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Expression of Zebra Fish 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 zebra fish *cyp19a* (zf*cyp19a*) and *cyp19b* (zf*cyp19b*) genes, encoding aromatase P450, an important steroidogenic enzyme. *In vivo* (mRNA quantification in exposed zebra fish larvae) and *in vitro* (activity of zf*cyp19*-luciferase reporter genes in cell cultures in response to chemicals and zebra fish transcription factors) assays were used. None of the treatments affected zf*cyp19a*, excluding the slight upregulation by E2 observed *in vitro*. Strong upregulation of zf*cyp19b* 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 zf*cyp19b* expression in the brain was also observed on the protein level, assessed by immunohistochemistry. TCDD alone did not affect zf*cyp19b* expression *in vivo* or promoter activity in the presence of zebra fish AhR2 and AhR nuclear translocator 2b (ARNT2b) *in vitro*. However, in the presence of zebra fish 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* zebra fish larvae and *in vitro* zf*cyp19b* reporter gene assays for evaluation of estrogenic chemical actions, provides data on the functionality of DREs predicted in zf*cyp19* promoters and shows the effects of cross talk between ER and AhR on zf*cyp19b* expression. The antiestrogenic effect of TCDD demonstrated raises further concerns about the neuroendocrine effects of AhR ligands.

Key Words: **endocrine disruption; aromatase CYP19; zebra fishestrogen 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 zebra fish (*Danio rerio*). Zebra fish *cyp19a* (zf*cyp19a*) is predominantly expressed in gonads, and zebra fish *cyp19b* (zf*cyp19b*) 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 zf*cyp19* promoters in zebra fish 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 zf*cyp19b* promoter, half-EREs, and aryl hydrocarbon responsive elements (also called dioxin-responsive elements, DREs) are predicted in

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zf*cyp19a* and zf*cyp19b* promoters (Kazeto *et al.*, 2001; Tong and Chung, 2003). Functionality of ERE in the zf*cyp19b* 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 zf*cyp19* 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 Bradfield, 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 to 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 Bradfield, 1996). The prototypic AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic compound among HAHs and also the most potent AhR activator (Schmidt and Bradfield, 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.

Zf*cyp19* 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 zf*cyp19* 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 zf*cyp19* genes expression and to examine the functionality of DRE sites predicted in the zf*cyp19* promoters, (2) to study the effects of AhR activators on the expression of an estrogen-regulated target gene in fish brain (zf*cyp19b*), and thus (3) to reveal the possible cross talk pathway between the ERs and the AhR/ARNT complex and its effects on zf*cyp19* 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 LGC Promochem SARL (Molsheim, France). Benzo[*a*]pyrene (B[*a*]P), α naphthoflavone (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 B[*a*]P (0.01 1μM) alone or E2 (10nM) with TCDD (0.0001 0.1nM) or B[*a*]P (0.01 1μM) with or without 0.5μM ANF. For each treatment, half of the exposure solution was renewed every day.

Measurement of zf*cyp19a *and zf*cyp19b *mRNA levels. At the end of the exposure period, the larvae were immediately placed at 4°C in RNA*later* (Sigma Aldrich, St. Quentin Fallavier, France), kept overnight at 4°C, and conserved at 20°C until further processing. The levels of zf*cyp19a* and zf*cyp19b* mRNA were measured by a branched DNA assay (QuantiGene, 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 zf*cyp19a* and zf*cyp19b* 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 Victor^2, Perkin Elmer, Courteboeuf, France). Zf*cyp19a* and zf*cyp19b* 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 (1:1500) and then with a streptavidin peroxidase complex (1:1500). 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 Axioskope photomicroscope (Zeiss, Gottingen, Germany) equipped with a DXC 390P digital camera (Sony, Tokyo, Japan). Visilog 6 software (Noesis, Vélizy 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 antimycotic solution, Sigma Aldrich) and 9% inactivated fetal calf serum (FCS; Life Technologies, Carlsbad, CA). U251 MG medium additionally contained 2mM of L 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 prt1A 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 zfERα contained the coding region of zebra fish ERα (zfERα) 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 re ceptor 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 DREdel luc (Menuet *et al.*, 2005) contained 486/+ 34 and 371/+ 34 regions of zf*cyp19b* promoter/exonI region in pGL2 basic (Promega Corporation, Madison, WI, USA), respectively. Cyp19b DREdel 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 zf*cyp19a* 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 zf*cyp19a* promoter previously published (Kazeto *et al.*, 2001), cyp19a fw 5′ GGTACcTATCAATAATGAGCCTGGA 3′, with nucleotide change (noted by lower case) introduced to create a *Kpn*I site (underlined), and cyp19a rev 5′ ACCTGCCATAAGAACGGATGGAGA 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 *Kpn*I/*Sac*I.

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 DREmut luc (mutated DRE site predicted at 238 bp), 5′ GCTGTAGAATAACTCGAGGAaaTGCCGCAGATGTCTAATATC 3′ primer (forward) was used (predicted site underlined, mutated bases denoted by lower case). Mutagenesis was also carried out to create two *Apa*I sites in the cyp19a luc (to cut at the positions 162 bp and 110 bp, for *Apa*I[1] and *Apa*I[2], respectively), which facilitated production of the cyp19a DREdel luc (with the deleted 52 bp region [162/ 110], containing a putative DRE site, position start 160bp [Kazeto *et al.*, 2001] or 154 [Tong and Chung, 2003]). The primers (forward) 5′ GAAAACCCAGAGATGACTTGCACGGgCcCGAGG GTTTGAGTGTCATGG 3′ and 5′ GAAACTCGACGCTGAAgggcCcAAAG GAGCACACAAGG 3′ were used to produce *Apa*I(1) and *Apa*I(2), respectively (created *Apa*I sites underlined, mutated bases denoted by lower case). Reverse primers used for site directed mutagenesis were reverse complementary to forward primers. All the inserted and mutated sequences were verified by sequencing using the Big Dye Terminator protocol (Synergene Biotech GmbH, Schlieren, Switzerland).

The normalization vector CMV gal, containing a β galactosidase coding sequence cloned after the constitutive promoter, was used to account for transfection efficiency from well to well.

Transfection experiments. The cells were trypsinized and seeded in 24 well plates in fresh medium 6 h before the transfection (concentration of cells 2×10^4/ml). All transfections were performed using a FuGene6 reagent according to manufacturer′s instructions (Roche Diagnostics, Indianapolis, IN). The DNA templates for transfection contained 150 ng of reporter vector, 15 ng of expression vectors, zfERα, zfAhR2, or zfARNT2b, or the same amount of Topo as control, and 50 ng of normalization vector CMV gal. The medium was replaced with fresh DMEM containing 2% of charcoal stripped FCS 12 h after the transfection. Vehicle control (DMSO 0.1%) with or without treatment chemicals was added to the medium. The cells were collected for reporter gene assays 48 h later. The medium was removed by vacuum aspiration, each well was washed with PBS, and 150 µl of Reporter Lysis Buffer (Promega) was added. Plates were frozen at 80°C and assayed at least 2 h later. Luciferase activity was assayed with BrightGlo luciferase substrate (Promega). The activity of β galactosidase was assayed with 2 nitrophenyl β D galactopyranoside (Fluka Chemica Biochemica, Buchs, Switzerland). Each experimental point was performed in triplicates, and the experiments were repeated three times on different days, unless otherwise noted.

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 [^{35}S]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 ^{32}P using T4 polynucleotide kinase and annealed to a three fold molar excess unlabeled rt DRErev to produce an rt DRE probe, followed by purification. Unlabeled competitor DNAs were similarly produced by annealing unlabeled rt DREfw:rt DRErev, zf a160fw:zf a160rev, zf a238fw:zf a238rev, 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). Briefly, approximately equal amounts of *in vitro* produced zfAhR2 and zfARNT2b proteins were incubated in the presence of TCDD 10nM 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 (20mM HEPES, pH 7.9, 100mM NaCl, 1mM DTT, 6% glycerol) were added and the incubation con tinued for additional 20 min at 22°C before the addition of approximately 1 ng of the labeled rt DRE probe with or without 50 fold molar excess of unlabeled rt DRE, zf a160, zf a238, zf b222, zf b399, or zf b453 competitor oligos. Following 20 min incubation at 22°C, complexes were resolved on a 0.5× TBE (90mM Tris, 64.6mM boric acid, and 2.5 EDTA, pH 8.3) 4% acrylamide gel at 4°C. The signal was detected on Hyperfilm MP exposed for 12 h.

Statistical analysis. To evaluate *in vivo* exposure data, nonparametric ANOVA followed by Mann Whitney *U* test was used. The differences were considered significant at $p < 0.05$. For evaluation of *in vitro* data, a normalized luciferase activity was determined for each data point by dividing the luciferase activity by β galactosidase activity and presented as a fold induction (mean ± SD) over control. The data were checked for normality (normal distribution of data was confirmed in all cases), and one way ANOVA followed by Tukey Honestly Significant Difference Test was applied to analyze the differences

TABLE 1
Oligonucleotide Probes Used for DNA-Binding Experiments

Oligo	Forward[a] sequence[b]
rt DREfw	ACCTTTGCACGCTATCGAAAT
zf a 160fw[c,d]	ACAGCTGAGGGTTTGAGTGTCATGGTAA
zf a 238fw	ACTCGAGGACGTGCCGCAGATG
zf b 222fw	GGAAAAGATGTGTGTTTCGCATTAA
zf b 399fw	CGCATTTCTCAGGCAACATTGT
zf b 453fw	AATAAAAACGGATTACATGTACCCCACC

Note. Predicted DRE sites are underlined.

[a]Reverse oligos were reverse complementary to forward oligos.

[b]Sequences are given in the 5′ 3′ order.

[c]The letters "a" or "b" correspond to zebra fish *cyp19a* or *cyp19b* genes from which the oligos were derived.

[d]The number indicates the position of start nucleotide of predicted DRE site in relation to exonI start in the respective promoter.

between data points, which were considered significant at $p < 0.01$, unless otherwise noted.

RESULTS

*Zf*cyp19a *and zf*cyp19b *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[*a*]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[*a*]P resulted in high mortality. Consequently, no data on the zf*cyp19* expression could be obtained for this treatment condition. All measurements of zf*cyp19* mRNA levels were performed on whole larvae extracts.

Exposure of zebra fish to 10nM E2 had no effect on zf*cyp19a* levels but significantly increased the expression of zf*cyp19b* (Figs. 1A and 1B). Exposure to graded concentrations of TCDD (0.001 0.1nM) or B[*a*]P (0.01 1μM) did not affect the expression of both zf*cyp19* genes (Figs. 1A and 1B, only effect of highest concentration shown). Coexposure to 10nM E2 and 0.1nM TCDD (or 1μM B[*a*]P) had no effect on the zf*cyp19a* 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 zf*cyp19b* expression: the addition of 0.1nM TCDD or 1μM B[*a*]P to 10nM E2 led to significant downregulation of zf*cyp19b* 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.

*Zf*cyp19a *and zf*cyp19b *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

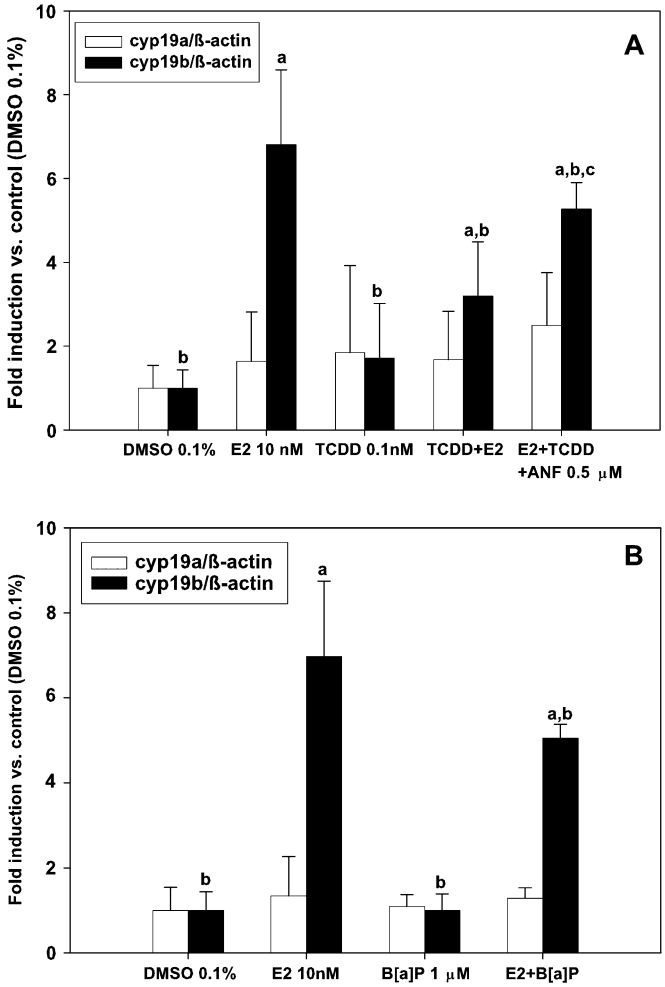

FIG. 1. The changes in *cyp19a* and *cyp19b* mRNA levels (normalized to β actin mRNA levels) in zebra fish larvae in response to exposure to 10nM E2 and 0.1nM TCDD (A) or 1μM B[*a*]P (B) alone or in combination, with or without 0.5μM ANF. Results shown are expressed as a fold induction (mean ± SD) rel ative to solvent control (DMSO 0.1%) and represent a mean of three indepen dent experiments, where each point was performed in duplicate measurements. The "a" in (A) and (B) indicates data points that are significantly different from solvent control; "b" in (A) and (B) indicates data points that are significantly different from E2 10nM; "c" in (A) indicates selected data point that is significantly different from E2 10nM + TCDD 1nM; $p < 0.05$ in all cases.

chemicals used in the *in vitro* experiments were below the cytotoxicity thresholds. The zf*cyp19b* 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 was about 10 times higher than that in U251-MG. Moreover, treatment with 10nM E2 in the presence of zfERα led to upregulation of zf*cyp19a* 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.

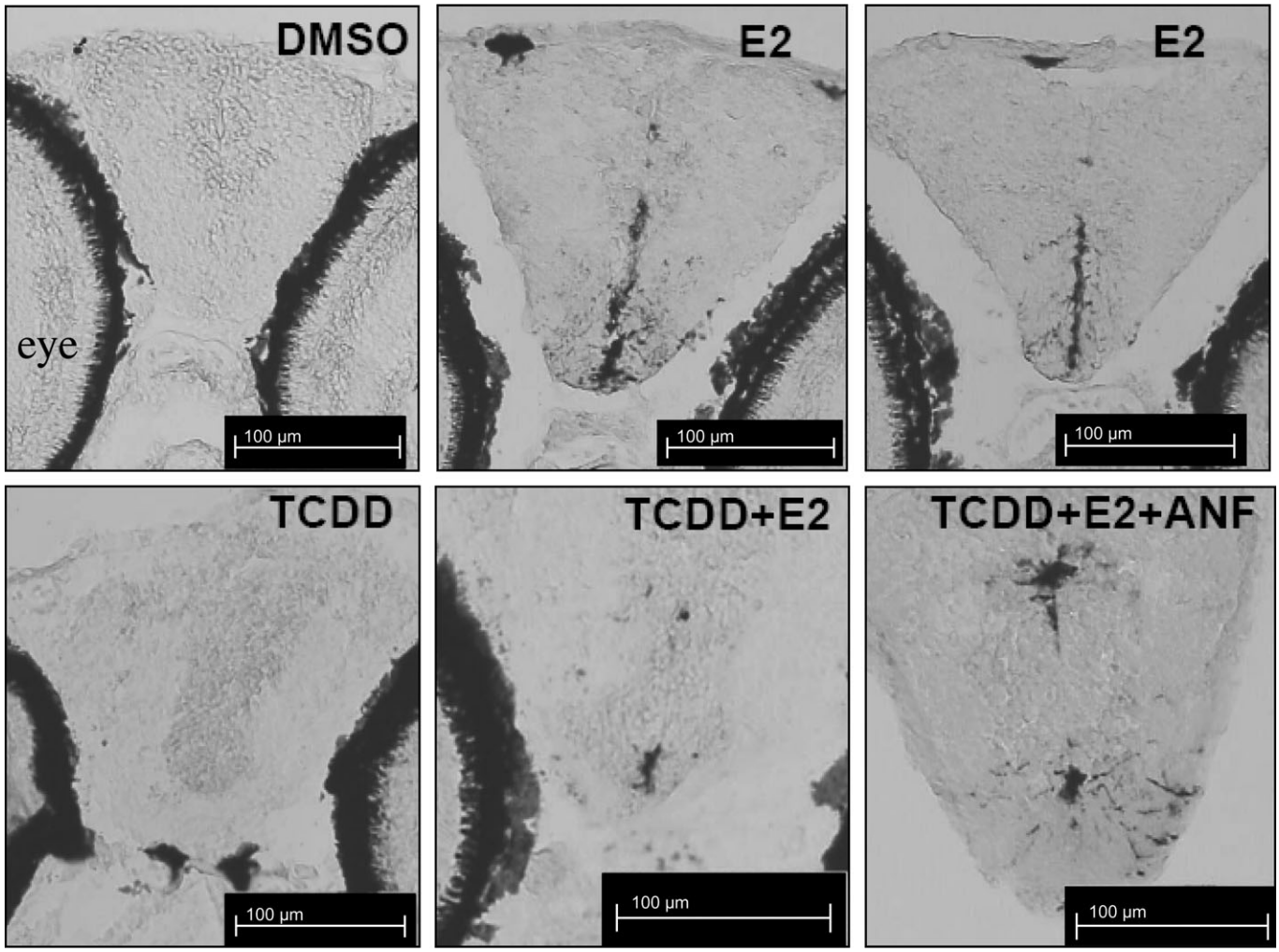

FIG. 2. CYP19B protein expression in the brain of zebra fish larvae (20 dpf) after 72 h exposure to DMSO 0.1% (DMSO), E2 10nM (E2), TCDD 0.1nM (TCDD), E2 10nM + TCDD 0.1nM (E2 + TCDD) or E2 10nM + TCDD 0.1nM + ANF 0.5µM (E2 + TCDD + ANF). Transverse sections (12 µm) were stained with zebra fish polyclonal CYP19B antibody. Four brains were analyzed per group; representative pictures are shown. No CYP19B immunoreactivity was observed in DMSO or TCDD exposed fish. Numerous CYP19B positive radial glial cells were observed in the brain of E2 treated fish, while only few cells were stained in fish exposed to E2 + TCDD. The addition of partial AhR antagonist ANF to the E2 + TCDD partially restored the levels of CYP19B expression.

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

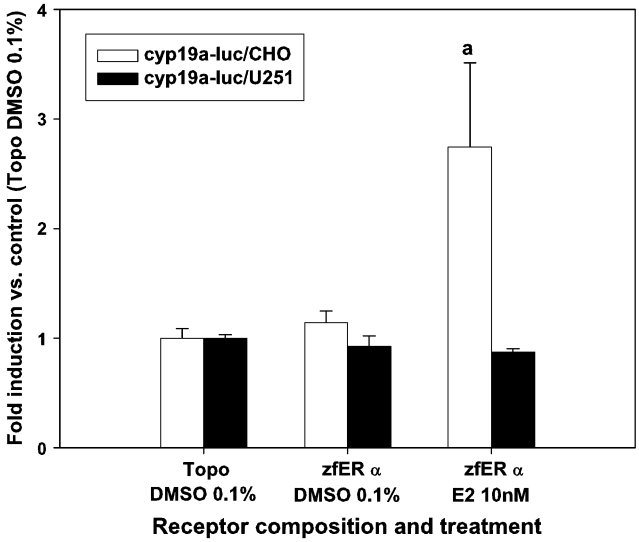

FIG. 3. The response of zebra fish *cyp19a* promoter to treatment with E2 in CHO K1 and U251 MG cells. Cells were transfected with the zf*cyp19a* luciferase reporter gene (cyp19a luc) and cotransfected with a control vector (Topo) or a vector expressing zfERα. Transfected cells were treated with DMSO 0.1% with or without E2 10nM. The data are expressed as a fold induction (mean ± SD) relative to control (Topo DMSO 0.1%) and represent a mean of three (in CHO K1) or two (in U251 MG) independent experiments, where each point was performed in triplicates. The data point significantly different from the control (Topo DMSO 0.1%) is marked with "a" ($p < 0.01$).

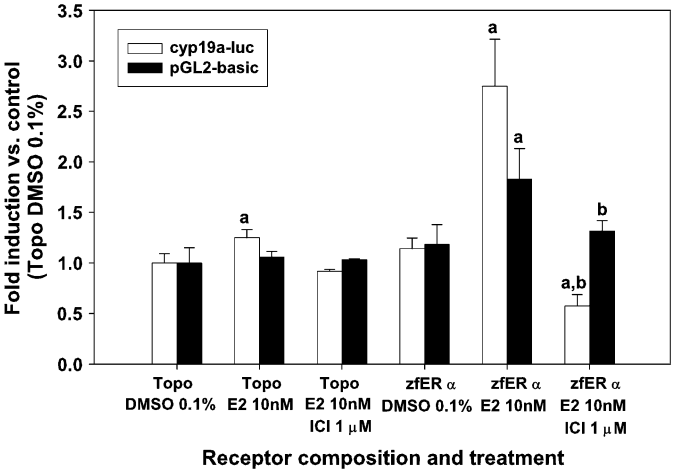

FIG. 4. The effect of zebra fish ERα and its agonist (E2) and antagonist (ICI) on the activity of zebra fish *cyp19a* promoter and pGL2 basic vector in CHO K1 cells. Cells were transfected with the zfcyp19a luciferase reporter gene (cyp19a luc) or with the pGL2 basic vector and cotransfected with a control vector (Topo) or a vector expressing zfERα. Transfected cells were treated with DMSO 0.1% with or without E2 10nM or E2 10nM and ICI 1μM. 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"; selected data points significantly different from zfERα E2 10nM are marked with "b"; $p < 0.01$ in all cases.

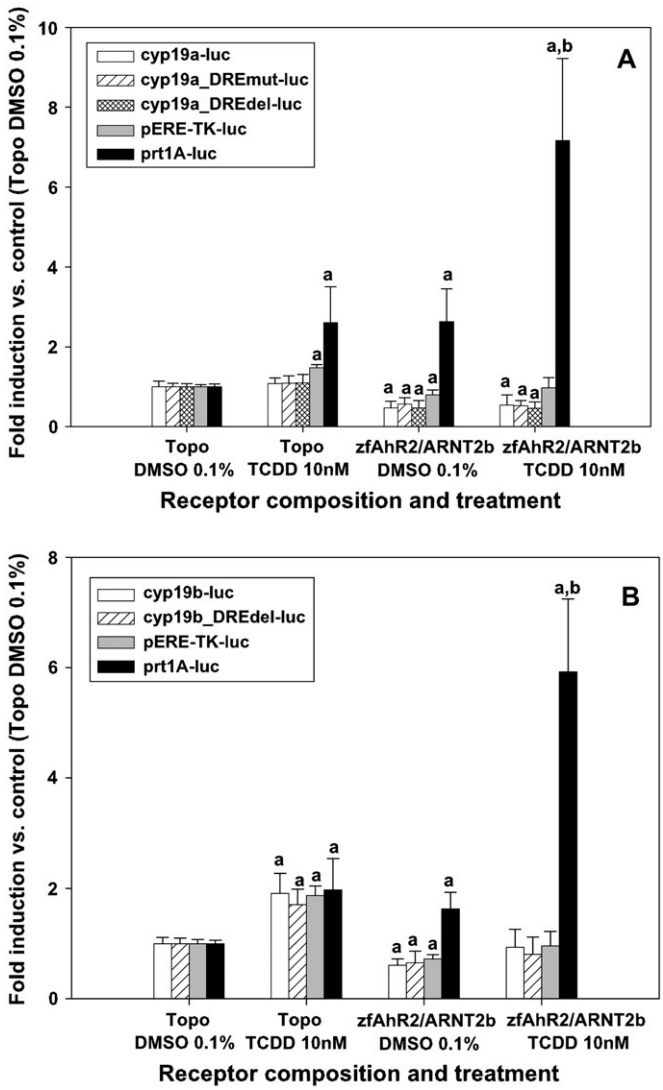

FIG. 5. The response of different reporter genes to treatment with TCDD in the presence of zebra fish AhR2 and ARNT2b in CHO K1 and U251 MG cells. Cells were transfected with different reporter genes (control dioxin responsive promoter luciferase [prt1A luc] and control estrogen responsive promoter luciferase [pERE TK luc] in CHO K1 [A] and U251 MG [B] cells; zfcyp19a luciferase [cyp19a luc] and its DRE deficient mutants [cyp19a DREmut luc and cyp19a DREdel luc] in CHO K1 cells [A]; zfcyp19b luciferase [cyp19b luc] and its DRE deficient mutant [cyp19b DREdel luc] in U251 MG cells [B]) and cotransfected with a control vector (Topo) or vectors expressing zfAhR2 and zfARNT2b. Transfected cells were treated with DMSO 0.1% with or without TCDD 10nM. 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"; selected data points significantly different from zfAhR2/zfARNT2b DMSO 0.1% are marked with "b"; $p < 0.01$ in all cases.

enhanced in the presence of these receptors (Fig. 5). The functionality of DRE sites predicted in the zf*cyp19a* and zf*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

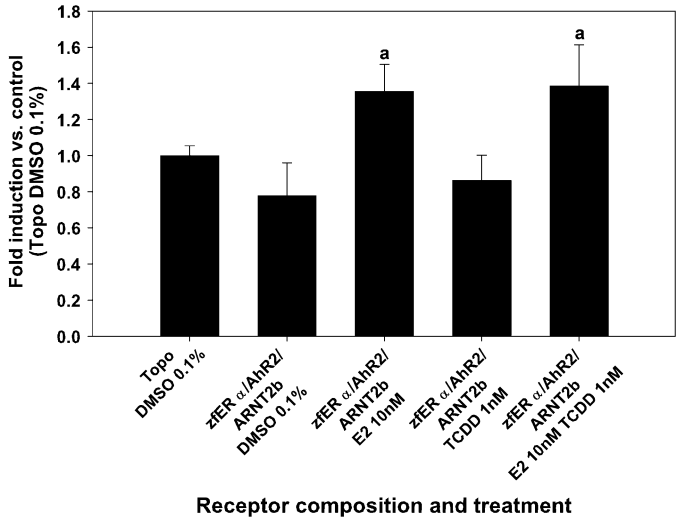

FIG. 6. The response of the zebra fish *cyp19a* luciferase reporter gene to treatment with E2 or TCDD, alone or in combination, in CHO K1 cells in the presence of zebra fish ERα, AhR2, and ARNT2b. Cells were transfected with the zf*cyp19a* luciferase reporter gene (cyp19a luc) and cotransfected with a control vector (Topo) or vectors expressing zfERα, 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" ($p < 0.01$).

mutant, cyp19b DREdel-luc, in U251-MG cells (Fig. 5B). The activity of the zf*cyp19a* promoter and its DRE-deficient mutants was not significantly affected by treatment with 10nM TCDD in the absence of zfAhR2/zfARNT2b (Fig. 5A). However, the activity of the zf*cyp19b* promoter was slightly upregulated in the presence of TCDD and absence of zfAhR2/zfARNT2b (Fig. 5B). The DRE-deficient mutant of zf*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*cyp19b* promoter (Fig. 5B). The activity of zf*cyp19a* and zf*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*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

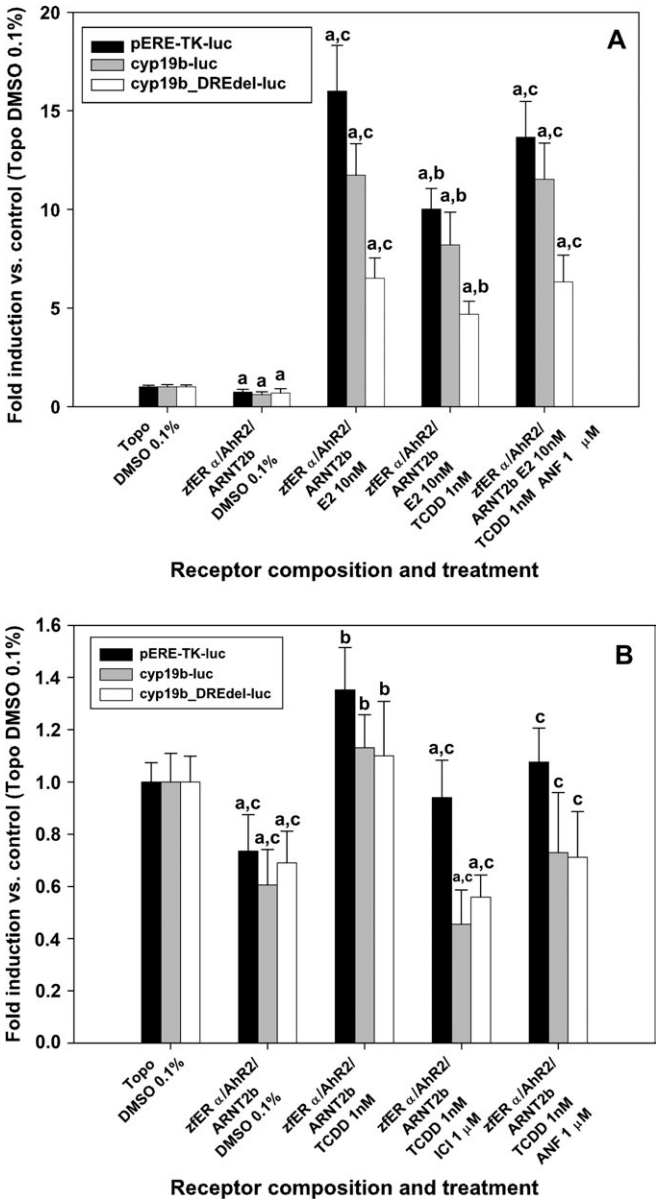

FIG. 7. The response of different reporter genes to treatment with the ligands of ER and/or AhR in U251 MG cells in the presence of zebra fish ERα, AhR2, and ARNT2b in U251 MG cells. The cells were transfected with different reporter genes (control estrogen responsive promoter luciferase [pERE TK luc], zf*cyp19b* luciferase [cyp19b luc], and its DRE deficient mutant [cyp19b DRE del luc]) and cotransfected with a control vector (Topo) or vectors expressing zfERα, zfAhR2, and zfARNT2b. Transfected cells were treated with 10nM E2 or 1nM TCDD alone or in mixture or in combination with 1μM ANF or 1μM 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 zfERα/AhR2/ARNT2b E2 10nM; and "c" indicates selected data points that are significantly different from zfERα/AhR2/ARNT2b E2 10nM TCDD 1nM. In (B), "a" indicates data points that are significantly different from the control (Topo DMSO 0.1%); "b" indicates data points that are significantly different from zfERα/AhR2/ARNT2b DMSO 0.1%; and "c" indicates data points that are significantly different from zfERα/AhR2/ARNT2b TCDD 1nM. $p < 0.01$ in all cases.

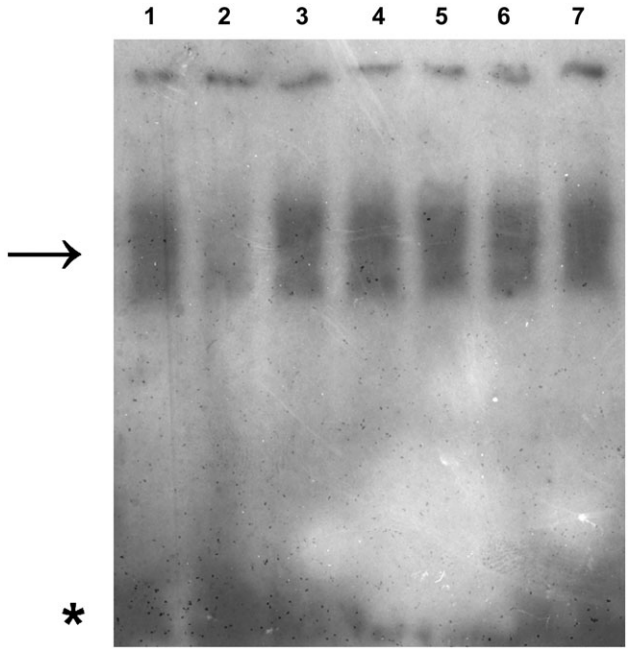

FIG. 8. Electrophoretic mobility shift assay of zebra fish AhR2 and ARNT2b interactions with DRE sites predicted in zebra fish *cyp19a* and *cyp19b* promoters. *In vitro* translated zfAhR2 and zfARNT2b proteins were incubated with 10nM TCDD and γ^{32}P labeled oligo rt DRE derived from a DRE in the rainbow trout *cyp1a* promoter. In lane 2, 50 fold molar excess of unlabeled competitor oligo rt DRE was added, and 50 fold molar excess of unlabeled competitor oligos derived from putative DREs in zf*cyp19a* and zf*cyp19b* promoters, zf a 160, zf a 238, zf b 222, zf b 399 and zf b 453, was added in lanes 3 7. The experiment was repeated two times on different days, representative picture is shown. The arrow indicates the position of specific zebra fish AhR2/ARNT2b rt DRE complexes. The asterisk indicates the position of the free probe.

type (Fig. 5). B[*a*]P (0.1 10μM) acted similarly to TCDD in these experiments (data not shown).

In the presence of zfERα, zfAhR2, and zfARNT2b, E2 response of zf*cyp19a* promoter activity was not significantly changed by the addition of 1nM TCDD (Fig. 6) or 1μM B[*a*]P (data not shown). However, coexposure to 10nM E2 and 1nM TCDD in the presence of zfERα, zfAhR2, and zfARNT2b led to significant downregulation of normal E2 response of the zf*cyp19b* promoter (Fig. 7A), which was rescued by the addition of 1μM ANF. In order to evaluate whether the effect of TCDD is dependent on the putative DRE sites present in the zf*cyp19b* promoter, we compared the responses of a wild-type zfcyp19b promoter and its DRE-deficient mutant. The two constructs exhibited the same general pattern of response to the treatments with ER and AhR ligands (Fig. 7A). The lower E2-induction magnitude of the cyp19b DREdel-luc is due to deletion of half-ERE site upstream of the full ERE, as was shown previously (Menuet *et al.*, 2005). Furthermore, we were able to show that a control E2-responsive promoter containing just an ERE site (pERE-TK-luc) also exhibited the same general response pattern to E2, TCDD, and ANF treatments (Fig. 7A).

TCDD alone led to slight but significant upregulation of the zf*cyp19b* promoter *in vitro* in U251-MG cells in the presence of zfERα, zfAhR2, and zfARNT2b (Fig. 7B). The addition of ER antagonist ICI (1μM) or AhR antagonist ANF (1μM) eliminated this slight upregulation (Fig. 7B). The DRE-deficient mutant of the zf*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 zebra fish AhR2/ARNT2b heterodimer to bind to the DRE sites predicted in the zf*cyp19a* and zf*cyp19b* promoters. Radioactively labeled probe rt DRE designed on the basis of the rainbow trout *cyp1a* promoter (Tanguay *et al.*, 1999) was used as a control. A strong complex was formed between the zfAhR2/zfARNT2b and rt DRE probe in the presence of TCDD (Fig. 8, lane 1). This complex was reported to migrate as duplets of unknown nature (Tanguay *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*cyp19a* (zf a 160, zf a 238) and zf*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).

DISCUSSION

*The Effects of Estrogens on the zf*cyp19a *Expression*

To establish the cellular system suitable for examining zf*cyp19a* promoter activity *in vitro*, two cell lines were transfected with a luciferase reporter driven by this promoter. Data demonstrated that the zf*cyp19a* promoter is more active in the ovarian cells context (CHO-K1) in comparison to glial cells (U251-MG). This finding corresponds well to *in vivo* observations since gonads (and especially ovary) were shown to be the main sites of zf*cyp19a* expression (Chiang *et al.*, 2001; Fenske and Segner, 2004; Goto-Kazeto *et al.*, 2004; Hinfray *et al.*, 2006; Sawyer *et al.*, 2006). We have observed a significant upregulation of the zf*cyp19a* promoter activity in E2-treated CHO-K1 cells in the presence of zfERα, in contrast to *in vivo* observations in zebra fish larvae where no upregulation of zf*cyp19a* by E2 was observed. *In vitro* E2 response was dependent on the presence of zfERα and also on the cellular context, as no upregulation was observed in U251-MG cells, even in the presence of zfERα. Surprisingly, the empty pGL2-basic vector, containing no promoter in front of luciferase-coding sequence, also responded to E2 treatment in the presence of zfERα, although the induction magnitude was significantly lower. Thus, although the observed induction of the zf*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 zf*cyp19a* promoter specific. The discrepancy between our *in vivo* and *in vitro* observations on the zf*cyp19a* 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 zf*cyp19a* 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 zf*cyp19a* expression in larvae (Hinfray *et al.*, 2006). In another study, downregulation of zf*cyp19a* 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 zf*cyp19a*. Exposure of adult female zebra fish to 10nM of E2 for 7 days also suppressed zf*cyp19a* expression and aromatase activity in the ovary (Hinfray *et al.*, 2006). On the contrary, in the protandrous black porgy (*Acanthopagrus schlegeli*), treatment of undifferentiated 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). C*yp19a* 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 zf*cyp19a *and zf*cyp19b *Gene Promoters*

Exposure to TCDD had no effect on the zf*cyp19a* or zf*cyp19b* mRNA levels in zebra fish larvae *in vivo*. We also examined the functionality of DRE sites predicted in zf*cyp19* 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 cofactors 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 zf*cyp19a* promoter activity. It did, however, slightly upregulate the activity of zf*cyp19b* 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 zf*cyp19a* and zf*cyp19b* 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 zf*cyp19a* and zf*cyp19b* 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 zf*cyp19a* and zf*cyp19b* 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 zf*cyp19a* and zf*cyp19b* promoters to bind to liganded zfAhR2/zfARNT2b heterodimer. The unresponsiveness of zf*cyp19a* and zf*cyp19b*

to TCDD may be due to low conservation of consensus sequence.

Observed unresponsiveness of zf*cyp19a* to AhR agonists stands in agreement with several reports (Hoffmann and Oris, 2006; Kazeto *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 *cyp19* transcription in ovarian granulosa cells (Baba *et al.*, 2005). TCDD decreases *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 *et al.*, 2000). TCDD reduced ovarian aromatase activity in adult female zebra fish (King Heiden *et al.*, 2006). B[*a*]P inhibited CYP19 in ovary tissue of flounder (*Platichthys flesus*) *in vitro* (Rocha Monteiro *et al.*, 2000) and in adult female killifish ovary *in vivo*, without affecting *cyp19a* mRNA levels (Patel *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 *cyp19a* expression, the evidence is equivocal.

Exposure to B[*a*]P was shown to increase mRNA levels of *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 *et al.*, 2004). The lack of zf*cyp19b* induction by B[*a*]P in our assay might be due to the lower concentrations used than in the study by Kazeto *et al.* (2004).

Altogether, these data provide evidence that TCDD and B[*a*]P may possibly alter the expression and/or activity of *cyp19* genes *via* several mechanisms, which might differ between different organisms. However, our data provide strong evidence that DRE sites predicted in the zf*cyp19a* and zf*cyp19b* promoters are not functional. Consequently, it is unlikely that either the isoform of the zf*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 zf*cyp19b *Expression*

E2 strongly upregulated the activity of the zf*cyp19b* promoter in the reporter gene assays and the expression of zf*cyp19b* in zebra fish larvae, leading to *de novo* synthesis of CYP19B protein in radial glial cells, in agreement with previous studies (Hinfray *et al.*, 2006; Kishida *et al.*, 2001; Menuet *et al.*, 2005). TCDD attenuated the normal E2-induced response of zf*cyp19b* expression. This was either partially (*in vivo*) or fully (*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 *in vivo* could be explained by the lower ANF concentration used compared to *in vitro* experiments (due to observed toxicity of higher concentrations in combination with TCDD). It might also reflect the complexity of *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 zf*cyp19b* response to E2 both *in vivo* and *in vitro* in our study and to EE2 in the other (Kazeto *et al.*, 2004). The effect of cotreatment with ANF on the B[*a*]P-induced changes in the E2 response of zf*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 *et al.*, 2006). However, *in vitro* observations suggested the involvement of AhR in this mechanism. Induced changes in zf*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 zf*cyp19b* expression.

In our *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 zf*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 *et al.*, 2003). Evidence of a similar mode of action in teleosts has also been observed in another study (Bemanian *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 *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 *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 *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 zf*cyp19b* mRNA levels in zebra fish larvae or on the zf*cyp19b* promoter driven luciferase expression in U251-MG cell line in the presence of zfAhR2/zfARNT2b *in vitro*. However, a slight upregulation of this gene's promoter activity by TCDD was observed in the presence of zfERα, 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 zf*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 zf*cyp19b* 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 zf*cyp19b* 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 zf*cyp19a* and zf*cyp19b* genes by *in vivo* exposure of zebra fish larvae and by *in vitro* luciferase reporter gene assays. The zf*cyp19a* 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 zf*cyp19a* and zf*cyp19b* 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 zf*cyp19b* 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 zf*cyp19b* promoter. This inhibition of E2-induced zf*cyp19b* 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 zf*cyp19b* 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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