the promoters that possess a functional ERE (Klinge, 2000). Ligand-activated AhR heterodimerizes with AhR nuclear translocator (ARNT) and activates transcription of target genes through binding to DREs (Schmidt and Bradford, 1996). Several isoforms of both ER and AhR/ARNT are found in teleost fish, including zebra fish (Andreasen et al., 2002; Karchner et al., 2005; Menuet et al., 2002; Prasch et al., 2006; Tanguay et al., 1999, 2000). 17β-estradiol (E2) is a natural ligand of ER. Many EDCs, for example, ethinylestradiol (EE2), nonylphenol (NP), or bisphenol A (BPA), can also bind and activate ER (Klotz et al., 1996). Similarly, the AhR can be activated by many environmental contaminants, including halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (Schmidt and Bradford, 1996). The prototypic AhR ligand 2,3,7,8-tetrachlorodibeno-p-dioxin (TCDD) is the most toxic compound among HAHs and also the most potent AhR activator (Schmidt and Bradford, 1996). In addition to their direct “classical” transcriptional actions, ER and AhR signaling pathways also converge at multiple points. Both estrogenic (Lind et al., 1999; Ohtake et al., 2003) and antiestrogenic (Chen et al., 2001; Navas and Segner, 2000; Ohtake et al., 2003) effects of AhR ligands have been reported.

Zfcyp19 genes may constitute a good model to study the molecular mechanisms of action and possible cross talk of estrogen- and dioxin-like EDCs. Characterization of interference of these EDC classes with zfcyp19 expression may also add to the understanding of the pathways leading to disruption of reproduction caused by these chemicals. Therefore, the aims of this study were (1) to clarify the role of AhR/ARNT in the regulation of zfcyp19 genes expression and to examine the functionality of DRE sites predicted in the zfcyp19 promoters, (2) to study the effects of AhR activators on the expression of an estrogen-regulated target gene in fish brain (zfcyp19b), and thus (3) to reveal the possible cross talk pathway between the ERs and the AhR/ARNT complex and its effects on zfcyp19 genes expression. This was performed by combining in vivo exposure of zebra fish larvae and in vitro cell-based luciferase reporter gene assays.

**Materials and Methods**

**Chemicals.** TCDD was obtained from LG C Promochem SARL (Molsheim, France). Benzo[a]pyrene (BaP), α-naphthol (ANF), and E2 were purchased from Sigma Aldrich Chemical Co. (St Louis, MO, USA). ICI 182 780 (ICI) was purchased from Tocris (Bristol, UK). DMSO solvent was used for all chemicals.

**Zebra fish handling and exposure.** Zebra fish larvae were reared in our breeding unit as previously described (Brion et al., 2004). At the age of 17 days postfertilization (dpf), they were exposed to chemical mixtures or to solvent alone (DMSO, 0.1% vol/vol) for 72 h (in 100 ml water, 20 fish per group for mRNA quantification or 4 fish per group for immunohistochemistry). Chemical mixtures contained E2 (10nM) or TCDD (0.001-0.1nM) or BaP (0.01-1 μM) alone or E2 (10nM) with TCDD (0.0001-0.1nM) or BaP(0.01-1 μM) with or without 0.5μM ANF. For each treatment, half of the exposure solution was renewed every day.

**Measurement of zfcyp19a and zfcyp19b mRNA levels.** At the end of the exposure period, the larvae were immediately placed at 4°C in RNAlater (Sigma Aldrich, St. Quentin Fallavier, France), kept overnight at 4°C, and conserved at -20°C until further processing. The levels of zfcyp19a and zfcyp19b mRNA were measured by a branched DNA assay (QuantigenGene, Genospectra, Fremont, CA, USA), as described previously (Hinfray et al., 2006). Briefly, for each exposure condition, two pools of 10 whole body zebra fish larvae were constituted, lysed, and incubated in a 96 well plate coated with synthetic oligonucleotide in the presence of a specific probe set designed according to the zfcyp19a and zfcyp19b mRNA sequences (gene bank accession numbers AF183906 and AF183908, respectively). The probe set consisted of a capture probe that anchored the target mRNA to the synthetic oligonucleotide, a blocking probe that linearized the target mRNA, and of a label probe that hybridized to the target mRNA and to a branched DNA coupled with alkaline phosphatase bound probes. Finally, a chemiluminescence substrate dioxetan that yields a luminescence signal proportional to the amount of mRNA present in the sample was added. Quantification of luminescence was made on a microplate luminometer (Wallac Victor2, Perkin Elmer, Courteboeuf, France). Zfcyp19a and zfcyp19b expression values were normalized to a housekeeping gene, zebra fish β actin (gene bank accession number NM 131031). For each pool, measurements were performed in duplicate. The experiments were repeated three times on different days.

**Immunohistochemistry.** For each exposure condition, four zebra fish brains were analyzed. At the end of the exposure period, larvae were euthanized in MS 222, fixed in PBS (pH 7.4) containing 4% paraformaldehyde and 0.2% picric acid for 24 h at 4°C, and processed for cryosections (12 μm). Immunohistochemistry was performed as previously described (Menuet et al., 2005). Briefly, tissue sections were incubated overnight at room temperature with the polyclonal zebra fish CYP19B antibody (directed against the synthetic polypeptide CNSNGETADNRTSKE corresponding to the last 15 residues of the protein sequence), diluted at 1:1000. Then the sections were rinsed and incubated with a biotinylated goat anti rabbit IgG (1:15000) and then with a streptavidin peroxidase complex (1:15000). Aromatase immunoreactivity was revealed by using a 3,3’-diaminobenzidine (DAB) nickel intensification protocol (Shu et al., 1988). Stained sections were observed and photographed under an Axioscope photomicroscope (Zeiss, Gottingen, Germany) equipped with a DXC 390P digital camera (Sony, Tokyo, Japan). Visilog 6 software (Noesis, Velizy Villacoublay, France) was used for image acquisition.

**Cell culture.** CHO K1 (Chinese hamster ovary) and U251 MG (human astrocytes) cells were maintained at 37°C under a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium without phenol red (DMEM; Sigma Aldrich) supplemented with 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 25 mg/ml of amphotericin (antibiotic antifungal solution, Sigma Aldrich) and 9% inactivated fetal calf serum (PCS; Life Technologies, Carlsbad, CA). U251 MG medium additionally contained 2μM of t. glutamine (Sigma Aldrich).
Plasmid constructs used for transfection. The following plasmids were obtained from Dr R. E. Peterson at the University of Wisconsin: the reporter plasmid pTrlA luc, containing a dioxin responsive promoter from the rainbow trout cyp1a gene (Abnet et al, 1999) in front of the luciferase coding sequence, and the expression vectors zfAhR2 (Tanguay et al, 1999) and zfARNT2b (Tanguay et al, 2000), containing the full length zebra fish AhR2 (zfAhR2) and ARNT2b (zfARNT2b) coding sequences, respectively, in pBK CMV. The expression vector zfERz contained the coding region of zebra fish ERz (zfERz) in Topo pCDNA3 vector (Menuet et al, 2002). An empty Topo pCDNA3 plasmid (Topo) containing no coding sequence for zebra fish receptors was used to equalize the amount of transfected DNA in the control (no exogenous repressor expression). The control estrogen responsive reporter plasmid pERE TK luc contained an ERE site and a TATA box in front of the luciferase coding sequence. The reporter plasmids cyp19b luc and cyp19b ΔDREdel luc (Menuet et al, 2005) contained 486/+ 34 and 371/+ 34 regions of zfcyp19b promoter/exonI region cloned into pGL2 basic (Promega Corporation, Madison, WI, USA), respectively. Cyp19b ΔDREdel luc was used as a DRE deficient mutant (lacking predicted DRE sites with start positions 453 and 399).

The reporter plasmid cyp19a luc contained 536/+ 37 region of zfcyp19a promoter/exonI, cloned into pGL2 basic vector. Total genomic DNA was obtained from adult zebra fish using conventional protocol (Nuesslein Volhard and Dahm, 2002). The desired fragment was amplified by PCR from genomic DNA using a specific primer set designed according to the sequence of the zfcyp19a promoter previously published (Kazeto et al, 2001), cyp19a fw 5’GGTGACATATAAATGAGCTGGAAG 3’, with nucleotide change (noted by lower case) introduced to create a KpnI site (underlined), and cyp19a rev 5’ACCTCGCATGAAAGCGATGGGAGAAG 3’. The PCR reaction was carried out under the following conditions: 35 cycles consisting of denaturation at 95°C for 1 min, annealing at 65°C for 45 s, extension at 72°C for 45 s, and final extension at 72°C for 10 min. The PCR product was purified and cloned into pGEM Teasy vector (Promega), then subcloned into pGL2 basic vector with KpnI/XbaI.

A QuickChange site directed mutagenesis kit from Promega was used according to manufacturer’s instructions to produce several mutated reporter constructs on the basis of cyp19a luc. Gene Runner (Hastings Software Inc., Hastings on Hudson, NY, USA) was used to reexamine promoter regions. To create the cyp19a ΔEREmut luc (mutated DRE site predicted at 238 bp), 5’GCTGTGATATACCTGGAGAATGCCCAGAGTTGTCTATATC 3’ primer (forward) was used (predicted site underlined, mutated bases denoted by lower case). Mutagenesis was also carried out to create two Apol sites in the cyp19a luc (to cut at the positions 162 bp and 110 bp, for Apol[1] and Apol[2], respectively), which facilitated production of the cyp19a ΔDREdel luc (with the deleted 52 bp region [162/110], containing a putative DRE site, position start 160bp) according to supplier’s recommendation (Tanguay et al, 2000). Briefly, approximately equal amounts of in vitro produced zfAhR and zfARNT2b proteins were incubated in the presence of TCDD 10 nM in DMSO 0.2% or DMSO 0.2% alone for 1 h at 22°C. Following incubation, 1.5 μg poly dI dC and binding buffer (20M HEPES, pH 7.9, 100M NaCl, 1mM DTT, 6% glycerol) were added and the incubation was continued for additional 20 min at 22°C before the addition of approximately 1 ng of the labeled ΔDRE probe with or without 50 fold molar excess of unlabeled ΔDRE probe, followed by purification. Unlabeled competitor DNAs were similarly produced by annealing unlabeled ΔDREwild DRErev, zf a160fw:zf a160rev, zf α238fw:zf α238rev, zf b222fw:zf b222rev, zf b399fw:zf b399rev, and zf b453fw:zf b453rev. An in vitro DNA binding assay was performed essentially as previously described (Tanguay et al, 2000).

Note. Reverse oligos were used reverse complementary to forward oligos. Sequences are given in the 5’ to 3’ order. The letters “a” or “b” correspond to zebra fish cyp19a or cyp19b genes from which the oligos were derived. The number indicates the position of start nucleotide of predicted DRE site in relation to exon1 start in the respective promoter.

In vitro translation and DNA-binding assays. ZfAhR2 and zfARNT2b proteins were produced from vectors zfAhR2 and zfARNT2b, respectively, in TNT rabbit reticulocyte lysate (Promega) according to supplier’s recommen-
dations. Side reactions containing [35S]methionine were performed to assess relative protein production. After the 90 min incubation at 30°C, radioactive translation products were resolved on a 7.5% SDS polyacrylamide gel, dried, and detected on Hyperfilm MP (Amersham Life Sciences, Buckinghamshire, UK), exposed for 12 h. Unlabeled reactions were stored at 70°C prior to functional studies. The sequences of oligonucleotides used for DNA binding assays are listed in Table 1. Oligonucleotide rt DREfw was 5’ end labeled with [32P] using T4 polynucleotide kinase and annealed to a three fold molar excess unlabeled rt DRErev to produce an rt DRE probe, followed by purification.

TABLE 1

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Forward* sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt DREfw</td>
<td>ACCCTTGACGCTATCGGAAT</td>
</tr>
<tr>
<td>zf a 160fw*a,b</td>
<td>ACAGCTGAGGGTTTGGAGTCATGGTAA</td>
</tr>
<tr>
<td>zf a 238fw</td>
<td>ACTCGGAGAGCTTGCGCAAGTG</td>
</tr>
<tr>
<td>zf b 222fw</td>
<td>GGAAAAAGATGTTTGGTCGATAA</td>
</tr>
<tr>
<td>zf b 399fw</td>
<td>CGCATTCTCAGGGCAACATGT</td>
</tr>
<tr>
<td>zf b 453fw</td>
<td>AATAAAAAACGGATTACAGTGACCCCCAC</td>
</tr>
</tbody>
</table>

Note. Predicted DRE sites are underlined.

aReverse oligos were reverse complementary to forward oligos.
bSequences are given in the 5’ to 3’ order.
cThe letters “a” or “b” correspond to zebra fish cyp19a or cyp19b genes from which the oligos were derived.
between data points, which were considered significant at \( p < 0.01 \), unless otherwise noted.

**RESULTS**

**Zfcp19a and zfcyp19b Expression in Zebra Fish Larvae**  
**In Vivo in Response to Exposure to Different Combinations of ER and AhR Ligands**

No increased mortality was observed in zebra fish exposed for 3 days to 10nM E2 or to graded concentrations of TCDD or B[α]P alone or in combination with E2. Exposure to 10nM E2, 0.1nM TCDD, and 0.5μM ANF also was not toxic to the fish. However, the addition of 0.5μM ANF to 10nM E2 and 1μM B[α]P resulted in high mortality. Consequently, no data on the zfcyp19 expression could be obtained for this treatment condition. All measurements of zfcyp19 mRNA levels were performed on whole larvae extracts.

Exposure of zebra fish to 10nM E2 had no effect on zfcyp19a levels but significantly increased the expression of zfcyp19b (Figs. 1A and 1B). Exposure to graded concentrations of TCDD (0.001 0.1nM) or B[α]P (0.01 1lM) did not affect the expression of both zfcyp19 genes (Figs. 1A and 1B, only effect of highest concentration shown). Coexposure to 10nM E2 and 0.1nM TCDD (or 1μM B[α]P) had no effect on the zfcyp19a expression (Figs. 1A and 1B), regardless of whether the coexposure was done in the presence or absence of 0.5μM ANF (Figs. 1A and 1B). A different response was observed for zfcyp19b expression: the addition of 0.1nM TCDD or 1μM B[α]P to 10nM E2 led to significant downregulation of zfcyp19b expression in comparison with the expression induced by E2 alone (Figs. 1A and 1B). The downregulating effect of TCDD could partly be rescued by adding 0.5μM of ANF, a partial antagonist of AhR, to the mixture of E2 and TCDD (Fig. 1A). Furthermore, the changes in the CYP19B protein expression in the brain in response to exposure to several combinations of ER and AhR ligands were assessed by immunohistochemistry using zebra fish CYP19B antibody. In radial glial cells, 10nM E2 strongly induced CYP19B expression, but 0.1nM TCDD did not (Fig. 2). In fish, coexposed to 10nM E2 and 0.1nM TCDD, only a few CYP19B-positive cells were observed. In the group exposed to 10nM E2, 0.1nM TCDD and 0.5μM ANF, the staining was much more intense compared to the E2 + TCDD group, nonetheless, it was still less intense compared to the E2 group (Fig. 2). The expression pattern of CYP19A could not be assessed due to unavailability of specific antibody for this zebra fish protein.

**Zfcyp19a and zfcyp19b Genes Promoter Activity In Vitro in Response to Treatment with Different Combinations of ER and AhR Ligands**

To gain deeper insights into the effects of E2 and TCDD on zebra fish promoters activity, appropriate luciferase reporter gene assays were performed. The concentrations of treatment chemicals used in the in vitro experiments were below the cytotoxicity thresholds. The zfcyp19b promoter was studied in U251-MG (glial) cell line, previously reported as the cell context favorable for its activity (Menuet et al., 2005). Cyp19a-luc was initially transfected in CHO-K1 (ovarian) and U251-MG cells to assess the possible influence of the cell context on the promoter activity. Basal luciferase activity produced by cyp19a-luc in CHO-K1 (ovarian) and U251-MG cells to assess the possible influence of the cell context on the promoter activity. Basal luciferase activity produced by cyp19a-luc in CHO-K1 was about 10 times higher than that in U251-MG. Moreover, treatment with 10nM E2 in the presence of zERz led to upregulation of zfcyp19a promoter activity in CHO-K1 cell line, while no response was observed in U251-MG (Fig. 3). Subsequent studies of this promoter were performed in CHO-K1 cells only.
Comparing the response of zf

**cyp19a**

promoter to E2 with and without cotransfected zfERα, and using E2 antagonist ICI, it was possible to show that ER is needed for the upregulation of this promoter by E2 (Fig. 4). As a control, the empty pGL2-basic vector was subjected to the same analysis. Unexpectedly, it also responded to E2 treatment in the presence of zfERα although the induction magnitude was lower (Fig. 4). Basal level of luciferase activity produced by cyp19a-luc in CHO-K1 cells was 26 ± 7 times higher than that produced by pGL2-basic. It was also higher (eightfold ± twofold) in comparison to cyp19b-luc in CHO-K1 cells. Furthermore, statistical analysis showed that the differences between the response of cyp19a-luc and pGL2-basic to E2 in CHO-K1 cells are significant (p < 0.05). This allowed us to presume that the response of cyp19a-luc to E2 in CHO-K1 cells is zf

**cyp19a**

promoter specific. Since no ERE site is found in the zf

**cyp19a**

promoter, we assumed that the indirect regulation by E2 might occur through the steroidogenic factor 1 (SF1) and half-ERE sites located in close proximity (SF1 at –127 bp and half-ERE at –101 bp from the transcription start). However, studies with mutated reporter gene constructs proved that this is not the case since the E2 response of constructs bearing mutations in the putative SF1 and half-ERE sites was not significantly different from that of the wild-type promoter (data not shown).

Next, the response of the zf

**cyp19a**

and zf

**cyp19b**

promoters to TCDD was investigated. The control dioxin-responsive promoter (prt1A-luc) exhibited dose-dependent response to TCDD treatment in the presence of zfAhR2/zfARNT2b in both CHO-K1 and U251-MG cells (Fig. 5; only one TCDD concentration [10nM] effect is shown). A slight upregulation in response to 10nM TCDD also occurred in the absence of zfAhR2/zfARNT2b. However, this effect was significantly
enhanced in the presence of these receptors (Fig. 5). The functionality of DRE sites predicted in the zf\textit{cyp19a} and zf\textit{cyp19b} promoters was examined using cyp19a-luc and its mutants, cyp19a DREmut-luc and cyp19a DREdel-luc, in CHO-K1 cells (Fig. 5A) and cyp19b-luc and its deletion mutants in U251 MG cells (Fig. 5B). The data are expressed as a fold induction (mean ± SD) relative to control (Topo DMSO 0.1%) and represent a mean of three independent experiments, where each point was performed in triplicates. Data points significantly different from the control (Topo DMSO 0.1%) are marked with “a” (p < 0.01).
mutant, cyp19b DREdel-luc, in U251-MG cells (Fig. 5B). The activity of the zf\textit{cyp19a} promoter and its DRE-deficient mutant was not significantly affected by treatment with 10nM TCDD in the absence of zfAhR2/zfARNT2b (Fig. 5A). However, the activity of the zf\textit{cyp19b} promoter was slightly upregulated in the presence of TCDD and absence of zfAhR2/zfARNT2b (Fig. 5B). The DRE-deficient mutant of zf\textit{cyp19b} promoter was upregulated similarly to wild type, and the E2-responsive promoter containing just an ERE site and a TATA box (pERE-TK-luc) responded to the TCDD treatment in the absence of zfAhR2/zfARNT2b similarly to zf\textit{cyp19b} promoter (Fig. 5B). The activity of zf\textit{cyp19a} and zf\textit{cyp19b} promoters, as well as of the control E2-responsive promoter, was significantly decreased in the presence of zfAhR2/zfARNT2b as compared to activity without expressed exogenous receptors (Fig. 5). The activity of the dioxin-responsive promoter was, on the contrary, upregulated in the presence of zfAhR2/zfARNT2b and absence of TCDD, and the addition of TCDD caused further significant upregulation of this response (Fig. 5). However, the addition of TCDD in the presence of zfAhR2/zfARNT2b did not further affect the activity of zf\textit{cyp19} promoters or that of the control E2-responsive promoter as it neither rescued the downregulation produced by expression of zfAhR2/zfARNT2b nor did it cause further downregulating effects (Fig. 5). The general response pattern of altered constructs did not significantly differ from that of the wild

FIG. 6. The response of the zebra fish \textit{cyp19a} luciferase reporter gene to treatment with E2 or TCDD, alone or in combination, in CHO K1 cells in the presence of zebra fish ER\textsubscript{a}, AhR2, and ARNT2b. Cells were transfected with the zf\textit{cyp19a} luciferase reporter gene (zf\textit{cyp19a} luc) and cotransfected with a control vector (Topo) or vectors expressing zfER\textsubscript{a}, zfAhR2, and zfARNT2b. Transfected cells were treated with DMSO (0.1%) with or without 10nM E2 or 1nM TCDD alone or in combination. The data are expressed as a fold induction (mean ± SD) relative to control (Topo DMSO 0.1%) and represent a mean of three independent experiments, where each point was performed in triplicates. Data points significantly different from the control (Topo DMSO 0.1%) are marked with “a” ($p < 0.01$).

FIG. 7. The response of different reporter genes to treatment with the ligands of ER and/or AhR in U251 MG cells in the presence of zebra fish ER\textsubscript{a}, AhR2, and ARNT2b in U251 MG cells. The cells were transfected with different reporter genes (control estrogen responsive promoter luciferase [pERE TK luc], zf\textit{cyp19b} luciferase [zf\textit{cyp19b} luc], and its DRE deficient mutant [zf\textit{cyp19b} DRE del luc]) and cotransfected with a control vector (Topo) or vectors expressing zfER\textsubscript{a}, zfAhR2, and zfARNT2b. Transfected cells were treated with 10nM E2 or 1nM TCDD alone or in mixture or in combination with 1\mu M ANF or 1\mu M ICI. The data are expressed as a fold induction (mean ± SD) relative to control (Topo DMSO 0.1%) and represent a mean of five independent experiments, where each point was performed in triplicates. In (A), “a” indicates data points that are significantly different from the control (Topo DMSO 0.1%); “b” indicates selected data points that are significantly different from zfER\textsubscript{a}/AhR2/ARNT2b E2 10nM; and “c” indicates selected data points that are significantly different from zfER\textsubscript{a}/AhR2/ARNT2b E2 10nM TCDD 1nM. In (B), “a” indicates data points that are significantly different from zfER\textsubscript{a}/AhR2/ARNT2b E2 10nM TCDD 1nM; “b” indicates data points that are significantly different from zfER\textsubscript{a}/AhR2/ARNT2b DMSO 0.1%; and “c” indicates data points that are significantly different from zfER\textsubscript{a}/AhR2/ARNT2b TCDD 1nM. $p < 0.01$ in all cases.
TCDD alone led to slight but significant upregulation of the zf\textit{cyp19b} promoter \textit{in vitro} in U251-MG cells in the presence of zf\textit{ERα}, zf\textit{AhR2}, and zf\textit{ARNT2b} (Fig. 7B). The addition of ER antagonist IC\textsubscript{1} (1\,\mu\text{M}) or AhR antagonist ANF (1\,\mu\text{M}) eliminated this slight upregulation (Fig. 7B). The DRE-deficient mutant of the zf\textit{cyp19b} promoter and the control E2-responsive promoter exhibited the same general pattern of response to these treatments (Fig. 7B).

DNA-binding assays were performed to further investigate the ability of zebrafish AhR2/ARNT2b heterodimer to bind to the DRE sites predicted in the zf\textit{cyp19a} and zf\textit{cyp19b} promoters. Radioactively labeled probe rt DRE designed on the basis of the rainbow trout \textit{cyp1a} promoter (Tanguay \textit{et al.}, 1999) was used as a control. A strong complex was formed between the zf\textit{AhR2}/zf\textit{ARNT2b} and rt DRE probe in the presence of TCDD (Fig. 8, lane 1). This complex was reported to migrate as duplexes of unknown nature (Tanguay \textit{et al.}, 2000). However, we have observed a single dispersed band migration. The complex was competed by a 50-fold molar excess of unlabeled rt DRE (Fig. 8, lane 2), indicating that DNA binding is specific. None of the competitor oligos containing sequences of DREs predicted in zf\textit{cyp19a} (zf a 160, zf a 238) and zf\textit{cyp19b} (zf b 222, zf b 399, zf b 453) promoters were able to compete with the active complex formation (Fig. 8, lanes 3–7).

\section*{DISCUSSION}

\textbf{The Effects of Estrogens on the zf\textit{cyp19a} Expression}

To establish the cellular system suitable for examining \textit{zf\textit{cyp19a}} promoter activity \textit{in vitro}, two cell lines were transfected with a luciferase reporter driven by this promoter. Data demonstrated that the \textit{zf\textit{cyp19a}} promoter is more active in the ovarian cells context (CHO-K1) in comparison to glial cells (U251-MG). This finding corresponds well to \textit{in vivo} observations since gonads (and especially ovary) were shown to be the main sites of \textit{zf\textit{cyp19a}} expression (Chiang \textit{et al.}, 2001; Fenske \textit{et al.}, 2004; Goto-Kazeto \textit{et al.}, 2004; Hinfray \textit{et al.}, 2006; Sawyer \textit{et al.}, 2006). We have observed a significant upregulation of the \textit{zf\textit{cyp19a}} promoter activity in E2-treated CHO-K1 cells in the presence of zf\textit{ERα}, in contrast to \textit{in vivo} observations in zebrafish larvae where no upregulation of \textit{zf\textit{cyp19a}} by E2 was observed. \textit{In vitro} E2 response was dependent on the presence of zf\textit{ERα} and also on the cellular context, as no upregulation was observed in U251-MG cells, even in the presence of zf\textit{ERα}. Surprisingly, the empty pGL2-basic vector, containing no promoter in front of luciferase-coding sequence, also responded to E2 treatment in the presence of zf\textit{ERα}, although the induction magnitude was significantly lower. Thus, although the observed induction of the \textit{zf\textit{cyp19a}} promoter by E2 may be an artifact induced by the empty vector, significantly higher basal luciferase activity and response to E2 of cyp19a-luc allowed us to presume that this
induction is zfcyp19a promoter specific. The discrepancy between our in vivo and in vitro observations on the zfcyp19a expression in response to E2 can be partially explained by the higher sensitivity of the in vitro reporter gene assays compared to the in vivo assay. Differences between the response of artificial reporter genes and endogenous target genes expression have been observed even in the same cell context (Shipley and Waxman, 2005). It is also possible that the induction of the zfcyp19a expression in vivo occurs only in specific cell types; thus, this effect is masked when measurements are performed on the whole-body homogenates. Moreover, it is well known that response to estrogens depends on several factors other than simple ligand-ER binding, such as ligand transport to target tissues or bioactivation, which may add to differences between in vitro and in vivo estrogenic responses.

Our in vivo results agree with previous studies that have shown the lack of estrogens’ effect on zfcyp19a expression in larvae (Hinfray et al., 2006). In another study, downregulation of zfcyp19a expression in larvae was observed in response to exposure to 1 100nM of EE2 (Kazeto et al., 2004), but the authors suggested that this effect is not mediated directly through the 5'-flanking region of zfcyp19a. Exposure of adult female zebra fish to 10nM of E2 for 7 days also suppressed zfcyp19a expression and aromatase activity in the ovary (Hinfray et al., 2006). On the contrary, in the protandrous black porgy (Acanthopagrus schlegeli), treatment of undifferen-
tiated fish with 6 mg E2/kg for 3 months resulted in an increased gonadal aromatase activity (Lee et al., 2004), but it was not established if the observed increase in aromatase activity was the result of enhanced expression of cyp19a or cyp19b. Cyp19a mRNA levels in the gonad of Rivulus marmoratus were upregulated in response to BPA, but downregulated by NP (Lee et al., 2006). Cyp19a expression was also upregulated in the brain of Atlantic salmon juveniles exposed to EE2 (Lyssimachou et al., 2006). Our results together with published data show that the estrogen effects on cyp19a expression may differ depending on the biological model used (species, in vitro vs. in vivo) as well as on the experimental design employed (life stage of development, mode of fish exposure, examined tissues, and ER ligand used). Further detailed investigation of in vivo effects of estrogens on cyp19a gene expression throughout the course of development might be of interest.

**Examination of the Functionality of DRE Sites**

**Predicted in the zfcyp19a and zfcyp19b Gene Promoters**

Exposure to TCDD had no effect on the zfcyp19a or zfcyp19b mRNA levels in zebra fish larvae in vivo. We also examined the functionality of DRE sites predicted in zfcyp19 promoters in vitro using zfAhR2 and zfARNT2b receptor proteins. ZfAhR2 was shown to be the functional receptor form both in vitro and in vivo (Prasch et al., 2003; Tanguay et al., 1999). ZfARNT1 splice proteins, but not zfARNT2b, were suggested to be the preferred dimerization partners for zfAhR2 in vivo (Prasch et al., 2004, 2006). However, zfARNT2b was shown to form a functional heterodimer with zfAhR2 in vitro that can specifically recognize DREs and induce DRE-driven transcription (Tanguay et al., 2000), which justifies the use of this form of zebra fish ARNT in the present in vitro assays. The activity of the control dioxin-responsive promoter was upregulated by TCDD (or B[a]P) treatment in the presence of zfAhR2/zfARNT2b in the CHO-K1 and U251-MG cells, which confirmed that these cellular systems contain all the co-factors necessary for the functioning of a classical AhR pathway. A slight upregulation in response to AhR agonists also occurred in the absence of zfAhR2/zfARNT2b. However, significantly higher upregulation in the presence of zfAhR2/zfARNT2b suggests that the low levels of endogenous receptors expressed in CHO-K1 and U251-MG cells do not hinder the observation of specific effects of exogenously expressed receptors. In the absence of zfAhR2/zfARNT2b, TCDD treatment did not affect zfcyp19a promoter activity. It did, however, slightly upregulate the activity of zfcyp19b promoter. The DRE-deficient mutant of this promoter was similarly upregulated under these conditions, suggesting the independence of the response observed from the predicted DRE sites. The possible mechanism of this upregulation is discussed in the next subsection. Expression of zfAhR2/zfARNT2b led to significant downregulation of the activity of zfcyp19a and zfcyp19b promoters, their DRE-deficient mutants, and of the control E2-responsive promoter containing only an ERE site. The addition of TCDD (or B[a]P) had no further effect on this downregulation. Thus, the downregulation of promoter activity caused by expression of zfAhR2/zfARNT2b seems to be an unspecific phenomenon, independent of DREs predicted in the zfcyp19a and zfcyp19b promoters. This could be due to the generally increased translation burden on the cells. Interestingly, the activity of the control dioxin-responsive promoter was significantly increased in the presence of zfAhR2/zfARNT2b even in the absence of TCDD, suggesting that functional DRE elements can promote higher basal activity of the promoter in the presence of high levels of AhR and ARNT, even without the ligand. In in vitro DNA-binding assays, zfAhR2/zfARNT2b heterodimer binds to DRE even in the absence of ligand (Tanguay et al., 2000). Thus, it is possible that in the cell culture the exogenously expressed unliganded zfAhR2/zfARNT2b were not readily bound by histones and therefore were able to exert some transcriptional effects on the activity of promoters containing functional DREs. Overall, the pattern of zfcyp19a and zfcyp19b promoters’ response to zfAhR2, zfARNT2b, and TCDD, which drastically differed from that of the control dioxin-responsive promoter, indicated the nonfunctionality of predicted DRE sites. We also demonstrated by electrophoretic mobility shift assays the inability of DREs predicted in the zfcyp19a and zfcyp19b promoters to bind to liganded zfAhR2/zfARNT2b heterodimer. The unresponsiveness of zfcyp19a and zfcyp19b
to TCDD may be due to low conservation of consensus sequence.

Observed unresponsiveness of zf\textit{cyp19a} to AhR agonists stands in agreement with several reports (Hoffmann and Oris, 2006; Kazeto \textit{et al.}, 2004). However, diverse effects of AhR agonists on aromatase in the ovary were observed in other studies. In mice, AhR cooperates with SF1 to activate \textit{cyp19} transcription in ovarian granulosa cells (Baba \textit{et al.}, 2005). TCDD decreases \textit{cyp19} mRNA levels in cultured rat granulosa cells, but the dependence of this effect on DRE-like sites in the promoter was not established (Dasmahapatra \textit{et al.}, 2000). TCDD reduced ovarian aromatase activity in adult female zebra fish (King Heiden \textit{et al.}, 2006). B[a]P inhibited CYP19 in ovary tissue of flounder (\textit{Platichthys flesus}) \textit{in vitro} (Rocha Monteiro \textit{et al.}, 2000) and in adult female killifish ovary \textit{in vivo}, without affecting \textit{cyp19a} mRNA levels (Patel \textit{et al.}, 2006). Thus, exposure to AhR ligands apparently may have an effect on aromatase activity in the ovary, but in respect to an effect on \textit{cyp19a} expression, the evidence is equivocal.

Exposure to B[a]P was shown to increase mRNA levels of \textit{cyp19b} in zebra fish larvae in 3-days exposure assay as well as in adult females exposed from immature stage for 56 days, but it was not the AhR activation but rather the weak estrogenic activity that was suggested to be primarily responsible for this effect (Hoffmann and Oris, 2006; Kazeto \textit{et al.}, 2004). The lack of \textit{zf\textit{cyp19b}} induction by B[a]P in our assay might be due to the lower concentrations used than in the study by Kazeto \textit{et al.} (2004).

Altogether, these data provide evidence that TCDD and B[a]P may possibly alter the expression and/or activity of \textit{cyp19} genes via several mechanisms, which might differ between different organisms. However, our data provide strong evidence that DRE sites predicted in the \textit{zf\textit{cyp19a}} and \textit{zf\textit{cyp19b}} promoters are not functional. Consequently, it is unlikely that either the isoform of the \textit{zf\textit{cyp19}} genes can be used as a biomarker of exposure to dioxin-like compounds.

### Cross Talk between ER and AhR and Its Effects on the \textit{zf\textit{cyp19b}} Expression

E2 strongly upregulated the activity of the \textit{zf\textit{cyp19b}} promoter in the reporter gene assays and the expression of \textit{zf\textit{cyp19b}} in zebra fish larvae, leading to \textit{de novo} synthesis of CYP19B protein in radial glial cells, in agreement with previous studies (Hinfray \textit{et al.}, 2006; Kishida \textit{et al.}, 2001; Menuet \textit{et al.}, 2005). TCDD attenuated the normal E2-induced response of \textit{zf\textit{cyp19b}} expression. This was either partially (\textit{in vivo}) or fully (\textit{in vitro}) rescued by the addition of AhR antagonist, ANF, suggesting the involvement of AhR in the downregulation mechanism. The inability to observe full rescue \textit{in vivo} could be explained by the lower ANF concentration used compared to \textit{in vitro} experiments (due to observed toxicity of higher concentrations in combination with TCDD). It might also reflect the complexity of \textit{in vivo} system setup, including the rates of uptake, metabolism and biodegradation of the chemicals, or the fact that ANF is a partial and not a full antagonist of AhR. Another AhR ligand, B[a]P, also downregulated the \textit{zf\textit{cyp19b}} response to E2 both \textit{in vivo} and \textit{in vitro} in our study and to EE2 in the other (Kazeto \textit{et al.}, 2004). The effect of cotreatment with ANF on the B[a]P-induced changes in the E2 response of \textit{zf\textit{cyp19b}} could not be measured because the exposure to E2, B[a]P, and ANF led to high mortality of the larvae, as was also observed by others (Billiard \textit{et al.}, 2006). However, \textit{in vitro} observations suggested the involvement of AhR in this mechanism. Induced changes in \textit{zf\textit{cyp19b}} mRNA levels were closely paralleled on the protein level, thus confirming the functional significance of our findings on the effects of ER and AhR ligands on the \textit{zf\textit{cyp19b}} expression.

In our \textit{in vitro} studies, TCDD downregulated the E2-induced response driven from a single ERE site on the promoter, independent of putative DRE sites or any other transcription sites, as was shown by experiments with the DRE-deficient mutant of the \textit{zf\textit{cyp19b}} promoter and with the E2-responsive promoter containing an ERE site, only. Similar results were recently obtained with mammalian receptors, showing that AhR agonists attenuate E2-induced transcription from a single ERE site, without binding directly to ERs or affecting expression levels of ERs (Ohtake \textit{et al.}, 2003). Evidence of a similar mode of action in teleosts has also been observed in another study (Bemanian \textit{et al.}, 2004).

Previous studies on the antiestrogenic effect of AhR ligands in fish focused mainly on hepatic E2 target genes involved in vitellogenesis (Anderson \textit{et al.}, 1996; Navas and Segner, 2000). To our knowledge, the present work provides the first report showing the antiestrogenic effect of an AhR agonist on an E2-regulated gene within a glial cell context. The biological significance of this effect is not known. However, radial glial cells are known to play a crucial role in embryonic and adult neurogenesis (Götz \textit{et al.}, 2002), and E2 is an important neurotrophic and neuroprotective factor. Further, it has been shown that AhR is expressed in the areas of fish brain involved in neuroendocrine regulation of reproductions, such as hypothalamus and gonadotropin cells (Ortiz-Delgado \textit{et al.}, 2002). Therefore, it can be hypothesized that disruption of the normal E2-induced expression of aromatase (and hence of local synthesis of E2) in radial glial cells may be a significant pathway for neuroendocrine effects of AhR ligands in vertebrate.

Exposure to TCDD or B[a]P alone had no effect on the \textit{zf\textit{cyp19b}} mRNA levels in zebra fish larvae or on the \textit{zf\textit{cyp19b}} promoter-driven luciferase expression in U251-MG cell line in the presence of zfAhR2/zfARNT2b \textit{in vitro}, however, a slight upregulation of this gene’s promoter activity by TCDD was observed in the presence of zfERx, zfAhR2, and zfARNT2b proteins. This effect was blocked by cotreatment with an excess of either ER antagonist ICI or AhR antagonist ANF, suggesting the involvement of both ER and AhR in the process. Experiments with the DRE-deficient mutant of the \textit{zf\textit{cyp19b}} promoter...
and the control E2-responsive promoter, containing one ERE site only, suggested that this mechanism is independent of predicted DRE sites, and only the ERE site is involved in this type of ER and AhR interaction. It was shown that liganded mammalian AhR/ARNT heterodimer can directly associate with unliganded ER, leading to stimulation of ERE-mediated transcription (Ohtake et al., 2003), in agreement with our present findings. In light of this, the slight upregulation of the zfcyp19b promoter activity and its DRE-deficient mutant as well as of the control E2-responsive promoter, observed in the presence of TCDD without any expressed zebra fish receptors, can be explained by the presence of low levels of mammalian ER, AhR, and ARNT, which were able to engage in the same kind of action. The differences between fold induction numbers observed in our study and the study of Ohtake et al. (2003) can be attributed to either the structural differences between mammalian and fish receptors, which may account for differences in relative potencies of ligands between mammals and fish (Abnet et al., 1999), or to the different cellular contexts used and the different promoters studied. We observed the weak estrogenic effect of TCDD in vitro only in the absence of the ER ligand, as was also shown by others (Ohtake et al., 2003). Thus, the inability to observe upregulation of endogenous zfcyp19b gene expression by TCDD in vivo in zebra fish larvae can be explained by the presence of endogenous estrogens, which preclude potential estrogenic actions of dioxins.

In summary, we have evaluated the effects of ER and AhR ligands on the activity of zfcyp19a and zfcyp19b genes by in vivo exposure of zebra fish larvae and by in vitro luciferase reporter gene assays. The zfcyp19a gene was not affected by treatment with E2 in vivo. However, a slight upregulation of this gene promoter was observed in vitro, the mechanism of which could not be clarified at the moment. In our study, AhR ligands, TCDD, or B[a]P did not affect zfcyp19a and zfcyp19b expression in vivo, and in vitro experiments suggested the nonfunctionality of predicted DRE sites. However, we have shown the attenuation of normal E2-induced upregulation of zfcyp19b expression by AhR ligands both in vivo and in vitro. We confirmed the involvement of AhR in this mechanism and were able to show in vitro its independence from the putative DRE sites predicted in the zfcyp19b promoter. This inhibition of E2-induced zfcyp19b expression in radial glial cells is a novel mode of AhR ligands action, which points to the possible disruption of neuroendocrine functions of estrogens as one of the toxic effects of this compound. We have also observed a slight induction of zfcyp19b promoter activity by TCDD in vitro, which seems to function in accordance with the mechanism where liganded AhR/ARNT associates with unliganded ER and triggers transcription from the ERE site. Thus, here we show that in fish, as it has been demonstrated in mammals, the levels of ER agonist estrogen might determine the estrogenicity or antiestrogenicity of AhR agonists in relation to estrogen-responsive genes, and that ER/AhR cross talk follows several pathways, not always depending only on the presence of DRE sites in the gene promoters. These findings should be taken into account while interpreting the results of studies investigating the estrogen-related actions of AhR ligands, especially, in mixtures. Further investigation of interactions between ER, AhR, their ligands, and gene promoters, as well as characterization of coregulators of this process, might greatly facilitate the research on the estrogen-related actions of dioxins.

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