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Detection of Thyroid System–Disrupting Chemicals Using *in Vitro* and *in Vivo* Screening Assays in *Xenopus laevis*

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We developed a thyroid hormone (TH) inducible primary screening assay for the identification and assessment of man-made chemicals that interfere with the TH-signalling pathway within target cells. The assay was developed in a *Xenopus laevis* cell line that was transduced with a self-inactivating (SIN) lentivirus vector (LV) containing a luciferase gene. The luciferase activation in this cell line was TH-specific: 3,3′,5-L-triiodothyronine (T_3) > 3,3′5-L-triiodothyroacetic acid (Triac) > 3,3′,5-D-triiodothyronine (D-T_3), > L-thyroxine (T_4) > 3,3′,5′-L-triiodothyronine (rT_3). The application of the ligand-dependent luciferase assay for screening for thyroid system-disrupting chemicals revealed that three phthalates (dicyclohexyl phthalate, *n*-butylbenzyl phthalate, and di-*n*-butyl phthalate), two herbicides (ioxynil and pentachlorophenol) and a miticide (dicofol) had 3,3′,5-L-triiodothyronine-T_3- antagonist activity at concentrations ranging from 10^{-6} to 10^{-5} M. These chemicals also inhibited the expression of the endogenous primary T_3-response TH nuclear receptor β (TRβ) gene. The inhibitory characteristics of these chemicals were similar for both assays performed, although the assay for T_3-dependent activation of TRβ gene was more sensitive than the luciferase assay. These results indicate that the luciferase assay was a rapid method with a small intra-assay variation for the primary screening of thyroid system-disrupting chemicals. Of the six chemicals, only *n*-butylbenzyl phthalate and pentachlorophenol exhibited T_3-antagonist activity in an *in vivo* metamorphosis-based assay. It should be noted that chemicals elicited thyroid system-disrupting activity in the luciferase assay did not always interfere with the thyroid system *in vivo*.

Key Words: **endocrine toxicology – endocrine; thyroid, gene expression/regulation – receptor; nuclear hormone, endocrine toxicology – endocrine disruptors.**

Over the past several decades amphibian populations worldwide have tended to decrease, the number of malformed frogs has increased, and some species at certain localities have been extirpated (Houlahan *et al.*, 2000; Ouellet *et al.*, 1997). Possible causes for these trends include natural population fluctuation, fungal pathogens, increased ultraviolet radiation, climate change, acid precipitation, loss of suitable habitats by human activity, and/or natural and man-made environmental contaminants (Baustein and Wake, 1995; Burkhart *et al.*, 2000; Wake, 1991). It is not clear what impact each of these possible causes has had upon amphibian populations; however, it is likely that environmental contaminants have had the greatest impact as amphibians are generally considered more sensitive to aquatic contaminants due to the permeability of their skin.

Xenopus laevis is a water-living amphibian (all of its life stages occur in water) that is used widely as a laboratory animal. Its development and the expression of its genes are well characterized. For these reasons, *X. laevis* has been approved as an experimental model for evaluating the effects of endocrine-disrupting chemicals (EDCs) in amphibians by the Organization for Economic Cooperation and Development. A critical process in the amphibian life cycle is metamorphosis that is obligatorily controlled by thyroid hormones (THs). This process is also influenced by several environmental factors, such as the density of the population, habitat dessication, temperature, and food availability (Denver, 1997).

THs are essential for normal brain development and behavior in higher vertebrates (Howdeshell, 2002; Zoeller *et al.*, 2002) and postembryonic development in lower vertebrates (Dickhoff *et al.*, 1990). However, there have been relatively few reports concerning the molecular mechanisms by which EDCs disrupt the thyroid system despite reports of structural lesions in thyroid follicular cells and irregular levels of THs in experimental animals and wildlife (Brouwer *et al.*, 1998; Colborn, 2002) exposed to EDCs. These results suggest that there may be thyroid system–disrupting chemicals in the environment not yet identified. Our laboratory has been interested in studying the effect of EDCs, especially those that interfere with the amphibian thyroid system *in vitro* including TH-binding to the major plasma TH-carrier protein, transthyretin (Ishihara *et al.*, 2003; Kudo and Yamauchi, 2005; Yamauchi *et al.*, 2000, 2002, 2003), cellular TH-uptake (Shimada and Yamauchi, 2004) and TH-binding to TH nuclear receptor (TR) (Ishihara *et al.*, 2003; Kudo and Yamauchi, 2005; Shimada and

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Yamauchi, 2004; Yamauchi *et al.*, 2002, 2003); and *in vivo* including *X. laevis* metamorphosis (Kudo and Yamauchi, 2005). Assay methods mechanistically distinct from ours have also been used by others to investigate thyroid system–disrupting chemicals (Crump *et al.*, 2002; DeVito *et al.*, 1999; Veldhoen and Helbing, 2001). Together, these studies revealed that EDCs target differentially several processes within the thyroid system in a chemical-dependent manner. Bioassay methods capable of identifying thyroid system–disrupting chemicals and their effects, as a total sum of each effect on individual processes, within the thyroid system are essential for the primary screening of thyroid system–disrupting chemicals. Furthermore, although there are advantages and limitations in each bioassay, as suggested by Zacharewski (1998), important criteria for primary screening bioassays may be high throughput with low cost.

In the present study, we established a permanent *X. laevis* recombinant cell line as a bioassay for screening thyroid system-disrupting chemicals using a self-inactivation (SIN) lentivirus vector (LV) containing a TH response element (TRE)-SV40 promoter-luciferase reporter gene. We chose nine suspect EDCs, most of which are known to affect at least one of the following processes: 3,3′,5-L-triiodothyronine- (T_3-) binding to transthyretins and TRs, and cellular T_3-uptake in amphibian systems (Ishihara *et al.*, 2003; Shimada and Yamauchi, 2004; Yamauchi *et al.*, 2002); and investigated their effects on the ligand-dependent luciferase assay, T_3-dependent activation of the TRβ gene using the same cell line and T_3-induced tadpole metamorphosis *in vivo*. Given the advantages of the luciferase assay, high sensitivity, rapidity and high reproducibility, its use as a high throughput primary screening system would provide large-scale assessment of environmental chemicals as thyroid system disruptors.

MATERIALS AND METHODS

Reagents. T_3, (>98% purity), L-thyroxine (T_4; >98%), 3,3′5-L-triiodothyroacetic acid (Triac; 95%), 3,3′,5-D-triiodothyronine (D-T_3; >98%), 3,3′,5′-L-triiodothyronine (rT_3; 95%), 17β-estradiol (E_2; >98%), and pentachlorophenol (>99% purity) were obtained from Sigma (St. Louis, MI). Bisphenol A (>98% purity), dicyclohexyl phthalate (>99% purity), dicofol (Kelthane, 2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol, analytical standard, >95% purity), and ioxynil (3,5-diiodo-4-hydroxybenzonitril, analytical standard, >99% purity) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and diethyl phthalate (>99% purity) and di(2-ethylhexyl) phthalate (>99% purity) were from Kanto Chemicals (Tokyo, Japan). Di-*n*-butyl phthalate (>98% purity) and *n*-butylbenzyl phthalate (>97% purity) were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals used in this study were either chromatography grade or the highest grade available and were purchased from Wako Pure Chemical Industries or Nacalai Tesque (Kyoto, Japan).

All chemicals tested as EDCs, except for E_2 which was dissolved in ethanol to a concentration of 1 mM, were dissolved in dimethylsulfoxide to a concentration of 10 mM. These chemicals were diluted with an appropriate buffer to give less than 0.5% (v/v) solvent. A control assay without the test chemicals was performed in the presence of the solvent alone and at less than 0.5% (v/v). The solvent did not affect the luciferase assay, the T_3-dependent activation of TRβ gene, cell viability nor T_3-induced tadpole metamorphosis.

Luciferase assay in a permanent* Xenopus laevis *recombinant cell line. A human immunodeficiency virus-1-based LV pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV-G) was prepared by transient transfection of the following plasmids: packaging construct, pMDLg/pRRE; VSV-G- and Rev- expressing construct, pCMV-VSV-G-RSV-Rev; and SIN LV construct, pCS-TRE-Luc-CG, as described elsewhere (Sugiyama *et al.,* in press). This LV contains the firefly luciferase gene under the control of the SV40 promoter and the *X. laevis* TREs. The structures of the TREs and pCS-TRE-Luc-CG are illustrated in Figure 1. Subconfluent monolayers of *X. laevis* XL58 cells were transduced with the viral vector at a multiplicity of infection of 10, and incubated in 70% Leibovitz's L-15 medium containing 10% fetal bovine serum (FBS) for 15 h at 25°C with air. A cell clone, XL58-TRE-Luc, with luciferase highly expressed in a T_3-dependent manner was isolated (Sugiyama *et al.*, in press).

XL58-TRE-Luc cells were cultured in FBS-free 70% Leibovitz's L-15 medium with or without 2 nM T_3 in the presence or absence (control) of each EDC for 24 h, following incubation in 70% Leibovitz's L-15 medium containing 10% resin-stripped FBS (Samuels *et al.*, 1979) for 15 h. Their cell lysate was assayed for firefly luciferase activity using the PicaGene Luminescence kit (Nippon Gene, Tokyo, Japan). Cell viability was examined using the Cell Count Reagent SF kit (Nacalai Tesque), according to the manufacturer's directions, based on the method described by Ishiyama *et al.* (1997). Protein concentration was measured by the Bradford dye-binding method using bovine γ-globulin as the standard (Bradford, 1976).

Metamorphosis assay. *Xenopus laevis* tadpoles were purchased from Akita Xenopus Co. (Ibaraki, Japan). Tadpoles were staged according to Nieuwkoop and Faber (1975). The metamorphosis assay was performed as described previously (Kudo and Yamauchi, 2005). In brief, five tadpoles in NF stages 51–52 were transferred into a 1 L glass beaker containing 0.5 L of FETAX buffer (625 mg/L NaCl, 96 mg/L $NaHCO_3$, 30 mg/L KCl, 15 mg/L $CaCl_2$, 60 mg/L $CaSO_4.2H_2O$ and 75 mg/L $MgSO_4.7H_2O$, pH 7.7) (Dumont *et al.*, 1983). The FETAX buffer contained dimethylsulfoxide, T_3 in dimethylsulfoxide or T_3 and each chemical in dimethylsulfoxide. Tadpoles were cultured in the FETAX buffer at 22–24°C. FETAX buffer containing the chemical applications was renewed every other day. Final dimethylsulfoxide concentrations were less than 0.02% in the chemical exposed and control

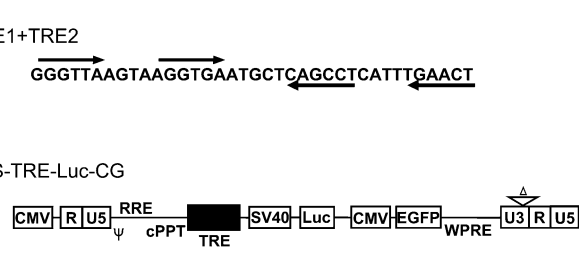

FIG. 1. Vector construction. (A) *Xenopus laevis* thyroid hormone response elements (TREs) located in the promoter region of TH/bZIP gene. Arrows indicate sequences related to the AGGTCA core half-site. The TREs (TRE1 + TRE2; Furlow and Brown, 1999) were introduced into pGL2 promoter vector to construct pGL2-TRE, as described in Materials and Methods. (B) Schematic representation of the construction of self-inactivating (SIN) LV plasmid. The RfA region of the parent SIN LV plasmid pCS-RfA-CG (Miyoshi *et al.*, 1998) was replaced with the TRE-SV40-Luc region in pGL2-TRE through pENTR4 to construct pCS-TRE-Luc-CG. CMV, cytomegalovirus promoter; ψ, packaging signal; RRE, rev responsive element; cPPT, central polypurine tract: SV40, SV40 promoter; Luc, firefly luciferase gene; EGFP, enhanced green fluorescent protein gene. WRRE, woodchuck hepatitis virus posttranscriptional regulatory element, Δ: deleting 133 bp in the U3 region of the 3′ long terminal repeat.

groups. After five days, all living tadpoles were frozen in liquid nitrogen and then stored at −80°C until RNA preparation.

Real-time polymerase chain reaction. Total RNA was extracted from XL58-TRE-Luc cells using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987) and from the frozen tadpoles using the LiCl-urea procedure (Auffray and Rougeon, 1980). To confirm its integrity, RNA (5 μg per lane) was electrophoresed in a 1% agarose gel containing 2.6 M formaldehyde, and 28S and 18S rRNA were visualized by ethidium bromide staining. The quantity of specific RNA species in each sample was estimated by real-time polymerase chain reaction (PCR) using SYBR Green Master Mix and ABI Prism 7000 (Applied Biosystems, Foster City, CA) after the RNA samples had been treated with reverse transcriptase (TaqMan Reverse Transcription Reagents, Applied Biosystems), as previously described (Kudo and Yamauchi, 2005). To standardize each experiment, the amount of TR transcript in each sample was divided by the amount of glyceraldehyde-phosphate dehydrogenase (GAPDH) transcript in the same sample. The primer sequences used were as follows: *X. laevis* TRα transcript (accession number: X17385) sense 5′-CAAGCACCAAGAACGAAAACC-3′ (nucleotide numbers 530–555) and antisense 5′-TTGGAAGGTCTGCTCATTCTTCTA-3′ (600–580); *X. laevis* TRβ transcript (accession number: M35356 and M35357) sense 5′-CAAGCACCAAGAACGAAAACC-3′ (nucleotide numbers 15–35) and antisense 5′-TTGGAAGGTCTGCTCATTCTTCTA-3′ (39–16); and *X. laevis* GAPDH transcript (accession number: V41753) sense 5′-CTCATGACAA-CAGTCCATGCTTTC-3′ (558–581) and antisense 5′-CTCTGCCATCTCTC-CACAGCTT-3′ (639–618).

Statistical analysis. The data are presented as mean ± SEM. Differences between groups were analyzed with either Student's *t*- test or Cochran-Cox test to evaluate the significance of the differences. $p < 0.05$ was considered statistically significant.

RESULTS

Ligand-Specific Luciferase Assay in XL58-TRE-Luc Cells

Figure 2 shows the specificity of the ligand-dependent luciferase assay in this recombinant cell line. Luciferase activity in XL58-TRE-Luc cells was induced 3.1- and 3.3-fold in the presence of 2 and 20 nM T_3, respectively. Triac was less potent than T_3 at the low concentration but was as potent as T_3 at the high concentration. T_4 and D-T_3 were moderately potent while rT_3 was less potent than the above chemicals. E_2 elicited no statistically significant effect on the induction of the luciferase activity in our cell culture system. These results indicate that this luciferase gene specifically responds to THs.

Effects of EDCs on the T_3-Dependent Luciferase Assay Using XL58-TRE-Luc Cells

To determine which phthalates affect the T_3-dependent luciferase activity, XL58-TRE-Luc cells were incubated with or without 2 nM T_3, in the presence or absence of various concentrations of each phthalate. In the absence of EDCs and presence of T_3, the luciferase activity increased by 3.0-fold. Figure 3 shows dose-effects of five phthalates on the T_3-dependent luciferase activity. Of the five phthalates, dicyclohexyl phthalate was the most potent antagonist with a 50% inhibitory concentration (IC_{50}) of 11 ± 3 μM ($n = 3$). The IC_{50} rank order of antagonist activity was dicyclohexyl phthalate > *n*-butylbenzyl phthalate (40 ± 6 μM, $n = 3$) = di-*n*-butyl phthalate (39 ± 1 μM, $n = 3$) > di(2-ethylhexyl) phthalate (>50 μM, $n = 3$) > diethyl phthalate (>50 μM, $n = 3$). The effect of ioxynil, pentachlorophenol, dicofol, and bisophenol A on the T_3-dependent luciferase assay was also investigated (Fig. 4). Of these chemicals, ioxynil was the most potent antagonist with an IC_{50} of 0.9 ± 0.3 μM ($n = 3$) with significant potency even at 0.2 μM. The antagonist activities of pentachlorophenol and dicofol were weaker with less than 50% inhibition at their

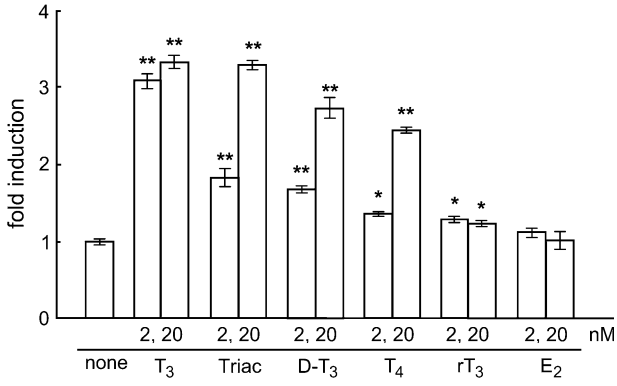

FIG. 2. TH-dependent luciferase assay in recombinant XL58-TRE-Luc cells. XL58-TRE-Luc cells were cultured in the FBS-free medium in the presence or absence (control) of T_3, Triac, D-T_3, T_4, rT_3, and E_2 at the indicated concentrations for 24 h at 25°C. The cell lysate was assayed for the luciferase activity. The vertical axis represents the ratio of the luciferase activity of the chemical-treated samples to that of the control samples. Experiments for each chemical were repeated at least twice with similar results. Each value is the means ± SEM of triplicate determinations. Statistically significant differences were $*p < 0.05$ and $**p < 0.001$, compared with the values of the control samples.

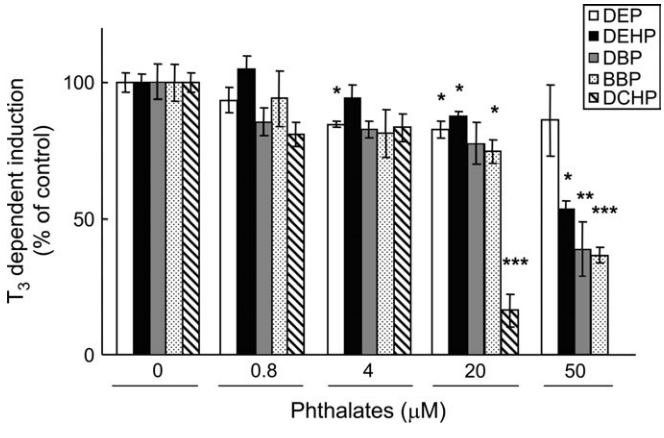

FIG. 3. Effect of phthalates on the T_3-dependent luciferase assay. XL58-TRE-Luc cells were treated with or without 2 nM T_3 for 24 h at 25°C, in the presence or absence (control) of various concentrations of each phthalate for 24 h. The vertical axis represents the T_3-dependent induction of luciferase activity as a percentage of control. DEP, diethyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DBP, di-*n*-butyl phthalate; BBP, *n*-butylbenzyl phthalate; DCHP, dicyclohexyl phthalate. Experiments for each chemical were repeated at least three times. Each value is the mean ± SEM of triplicate determinations. Statistically significant differences were $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

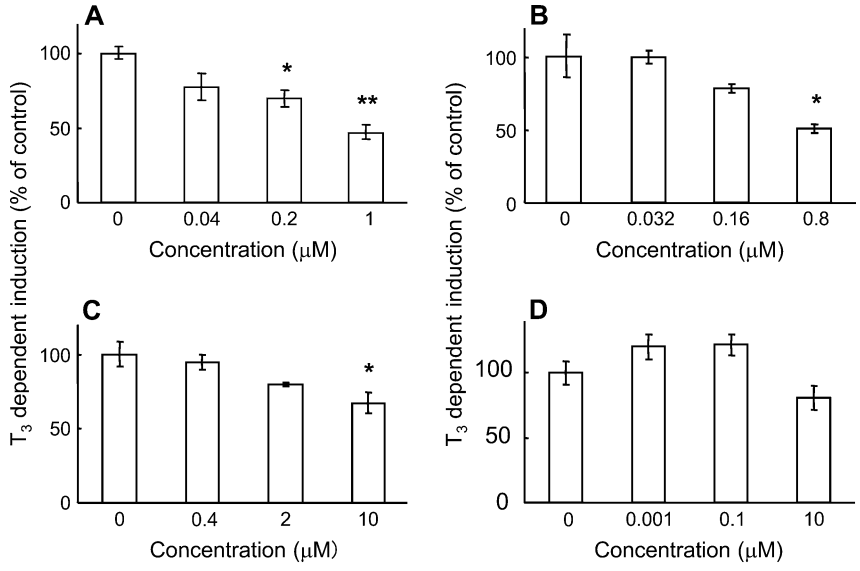

FIG. 4. Effect of other EDCs on the T_3-dependent luciferase assay. XL58-TRE-Luc cells were treated with or without 2 nM T_3 for 24 h at 25°C, in the presence or absence (control) of various concentrations of each EDC for 24 h. The vertical axis represents the T_3-dependent induction in the luciferase activity as a percentage of control. EDCs include ioxynil (A), pentachlorophenol (B), dicofol (C), and bisphenol A (D). Experiments for each chemical were repeated at least three times. Each value is the mean ± SEM of triplicate determinations. Statistically significant differences were $*p < 0.05$ and $**p < 0.01$.

maximum concentrations, 0.8 and 10 μM, respectively. Bisphenol A failed to elicit statistically significant responses at the concentrations tested (0.001–10 μM). Activation of luciferase activity was not detected when the chemicals were applied in the absence of T_3. All of these chemicals did not affect cell viability at the highest concentrations tested under our experimental conditions (data not shown).

*Effects of EDCs on the T_3-Dependent Activation of TR*β *Gene in XL58-TRE-Luc Cells*

To investigate whether EDCs that disrupted the cellular T_3-signaling pathway in the T_3-dependent luciferase assay also affected other T_3-response genes, the transcripts of endogenous primary T_3-response TR genes were analyzed by real-time PCR. XL58-TRE-Luc cells were grown with or without 2 nM T_3 in the presence or absence of various concentrations of each EDC. The TRα transcript was not induced by T_3 in XL58-TRE-luc cells under our experimental conditions. In the absence of EDCs and presence of T_3, the amount of TRβ transcript increased by 13.3 ± 0.5 ($n = 5$) fold. This T_3-dependent activation was significantly inhibited by all chemicals tested except for bisphenol A at the indicated or higher concentrations (Fig. 5). The inhibition percentages obtained from independent repeated experiments were 30 ± 7% diethyl phthalate (50 μM, $n = 3$), 29 ± 5% di(2-ethylhexyl) phthalate (50 μM, $n = 3$), 32 ± 5% di-*n*-butyl phthalate (50 μM, $n = 3$), 45 ± 4% *n*-butylbenzyl phthalate (50 μM, $n = 3$), 42 ± 6% dicyclohexyl phthalate (20 μM, $n = 3$), 45 ± 4% ioxynil (0.1 μM, $n = 3$), 63 ± 4% pentachlorophenol (0.08 μM, $n = 3$), and 32 ± 5% dicofol (10 μM, $n = 3$). *n*-Buthylbenzyl phthalate, even at the concentration of 4 μM, were still potent, with more than 50% inhibition. Activation of the TRβ gene was not detected when the chemicals were applied in the absence of T_3.

*Effects of EDCs on the T_3-Dependent Activation of TR*β *Gene in* Xenopus laevis *Tadpoles*

To examine whether the potent chemicals selected from the above *in vitro* assays affect the thyroid system *in vivo*, *X. laevis*

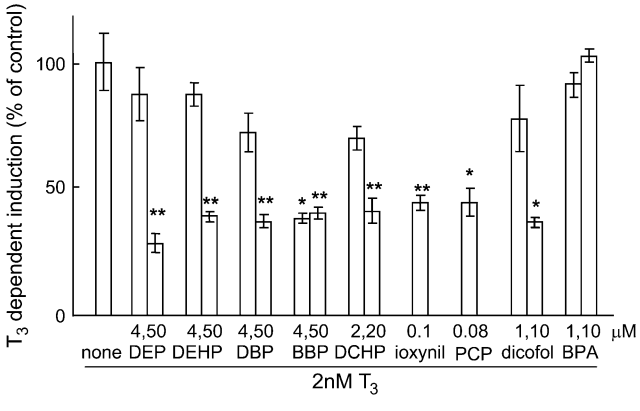

FIG. 5. Effect of EDCs on the T_3-dependent activation of TRβ gene in XL58-TRE-Luc cells. Cells were treated with or without 2 nM T_3 for 24 h at 25°C, in the presence or absence (control) of each EDC at the defined concentrations. RNA was extracted from the cells, and TRβ transcript levels were analyzed by real-time PCR. The vertical axis represents a T_3-dependent induction in the amount of TRβ transcript as a percentage of control. DEP, diethyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DBP, di-*n*-butyl phthalate; BBP, *n*-butylbenzyl phthalate; DCHP, dicyclohexyl phthalate; PCP, pentachlorophenol; BPA, bisphenol A. Experiments for each chemical were repeated at least three times. Each value is the mean ± SEM of triplicate determinations. Statistically significant differences were $*p < 0.05$ and $**p < 0.01$.

tadpoles in NF stages 51–52 tadpoles were immersed in FETAX buffer alone or in FETAX buffer containing 2 nM T_3 with or without each chemical, and cultured for five days at 22–24°C. Total RNA was then extracted from tadpoles in each group and the amount of TRβ transcript was quantified by real-time PCR (Fig. 6). At the end of the experiments, positive control (T_3-treated), test (T_3 and chemical-treated), and negative control (T_3-untreated) tadpoles were in NF stages 54, 53–54, and 51–52, respectively. No tadpoles died during the culture period. The amount of TRβ transcript increased 12.4 ± 0.7 times (data not shown) in T_3-treated tadpoles, which was less than half of the increase reported in the previous *in vivo* study (Kudo and Yamauchi, 2005). Co-treatment of T_3 with *n*-butylbenzyl phthalate (4 μM) or pentachlorophenol (0.1 μM) significantly inhibited the T_3-induced increase in the amount of TRβ transcript. The inhibition percentages obtained from independent repeated experiments were 48 ± 3% *n*-butylbenzyl phthalate ($n = 3$) and 62 ± 6% pentachlorophenol ($n = 3$). No significant effects were detected for di-*n*-butyl phthalate (4 μM), ioxynil (0.1 μM), dicofol (0.1 μM), and bisphenol A (5 μM). When tadpoles were treated with di-*n*-butyl phthalate or dicofol, at concentrations several times higher, some of the test tadpoles died due to toxicity. Therefore, it was not possible to compare their thyroid system-disrupting activity in tadpoles with that in cultured cells at similar concentrations. These results indicated that of the six chemicals showing T_3-antagonist activity in the *in vitro* assays only *n*-butylbenzyl phthalate and pentachlorophenol exhibited T_3-antagonist activity *in vivo* at micromolar and sub-micromolar concentrations, respectively.

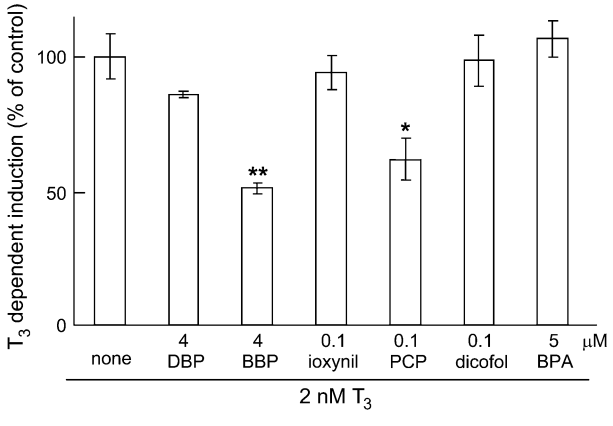

FIG. 6. Effect of EDCs on the T_3-dependent activation of TRβ gene in T_3-induced metamorphosing tadpoles. Tadpoles were treated with or without 2 nM T_3 for five days at 22–24°C in the presence of each EDC at the defined concentrations. They are also treated with 2 nM T_3 alone as a positive control, without T_3 as a negative control. Total RNA was extracted from these tadpoles and analyzed by real-time PCR. DBP, di-*n*-butyl phthalate; BBP, *n*-butylbenzyl phthalate; PCP, pentachlorophenol; BPA, bisphenol A. Vertical axis represents the ratio of the amount of TRβ transcript in T_3-treated samples to that in T_3-untreated samples as a percentage of the maximum induction, which corresponds to the ratio of the amount of TRβ transcript in positive control to that in negative control. Experiments for each chemical were repeated at least three times. Each value is the mean ± SEM of triplicate determinations. Statistically significant differences were $*p < 0.05$ and $**p < 0.01$.

DISCUSSION

We have used a recombinant *X. laevis* cell line containing a T_3-dependent reporter gene, XL58-TRE-Luc, to detect the thyroid system-disrupting activity of several suspect EDCs. This luciferase assay was highly sensitive to T_3 with an ED_{50} of 10^{-11} M, in the absence of FBS (Sugiyama *et al.*, in press) and was specific to THs: T_3 > Triac > D-T_3 > T_4 > rT_3 (Fig. 2). This specificity was in agreement with the binding specificity of amphibian and mammalian TRs (Mukku *et al.*, 1983; Thomas *et al.*, 1992). In the present study, the ligand-dependent luciferase assay revealed the TH-antagonist activities of at least three phthalates, dicyclohexyl phthalate, *n*-butylbenzyl phthalate, and di-*n*-butyl phthalate, and three agricultural chemicals, ioxynil, pentachlorophenol, and dicofol. The T_3-dependent activation of the TRβ gene confirmed the T_3-antagonist activities of these chemicals in the same cells, and indicated that di(2-ethylhexyl) phthalate and diethyl phthalate were also potent. Of the above chemicals, only *n*-butylbenzyl phthalate and pentachlorophenol elicited T_3-antagonist activity in tadpoles.

Of the six chemicals found to have T_3-antagonist activity using the luciferase assay, pentachlorophenol and ioxynil were previously identified as the most potent inhibitors of T_3-binding to *X. laevis* transthyretin (IC_{50} values of 180–260 nM); however, none of them, at 1 μM, were potent inhibitors of T_3-binding to the *X. laevis* TRβ gene (Yamauchi *et al.*, 2002). Di(2-ethyhexyl) phthalate and pentachlorophenol are known to decrease the plasma thyroxine level and the plasma T_3 and thyroxine levels, respectively, in rat (Brucker-Davis, 1998). These effects are probably due to alterations in thyroidal activity (Hinton *et al.*, 1986) or the control of the hypothalamus/pituitary/thyroid axis (Jekat *et al.*, 1994). These findings suggest that these chemicals target TH system processes, extracellular TH-transport system, TH-synthesis, and/or control system of TH-synthesis, before THs access the peripheral target cells. Together with our previous findings, the present study opened the possibility that some of the six chemicals interfere with a process or several processes other than T_3-binding to TR in the T_3-signaling pathway within peripheral target cells. This possibility has been proposed for polychlorinated biphenyls (PCBs) or hydroxylated PCBs. These compounds affected TH-dependent extension of Purkinje dendrite in mouse cerebellar culture at 10^{-12}–10^{-11} M (Kimura-Kuroda *et al.*, 2005) and suppressed T_3-induced transcriptional activation of TR at 10^{-10} M (Iwasaki *et al.*, 2002), although they slightly competed with T_3-binding to TR at the concentrations of more than 10^{-6} M (Cheek *et al.*, 1999b; Gauger *et al.*, 2004). Miyazaki *et al.* (2004) found that these compounds induced partial dissociation of TR/retinoid X receptor heterodimer

complex from the TRE. It remains to be clarified how the three phthalates and the three agricultural chemicals affected T_3-dependent transactivation in our culture system.

Using the luciferase assay, the significant effective doses of the three phthalates and the three agricultural chemicals were 10^{-7}–10^{-5} M (Figs. 2 and 3). Most of these EDCs have been shown to interfere with the estrogen-signaling pathway by competitive binding to estrogen receptors (Jobling *et al.*, 1995; Matthews *et al.*, 2000; Nakai *et al.*, 1999; Zacharewski *et al.*, 1998), the MCF-7 proliferation assay (Harris *et al.*, 1997; Okubo *et al.*, 2003; Soto *et al.*, 1995), yeast and mammalian two-hybrid systems (Nishihara *et al.*, 2000; Zacharewski *et al.*, 1998), and estrogen-dependent reporter gene assays (Harris *et al.*, 1997; Jobling *et al.*, 1995; Zacharewski *et al.*, 1998), with effective concentrations of 10^{-6}–10^{-3} M. Pentachlorophenol (10^{-6} M) exhibited antagonist activity in a yeast progesterone-dependent reporter gene assay (Tran *et al.*, 1996) whereas dicofol (10^{-6}–10^{-5} M) exerted estrogenic activity in the yeast estrogen-dependent reporter gene assay and the MCF-7 proliferation assay (Vinggaard *et al.*, 1999). The effective concentrations of these EDCs for our luciferase assay and most of the above assays for steroid system disruption were within a similar range. However, our cell culture system specifically responded to THs but not to E_2 (Fig. 1). It is likely that these EDCs interfere with several hormonal signalling pathways simultaneously within a cell, and/or that they target a cellular site that affects several hormonal signalling pathways. The rank orders of their steroidgenic or anti-steroidgenic activities showed occasional discrepancies, which could be due to differences in the cellular environment of the cell lines and/or the species used (Matthews *et al.*, 2000; Zacharewski *et al.*, 1998) or differences in the assay methods and/or in the assay conditions used (Harris *et al.*, 1997; Okubo *et al.*, 2003; Zacharewski *et al.*, 1998). These rank orders did not compare with that of their TH-antagonist activities as determined by our luciferase assay. Therefore, it is not possible to conclude whether the chemicals potent in the luciferase assay act on a single cellular site that affects several hormonal signalling pathways.

The inhibitory effect of the EDCs on the T_3-dependent activation of the TRβ gene (Fig. 5) was similar to that determined using the luciferase assay. The fold induction of TRβ gene expression by T_3 and inhibition of its T_3-dependent induction by the EDCs indicated that the assay for T_3-dependent activation of the TRβ gene was more sensitive to T_3 and the EDCs used than the luciferase assay. Of the five phthalates, dicyclohexyl phthalate (20 μM) had the most potent T_3-antagonist activity in the luciferase assay whereas *n*-butylbenzyl phthalate (4 μM) was the most potent inhibitor of the T_3-dependent activation of the TRβ gene. This discrepancy may be, in part, due to the difference in the structure of the TREs located in the two genes.

N-Butylbenzyl phthalate (4 μM) and pentachlorophenol (0.08–0.1 μM), exhibited T_3-antagonist activity in both the *in vitro* and *in vivo* assays in *X. laevis*. Phthalates including *n*-butylbenzyl phthalate are widespread in food and the environment due to their use as plasticizers in consumer products, food packaging materials and biomedical devices. *n*-Butylbenzyl phthalate is one of the most abundant man-made chemicals and is used in the production of vinyl floor tiles, adhesives, and synthetics leather. Phthalates can leach out of these materials into water, soil, or food over time, after use. Pentachlorophenol had been used commonly as a fungicidal substance for wood protection and leather impregnation and as a herbicide. It is also present in the gases of municipal waste incinerators. Due to its highly persistent nature, pentachlorophenol is still one of the dominant phenolic compounds in blood (Sandau *et al.*, 2000). However, little is known about their absorption, bioaccumulation, and metabolism, and their chronic effects on humans or wildlife if administered at low concentrations over long time periods. Considering the prevalence of phenolic compounds in the environment, more research should be focused on their thyroid system-disrupting activities as well as steroid system-disrupting activities, and their cellular target sites, i.e., metabolic enzymes that activate or inactivate the chemicals and endogenous hormones, hormone receptors and/or nuclear co-regulators.

Recently, it was reported that bisphenol A (10^{-5}–10^{-4} M) inhibited T_3-induced metamorphosis (Iwamuro *et al.*, 2003). In a mammalian two-hybrid system, bisphenol A (10^{-5} M) recruited the nuclear corepressor N-CoR to the TR then elicited T_3-antagonist activity (Moriyama *et al.*, 2002). However, in the present *in vitro* and *in vivo* assays, bisphenol A (10 μM and 5 μM, respectively) exhibited no T_3-antagonist activity in *X. laevis*. Although we can not fully explain this discrepancy, higher concentrations of bisphenol A than we used would be necessary to elicit significant T_3-antagonist activity under our experimental conditions.

The luciferase assay using XL58-TRE-Luc cells has several advantages. This cell line can produce enough endogenous TRβ in response to exogenously added T_3. It is easy to grow and work with, and the data derived from the assay had a small intra-assay variation when compared with the transient transcription assay (Sugiyama *et al.*, in press). Although there have been a number of reports on the effects of environmental chemicals on amphibian metamorphosis (Cheek *et al.*, 1999a; Crump *et al.*, 2002; Larson *et al.*, 1998; Veldhoen and Helbing, 2001), most of these assays were not suitable for the primary screening of potential thyroid system-disrupting chemicals because of the time required and the high costs involved. Our luciferase assay would provide high throughput primary screening for chemicals with thyroid system-disrupting activity in the water environment where amphibians habitat. However, it should be noted that the luciferase assay would not provide the potential intracellular sites for thyroid disruption by EDCs, and that the *in vitro* assays, including the assay for T_3-dependent activation of TRβ gene, and the transthyretin and TR binding assays, can not reproduce the pharmacokinetic and pharmacodynamic interactions and sensitive developmental

windows that may occur *in vivo*. We actually found that different concentrations of EDCs were required to elicit a toxic effect in cultured cells and metamorphosing tadpoles. Of the chemicals that were T_3-antagonistic in the luciferase assay and T_3-dependent TRβ gene activation in cultured cells, only *n*-butylbenzyl phthalate and pentachlorophenol elicited T_3-antagonist activity in tadpoles reflecting in part differences in the action of EDCs in the *in vitro* and *in vivo* assays, as described above. Despite these limitations, the results from the *in vitro* assays may indicate potential mechanisms of EDC action. Furthermore, our study indicates the importance of the combination of several assays to assess the thyroid system-disrupting activity of EDCs. The transgene by the LV system can be introduced into organisms (Miyoshi *et al.*, 1998; Zufferey *et al.*, 1998) as well as cell lines (Gatlin *et al.*, 2001; Katayama *et al.*, 2004). Therefore, the same DNA construct can be introduced into eggs, embryos, or some tissues of tadpoles. This application of the transgene would allow, to some extent, the connection of data obtained from the various *in vitro* assays with data obtained from *in vivo* metamorphosis assays.

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