1 **Development of yeast reporter assays for the enhanced detection of**
2
3
4 **environmental ligands of thyroid hormone receptors α and β from *Xenopus***
5
6
7
8 ***tropicalis***
9

10
11
12
13
14
15 **Satoko Matsui¹, Sayoko Ito-Harashima¹, Yuji Sugimoto¹, Eiji Takada¹, Kazuhiro**
16
17
18 **Shiizaki^{1, 2}, Masanobu Kawanishi¹, Takashi Yagi^{1, 3}**
19
20
21
22
23
24
25

26 ¹Department of Biology, Graduate School of Science, Osaka Prefecture University,
27
28
29
30 Naka-ku, Sakai, Osaka 599-8570, Japan
31
32

33 ²Present address: Department of Life Science, Toyo University, Itakura-cho, Gunma
34
35
36
37 374-0193, Japan
38
39
40

41 ³Department of Life Science, Dongguk University, Jung-gu, Seoul 100-715, Republic
42
43
44 of Korea
45
46
47
48
49
50

51 *Correspondence to: T. Yagi; yagi-t@riast.osakafu-u.ac.jp
52
53
54
55
56
57
58
59
60
61
62

ABSTRACT

Thyroid hormones (THs) are involved in the regulation of metabolic homeostasis during the development and differentiation of vertebrates, particularly amphibian metamorphosis, which is entirely controlled by internal TH levels. Some artificial chemicals have been shown to exhibit TH-disrupting activities. In order to detect TH disruptors for amphibians, we herein developed a reporter assay using yeast strains expressing the thyroid hormone receptors (TRs) α and β together with the transcriptional coactivator SRC-1, all of which were derived from the frog *Xenopus tropicalis* (XT). These yeast strains responded to endogenous THs (T_2 , T_3 , and T_4) in a dose-dependent manner. They detected the TR ligand activities of some artificial chemicals suspected to exhibit TH-disrupting activities, as well as TR ligand activity in river water collected downstream of sewage plant discharges, which may have originated from human excrement. Moreover, the responses of XT TR strains to these endogenous and artificial ligands were stronger than those of yeast strains for human TR α and β assays, which had previously been established in our laboratory. These results indicate that the yeast reporter assay system for XT TR α and β is valuable for assessing TR ligand activities in environmental samples that may be particularly

1 potent in amphibians.
2
3
4
5
6
7

8 **KEY WORDS**
9

10
11
12 Thyroid hormone; amphibian metamorphosis; endocrine-disrupting substance; yeast
13
14

15 reporter assay; water pollutant
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1. INTRODUCTION

Thyroid hormones (THs) are essential endocrine signaling molecules that control metabolic homeostasis during the development and differentiation of vertebrates (Yen, 2001; Warner and Mittag, 2012; Kulkarni and Buchholz, 2013). Two THs, 3,5,3',5'-tetraiodothyronine (thyroxine, T_4) and 3,5,3'-triiodothyronine (T_3) are synthesized in the thyroid gland under the control of thyroid-stimulating hormone. T_4 is primarily secreted into the blood, and is converted to the active form T_3 by deiodinases in cells. T_4 is also metabolized into other inactive iodothyronine products, such as 3,3',5'-triiodothyronine (reverse T_3), 3,3'-diiodothyronine (T_2), 3'-monoiodothyronine (T_1), and thyronine (T_0). THs are metabolized to water-soluble conjugated forms by sulfation or the formation of glucuronides in the liver, and are excreted by the kidney or in bile. This process contributes to the conservation of body iodine stores (Kogai and Brent, 2005; Morvan-Dubois et al., 2008).

TH signaling requires thyroid hormone receptors (TRs), which are members of the nuclear receptor superfamily. TRs are transcription factors that regulate gene expression by binding to DNA at specific sites qualified as thyroid hormone response elements (TRE). TREs are generally composed of two half sites with the consensus

motif sequence 5'-AGGTCA-3' (Brent et al., 1989). A representative TRE with a direct repeat of the half sites separated by 4 bp is referred to as the DR-4 element (Suen et al., 1994). Other types of TREs are IR-0 (an inverted repeat with no spacer between the half sites) and ER-6 (an everted repeat with a 6-bp spacer) (Glass et al., 1988). Liganded TRs recruit coactivator complexes on TRE in order to facilitate transcription. In contrast, in the absence of T_3 , TRs repress transcription by recruiting corepressor complexes (Damm et al., 1989; Paul et al., 2007). Two TR genes, *TR α* and *TR β* , are conserved in vertebrates (Lazar, 1993), and the relative expression of these two TR genes and the distribution of their products vary among tissues and during different stages of development (Hodin et al., 1990; Yaoita and Brown, 1990). Moreover, cross-regulation has been demonstrated between the TR isoforms. The regulation of *TR β* expression in the liver and heart is known to be controlled by *TR α* (Sadow et al., 2003).

Amphibian metamorphosis shares a number of similarities with postembryonic development in mammals (Tata, 1999). Amphibian metamorphosis is divided into three developmental periods: premetamorphosis, prometamorphosis, and climax (Dodd and Dodd, 1976). Premetamorphosis is the truly larval period, during which the tadpole

grows and develops under the control of maternal T_3 , prior to the formation of a functional thyroid gland. In prometamorphosis, the most prominent change is the development of both sets of limbs, and morphological alterations to the adult frog are almost complete by climax. In climax, the cloacal tailpiece begins to shrink, and upon tail resorption, all radical changes end. These metamorphic processes are entirely controlled by T_3 and TRs. In premetamorphosis, $TR\alpha$ mRNA levels increase and peak by prometamorphosis. $TR\beta$ mRNA is barely detectable during premetamorphosis. In prometamorphosis, endogenous T_3 is synthesized by the thyroid gland, and $TR\beta$ mRNA is up-regulated by endogenous T_3 . Endogenous T_3 and $TR\beta$ mRNA levels peak at climax, and then rapidly decrease to a minimum in parallel with $TR\alpha$ mRNA levels (Yaoita and Brown, 1990).

Population decreases, reproductive anomalies, and malformations have been increasingly reported in highly aquatic animals since the 1990s (Kingsford et al., 1996; Houlahan et al., 2000; Vandenlangenberg et al., 2003). One possible cause of these events is considered to be the endocrine disruptive effects induced by environmental contaminants through direct actions on nuclear receptors (Burkhart et al., 1998; Vos et al., 2000). Polychlorinated biphenyls (PCBs), which are industrial chemicals used as

1 coolants and lubricants in electrical equipment, have been suggested to interact with
2
3
4 TR because of their structural similarities to THs ([McKinney et al., 1994](#)). Bisphenol A
5
6
7
8 (BPA), which is used to produce polycarbonate plastic, has been shown to bind to TR
9
10
11 as an antagonist ([Moriyama et al., 2002](#)). The detergent component nonylphenol has
12
13
14
15 been reported to weakly interfere with TH binding to the TH transport protein
16
17
18 transthyretin (TTR) (Ishihara et al., 2003). Chlorinated derivatives of BPA and NP have
19
20
21
22 been shown to inhibit TH binding not only to TTR, but also to the ligand binding domain
23
24
25
26 (LBD) of chicken and bullfrog TR (Yamauchi et al., 2003). *o*-*t*-Butylphenol (OBP),
27
28
29 2-isopropylphenol (IPP), tetrabromobisphenol A (TBBPA), and tetramethylbisphenol A
30
31
32
33 (TMBPA), other industrially produced substances, also exhibit TH-disrupting activities
34
35
36
37 ([Kitamura et al., 2005](#); [Kudo et al., 2005](#); [Shiizaki et al., 2010](#)).

40
41 We have been attempting to establish yeast reporter assay systems to screen
42
43
44 nuclear receptor ligands ([Kawanishi et al., 2003, 2006](#); [Chu et al., 2009a, b](#); [Shiizaki et](#)
45
46
47 [al., 2010, 2014](#); [Ito-Harashima et al., 2015](#)), and they have been applied in various
48
49
50
51 studies on environmental contaminants ([Kawanishi et al., 2004](#); [Chu et al., 2009a](#)).
52
53
54
55 These bioassays are based on the quantification of reporter enzymes that reflect
56
57
58
59 ligand-dependent nuclear receptor activation in yeast cells. They are simpler, easier to
60
61
62
63
64
65

1 handle, and less expensive than mammalian cell-based bioassays. Moreover, since
2
3
4 yeasts do not express endogenous nuclear receptor homologs ([Goffeau A et al., 1997](#)),
5
6
7
8 they may be used to detect ligands that are specific to individual receptor subtypes.
9

10
11 In the present study, we developed reporter assay systems using yeast strains
12
13 expressing TR α and TR β along with the transcriptional coactivator SRC-1, all of which
14
15
16 were derived from the frog *Xenopus tropicalis* (XT). Since amphibian metamorphosis
17
18
19 is entirely controlled by TRs (reviewed by Brown and Cai, 2007), a yeast system that
20
21
22
23 mimics TR-mediated gene signaling in the amphibian is considered advantageous for
24
25
26 evaluating the ecotoxicological potencies of environmental samples. We examined the
27
28
29 responses of these frog TR strains to endogenous THs, chemicals suspected to be TR
30
31
32
33 ligands, and several environmental samples. Furthermore, we compared the ligand
34
35
36 responses of XT TR strains with those of our human TR strains that had been
37
38
39 established previously ([Shiizaki et al., 2010](#)).
40
41
42
43
44
45
46
47
48
49
50

51 **2. MATERIALS AND METHODS**

52 **2.1. Chemicals**

53
54
55 Dimethyl sulfoxide (DMSO), *o*-nitrophenyl- β -D-galactopyranoside (ONPG), T₃, and
56
57
58

1 TMBPA were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).
2
3
4 3,5-Diiodo-L-thyronine (T₂) and L-thyroxine (T₄) were obtained from MP Biomedicals,
5
6
7
8 LLC (Santa Ana, CA, USA). TBBPA, IPP, and BPA were obtained from Tokyo-Kasei
9
10
11 Co., Ltd. (Tokyo, Japan). OBP and dithiothreitol (DTT) were purchased from Nacalai
12
13
14
15 Tesque (Kyoto, Japan). *p*-*n*-Nonylphenol (NP) was purchased from WAKO Pure
16
17
18
19 Chemicals (Osaka, Japan).
20
21
22
23
24
25
26
27
28
29

30 **2.2. Cloning of XT TR α , TR β , and SRC-1 cDNAs**

31
32

33 XT frogs were provided by the National BioResource Project (NBRP) of the Ministry
34
35
36
37 of Education, Culture, Sports, Science and Technology (MEXT), XenoBiores at
38
39
40
41 Hiroshima University, Japan. Total RNA was extracted from the livers of adult frogs
42
43
44
45 using ISOGEN (Nippon Gene, Tokyo, Japan). The DNA fragments containing the open
46
47
48
49 reading frames (ORFs) of XT TR α (NM_001045796), TR β (NM_001045805), and
50
51
52 SRC-1 (NM_001112912) were obtained by a reverse-transcription polymerase chain
53
54
55
56 reaction (RT-PCR). The synthesis of cDNA was performed using SuperScript III
57
58
59 Reverse Transcriptase (Invitrogen, Carlsbad, CA) with 1.8 μ g of total RNA as the
60
61
62
63
64
65

1 template. The ORFs of these genes were amplified by PCR using KOD-Plus
2
3
4 (TOYOBO Co., Ltd., Osaka, Japan) from cDNA with the primer pairs shown in Table 1.
5
6
7
8 Primers containing a restriction site and/or yeast ribosomal binding consensus
9
10
11 sequence near the initiation codon were designed based on the nucleotide sequences
12
13
14
15 registered in the DDBJ/EMBL/GenBank database. Regarding XT TR α cDNA, nested
16
17
18 PCR was performed using xTR α 1F and xTR α 1R in first-step PCR, and xTR α 2F and
19
20
21 xTR α 2R in second-step PCR. The primer pairs xTR β F and xTR β R, and xSRC1F and
22
23
24 xSRC1R were used to amplify XT TR β and SRC-1 cDNA, respectively.
25
26
27
28
29
30
31
32
33
34
35
36

37 **2.3. Plasmids**

38
39
40
41 Plasmids expressing nuclear receptors and coactivators were constructed for XT
42
43
44 TR α and TR β ligand assays. Detailed maps of the plasmids are shown in Figure 1. The
45
46
47 nuclear receptor expression plasmids pUdp6-xTR α and pUdp6-xTR β (Fig. 1A) were
48
49
50 generated by inserting XT TR α and TR β cDNAs, respectively, into the *Xma*I-*Eco*RI
51
52
53 sites of multiple cloning site 2 (MCS2) of the pUdp6 vector without a yeast replication
54
55
56 origin, which had previously been constructed in our laboratory ([Shiizaki et al., 2010](#)).
57
58
59
60
61
62
63
64
65

1 In order to construct the coactivator expression plasmid pESC-Leu-xSRC1, XT SRC-1
2
3
4 cDNA was cloned into the *PacI*-*NotI* sites of MCS1 of the pESC-leu vector (Stratagene,
5
6
7
8 La Jolla, USA) (Fig. 1B). The expression of these genes was under the control of the
9
10
11 yeast *GAL1, 10* promoter, which was induced by the addition of galactose as a carbon
12
13
14
15 source in media. The nucleotide sequences of XT TR α , XT TR β , and XT SRC-1 ORFs
16
17
18
19 were confirmed using the ABI DNA sequencer.
20
21

22 We found two and five sequence alterations in XT TR β and XT SRC-1 ORFs,
23
24
25
26 respectively, which were distinct from the sequences registered in the
27
28
29
30 DDBJ/EMBL/GenBank database. Most of these alterations were synonymous
31
32
33
34 mutations, whereas the G to A change at the nucleotide position 536 in the XT
35
36
37 TR β ORF was associated with an amino acid substitution, serine (AGT) to asparagine
38
39
40 (AAT) at codon 179. The expression vector for XT TR β^{Ser179} was constructed by fusion
41
42
43
44 PCR using mutagenic primers. In the first PCR, the 5'- and 3'-fragments of XT TR β
45
46
47
48 were amplified from pUdp6-xTR β^{Asn179} by PCR using KOD-Plus with the mutagenic
49
50
51
52 primers xTR β fPCRF and xTR β fPCRR, in combination with xTR β F and xTR β R,
53
54
55
56 respectively (Table 1). The PCR products were used as templates in the second PCR
57
58
59
60 with the primer pair xTR β F and xTR β R to obtain the full-length XT TR β^{Ser179} ORF. The
61
62
63
64
65

1 amplified DNA fragment was digested by *Bst*XI and *Spe*I, and inserted into the
2
3
4 *Bst*XI-*Spe*I sites of pUdp6-xTR β ^{Asn179} to replace a large part of the XT TR β ORF with
5
6
7
8 the fusion PCR product. The introduction of the Asn¹⁷⁹ (AAT) to Ser¹⁷⁹ (AGT) mutation
9
10
11
12 was confirmed by sequencing. In order to reconfirm the nucleotide sequence of codon
13
14
15 179 in XT TR β , the DNA fragment surrounding this position was amplified from cDNA
16
17
18
19 by PCR using KOD-Plus in eight independent experiments, and was then purified and
20
21
22
23 sequenced.
24

25
26 The reporter plasmids pYT-IR0x3, pYT-IR0x5, and pYT-DR4x7 previously
27
28 constructed in our laboratory ([Shiizaki et al., 2010](#)) were used in the present study.
29
30
31
32
33 pYT-IR0x3 and pYT-IR0x5 contained three and five copies of IR0 (AGGTCATGACCT),
34
35
36
37 respectively, and pYT-DR4x7 contained seven copies of DR-4
38
39
40
41 (AGGTCACAGGAGGTCA) as the TREs. These TREs were integrated into the MCS
42
43
44 immediately upstream of the *CYC1* minimal promoter-*lacZ* fusion in the pRW95-3
45
46
47
48 vector (Fig. 1C).
49

50 51 52 **2.4. Yeast strain and transformation** 53

54
55 The method used to construct yeast strains for the XT TR α and TR β assay was the
56
57
58
59 same as that described by Ito-Harashima et al. ([2015](#)). *Saccharomyces cerevisiae*
60
61
62

strain W303a (*MATa*, *ade2-1*, *trp1-1*, *leu2-3*, *his3-11, 15*, *ura3-1*) was used as a host to introduce the plasmids described above. Yeast cells were cultured in yeast extract peptone dextrose (YPD) medium or synthetic complete medium comprising 0.67% yeast nitrogen base without amino acids (Difco, Detroit, MI), 2% glucose, and appropriate supplements ([Bruke et al., 2000](#)). Drop-out medium was prepared by excluding the indicated nutrients from the synthetic complete medium. All transformations were performed according to the lithium acetate method ([Gietz et al. 1995](#)). In brief, the SRC-1 expression plasmid, pESC-Leu-xSRC1 and the reporter vectors were introduced into yeast strain W303a, and transformants were selected on *leu⁻ trp⁻* agar plates. Transformants were isolated and cultured in *leu⁻ trp⁻* medium for subsequent transformation. The nuclear receptor expression plasmids, pUdp6-xTR α and pUdp6-xTR β , were linearized by *Apa* I and *Eco*R V digestion, respectively, and then introduced into the *ura3-1* locus on chromosome V by homologous recombination. Transformed cells were selected on *leu⁻, trp⁻, ura⁻* agar plates, and the yeast strains for the reporter assay were finally established. The human TR α and TR β strains that had been previously constructed in our laboratory ([Shiizaki et al., 2010](#)) were also used in the assays.

2.5. Reporter assay

The assay procedure used in this study was similar to that described by Miller (1998) and Shiizaki et al. (2010). In brief, yeast strains were grown overnight at 30°C in synthetic glucose medium lacking tryptophan, uracil, and leucine. The optical density (OD) of each culture was adjusted to 1.0 with the same medium, and 10 µL of yeast culture was added to 90 µL of synthetic medium containing 2% galactose (leu⁻, trp⁻, ura⁻) in 96-well microtiter plates. A 1-µL aliquot of ligand substances dissolved in DMSO was added to each well, and the microtiter plates were incubated at 30°C for 18 h. In order to examine agonistic effects, various concentrations of THs, chemicals, and environmental samples were used: 0.0001-100 µM T₂, T₃, and T₄; 0.01-100 µM OBP, IPP, TBBPA, and TMBPA. After being incubated with these chemicals, a 10-µL suspension of each yeast cell was transferred to a new 96-well microtiter plate, and 100 µL of Z-buffer (60 mM Na₂PO₄, 40 mM NaH₂O₄, 1 mM MgCl₂, 10 mM KCl, 2 mM DTT, and 0.2% sarcosyl; pH 7.0) containing 1 mg/mL ONPG was added to the plate, which was then incubated at 37°C for 60 min (90 min for assays of environmental samples). Absorbances were measured at wavelengths of 405 and 595 nm in order to

1 estimate o-nitrophenol production and yeast cell density, respectively. Increases of
2
3
4 induction were represented using the formula $(OD_{405}/OD_{595}$ in exposed wells) -
5
6
7
8 $(OD_{405}/OD_{595}$ in control wells). EC_{50} values were calculated using dose response
9
10
11 curves from the assay of each strain. The β -gal activity detected in each sample was
12
13
14
15 converted to the T_3 equivalent concentration, which was calculated from the T_3 -dose
16
17
18
19 response curve of each TR strain as the standard.
20
21
22
23
24
25

26 **2.6. Extraction of organic compounds from environmental samples and human** 27 28 29 **body waste** 30 31 32

33
34 Water samples were collected from four locations along the Yamato River on 23 July
35
36
37 2012. Each water sample (3 L) was filtered with a Whatman GF/C 47 mm Φ filter
38
39
40
41 (Whatman International Ltd., England), and passed through a Waters Sep-Pak® Plus
42
43
44 C18 Environmental Cartridge (Waters Corporation, USA) ([Kawanishi et al., 2004](#)). The
45
46
47
48 cartridge was washed with 10 mL of pure water, and bound substances were eluted
49
50
51
52 from the cartridges with 6 mL of DMSO and then dried in a vacuum concentrator. The
53
54
55
56 extract was redissolved in 60 μ L of DMSO and stored at -20°C . The concentration
57
58
59
60 factor of this stock was 50,000 and the stock was further diluted (final concentration
61
62
63
64
65

factors: 1,000, 2,500, and 5,000) for yeast reporter assays.

Human biological samples, urine and feces, were provided from three independent donors, respectively. All donors gave written informed consent. Each urinary sample (500 mL) was centrifuged, and the resulting supernatant was filtered through a Whatman GF/C 47 mm Φ filter. Dilute sulfuric acid (final concentration 0.5 N) was added to the filtered sample, which was then heated at 60°C for 1 hour in order to deconjugate the excreted hormones. The sample was adjusted to pH 6-7 with 10 N NaOH, subjected to solid-phase extraction, and dried, similar to river water samples. The dried extract was redissolved in 1 mL of DMSO and stored at -20°C. The concentration factor of this stock was 500 and the stock was further diluted (final concentration factors: 5, 50, and 100) just before the yeast reporter assays.

Each fecal sample (approximately 3 g) was mixed with 20 mL methanol using a vortex and centrifuged. The supernatant was deconjugated and neutralized, similar to the urinary samples. The sample was dried, redissolved in 1 mL of DMSO, and stored at -20°C. This stock and 2-, 5-, 10-, 15-, and 20-fold diluted samples were used for the yeast reporter assays.

TH-like activities detected in river water, urine, and feces were represented as T_3

equivalent concentrations using the data of diluted samples, which showed β -gal activity in a dose-dependent manner.

2.7. Statistical analysis

Data were analyzed using the Student's *t*-test to assess significance between two sets of values. Probability (*p*) values < 0.05 were considered significant.

3. RESULTS

3.1. Optimization of the yeast reporter assay for XT TRs

In order to optimize the reporter assay for XT TRs, we tested reporter plasmids carrying DR-4 and IR-0 as TREs. Although XT TR α and XT TR β (Asn179, see below) both activated T₃-induced reporter gene expression in yeast via these TREs, IR-0 was more responsive than DR-4 at a low T₃ concentration. Increases in the copy number of the IR-0 motif potentiated the response to T₃ (Fig. 2A). Therefore, we used pYT-IR0x5 containing five copies of IR-0 as a reporter plasmid in this study. We also investigated whether the coactivator XT SRC-1 had the ability to enhance the responses of XT TRs. As shown in Figure 2B, the expression of XT SRC-1 markedly enhanced the response

1 against T₃ in the XT TR strains. Reporter activity increased 62- and 74-fold in the XT
2
3
4 TR α and XT TR β strains, respectively, following the addition of 1 μ M T₃. We selected
5
6
7
8 typical clones exhibiting higher β -galactosidase activity induced by the exposure to T₃,
9
10
11
12 and further investigated their responses to endogenous and environmental TR ligands.
13
14

15 We detected a nonsynonymous alteration in the cloned XT TR β ORF, which was
16
17
18 different from the sequence registered in the DDBJ/EMBL/GenBank database
19
20
21 (NM_001045805). This alteration was a serine (AGT) to asparagine (AAT) substitution
22
23
24
25
26 at codon 179 in the ligand-binding domain (LBD). Since this mutation may have the
27
28
29 ability to affect the ligand response of the yeast strain, we constructed an expression
30
31
32 vector for XT TR β ^{Ser179} by fusion PCR using mutagenic primers, and introduced it into
33
34
35
36
37 the yeast. Unexpectedly, the response of the XT β ^{Ser179} strain against T₃ was markedly
38
39
40 weaker than that of the XT β ^{Asn179} strain (Fig. 3A). The amino acid sequences of the
41
42
43
44
45 LBD were highly conserved across five amphibian species [XT, *X. laevis*
46
47
48 (NP_001090182), *Rana catesbeiana* (AAA53658), *Necturus maculosus* (AAO62999),
49
50
51 and *Ambystoma mexicanum* (AAO47436)] and humans (NP_001239563), and was
52
53
54
55 asparagine at this position, except for that in XT (Fig. 3B). By sequencing DNA
56
57
58
59 fragments amplified from cDNA in eight independent PCR experiments, we confirmed
60
61
62
63
64
65

that codon 179 of the XT TR β gene encoded asparagine (AAT), similar to other species. Therefore, we used the yeast strain expressing XT TR β^{Asn179} as the XT TR β strain in this study.

3.2. Responses to endogenous ligands

In order to confirm whether the established yeast strains respond to the endogenous THs, T₂, T₃, and T₄, reporter assays were performed, and the responses of the XT TR strains were compared with those of human TR strains. Figure 4 shows the dose-response curves of reporter expression against THs in the human and XT TR α and TR β strains. The EC₅₀ values of THs calculated from the dose-response curves are shown in Table 2. T₃ and T₄ were more potent ligands than T₂ for all strains. In XT TR α and TR β strains and the human TR β strain, transactivation by T₃ and T₄ was observed at 0.01 μ M, while these ligands activated human TR α at 0.1 μ M. The XT TR α and TR β strains showed 2.0 to 2.5-fold higher reporter activities to 1 μ M T₃ and T₄ than both of the human TR strains. The most prominent differences in ligand responses between XT and human TRs, and the TR subtypes were observed against T₂, a T₃ metabolite *in vivo* with a very low affinity to TRs (Moreno et al., 2008). Although the

agonist activity of T₂ was detected at a higher concentration than that of T₃ or T₄ (Table 2), XT TR α and TR β showed 7.3- and 3.5-fold higher reporter activities, respectively, at 1 μ M T₂ than their human counterparts. TR β from both species showed 4- to 7-fold lower EC₅₀ values than TR α in yeasts. The established XT TR strains may have the ability to detect TR ligands in a species-specific manner.

3.3. Response to industrial chemicals

We tested several chemicals suspected of being agonists of TRs using the established strains, and the responses of the XT TR strains were compared with those of human TR strains (Fig. 5). All strains detected the agonist activities of OBP, IPP, TBBPA, and TMBPA. In contrast to endogenous THs, the ligand activities of these test chemicals were only observed at high concentrations (Fig. 5, Table 3). The XT TR α and TR β strains showed higher sensitivities to all chemicals than the human TR strains. Among the four TR strains, the XT TR β strain exhibited the highest sensitivity to these chemicals, except for TBBPA. The maximum activity of TBBPA was observed in the XT TR α strain. The most potent ligand was TBBPA in all strains because the concentration of the detection limit and the EC₅₀ value of this chemical were significantly lower than

those of the other chemicals tested (Table 3). The agonist activity of TBBPA was more potent than that of TMBPA ($p < 0.05$ for XT TRs, and $p < 0.01$ for human TRs), which has methyl groups instead of bromides in the bisphenol structure. Higher concentrations of TBBPA (10 μ M) and TMBPA (100 μ M) significantly reduced T_3 -induced reporter gene activity in all strains, possibly due to the inhibition of β -gal activity or the cytotoxicities of the chemicals.

In order to compare the effects of these chemicals on each TR, the maximum β -gal activity detected in each assay was converted to T_3 equivalent concentrations. As shown in Table 4, the potencies of these chemicals for XT TR strains corresponded to higher T_3 concentrations than those for human TR strains.

3.4. Assay of river water and human body waste

We measured the ligand activity of river water as a representative environmental sample using TR α and TR β strains for XT and humans (Fig. 6). Water samples were collected from four locations along the Yamato River, one of the biggest watersheds in the western part of Japan with the lowest water quality in the Japanese environmental standard. The river sampling locations are shown in Figure 6A. The responses of XT

1 and human TR strains were compared in Figure 6B. All TR strains exhibited
2
3
4 significantly high reporter activities at ST3 ($p < 0.01$). Among the four TR strains tested,
5
6
7
8 the XT TR β strain showed the highest sensitivity. The T₃ equivalent concentrations of
9
10
11
12 the TH-like activities of the water samples were calculated and shown in Table 5.
13
14

15 A sewage plant exists immediately upstream of ST3. Sewage-treated water includes
16
17
18 household wastewater containing human excrement. Since the high TH-like activity of
19
20
21
22 ST3 water samples may be due to human excrement, we assayed the fecal and
23
24
25
26 urinary samples of three independent donors using XT and human TR strains. The
27
28
29
30 organic compounds in excrement were extracted and concentrated using a similar
31
32
33
34 method to that of river samples, except for deconjugation as an additional step. The
35
36
37 agonistic activities of excrement were detected in the yeast reporter assay. The
38
39
40
41 responses of XT TR α and TR β strains against both excrement samples were stronger
42
43
44 than those of human TR strains (data not shown). The T₃ equivalent concentrations of
45
46
47
48 the ligand activities of both excrement samples (Table 6) indicated that XT TR strains
49
50
51
52 detected TH-like activity more sensitively than human TR strains.
53
54
55
56
57
58

59 4. DISCUSSION 60 61 62 63 64 65

1 In our laboratory, several yeast reporter assay systems with intact mammalian
2
3
4 nuclear receptors, e.g., aryl hydrocarbon, estrogen, THs, retinoic acid, retinoid X, and
5
6
7
8 glucocorticoid and mineralocorticoid receptors, have been developed for the
9
10
11 comprehensive screening of endocrine disruptors ([Kawanishi et al., 2003, 2006; Chu](#)
12
13
14
15 [et al., 2009a, b; Shiizaki et al., 2010, 2014; Ito-Harashima et al., 2015](#)). These reporter
16
17
18
19 assay systems have the ability to detect the ligands that bind to each mammalian
20
21
22
23 nuclear receptor. In environmental or ecological assessments, it is desirable to use
24
25
26
27 assay systems for nuclear receptors derived from various aquatic animals such as fish,
28
29
30 crustaceans, and amphibians. In the present study, in consideration of amphibian
31
32
33
34 metamorphosis being entirely regulated by THs, a reporter assay system using yeast
35
36
37
38 strains expressing homodimers of frog (XT) TR α and TR β along with the coactivator
39
40
41 SRC-1 was developed to screen environmental TR ligands.
42
43

44 In the course of our study, we found that the regulation of the expression levels of
45
46
47
48 cellular components was important for the optimization of the yeast reporter assay
49
50
51
52 system. In our recent systems, the nuclear receptor expression vector was integrated
53
54
55
56 into the *ura3-1* locus on chromosome V by homologous recombination. The yeast
57
58
59 strains showed stable ligand responses in receptor/subtype-specific manners ([Chu et](#)
60
61
62
63
64
65

al., 2009a; Shiizaki et al., 2010, 2014; Ito-Harashima et al., 2015). These findings imply the stable expression of nuclear receptors at the *ura3-1* locus. Since our TR strains have the same genetic background, differences in reporter activities may be TR subtype-specific.

As shown in Figure 2A, the T_3 -dependent expression of the reporter gene was induced via IR-0 and DR-4 in XT TR α - and TR β -expressing yeast strains. This was different from the human TR homodimer-expressing yeast strains in which DR-4 did not act as TRE (Shiizaki et al., 2010). The DR-4 element is a representative TRE in mammals, and TRs bind as heterodimers with retinoid X receptor α (RXR α). In XT and *X. laevis*, TR β -RXR was found to bind to the DR-4-type elements identified in the earliest TH-regulated genes during metamorphosis (Das et. al., 2009). In our XT TR-expressing yeast strains, IR-0-type TRE induced β -gal expression more efficiently than DR-4-type TRE at a lower T_3 concentration (Fig 2A). Therefore, we selected the IR-0 element as an enhancer of the reporter vector, although it is an uncommon TRE. Since our human TR strains also carry IR-0-type TRE (Shiizaki et al., 2010), it enabled us to compare differences in ligand responses between two species in yeast reporter assays. We further showed that the expression of the transcriptional coactivator XT

SRC-1 greatly enhanced T_3 -induced reporter gene activity in XT TR α - and TR β -expressing yeast strains (Fig. 2B). We herein found a sequence alteration in XT TR β cDNA, which was associated with the serine to asparagine substitution at codon 179 in LBD. We confirmed that codon 179 of XT TR β was asparagine, which is the same as that in other species. We also showed that XT TR β^{Asn179} was a highly active TR β ortholog of XT in yeast. XT TR β^{Ser179} , which is registered in the DDBJ/EMBL/GenBank database (NM_001045805), is likely to be a minor variant with lower activity (Fig. 3).

The endogenous THs, T_2 , T_3 , and T_4 were assayed using the established yeast strains. In general, the binding affinity of T_3 to TRs is stronger than T_4 , and T_2 is an inactivated form of THs with significantly low affinity to TRs ([Cheek et al., 1999](#); [Sandler et al., 2004](#); [Lechan et al., 2010](#)). Our XT TR strains detected all THs and responded to T_3 and T_4 similarly. Although their responses to T_2 were less sensitive, reporter activities were similar to T_3 and T_4 at high doses (Fig. 4). The XT TR β strain showed higher sensitivity to all THs, particularly T_2 , than the XT TR α strain, which was similar to the responses of human TR strains (Fig. 4, Table 2). Our yeast data appeared to be consistent with previous findings showing that *in vitro* translated

human TR β was more responsive to THs than that of TR α in binding assays (Ball et al., 1997). Furthermore, XT TR α and TR β strains responded to these ligands more strongly than the respective human TR strains (Fig. 4). Since few differences were observed in EC₅₀ values among the XT and human TR strains (Table 2), both TRs may have similar affinities to THs. However, the ligand-dependent transactivation activities of the TRs may be different between two species. Such species-specific ligand responses have also been described in reporter assay systems for *Oryzias latipes* (Japanese medaka fish) and human glucocorticoid receptors (GR), amphibian estrogen receptors (ER), and TRs from various vertebrates, all established in mammalian cultured cells (Katsu et al., 2010; Oka et al., 2012; Miyagawa et al., 2015). We previously reported that the responses of the aryl hydrocarbon receptor (AhR) from various rodent species to dioxins in yeast AhR reporter assays correlated with the sensitivities of animals to dioxins (Kawanishi et al., 2006). Therefore, it is conceivable that the responses of TRs in yeasts may correspond to the *in vivo* receptor responses of two species.

Nuclear receptors and their regulatory factors were previously reported to be evolutionally conserved, and, thus, the amino acid sequences of XT and human TRs

LBDs are highly homologous (TR α and TR β have 93% and 94% homologies, respectively). With respect to the receptor-interacting domains of two SRC-1s, the sequences were 100% identical. In contrast, the histone acetyltransferase domains of two SRC-1s, which are relevant to chromatin remodeling during the receptor-directed initiation of transcription, are relatively divergent (70% homology) (Leo and Chen, 2000; Xu and Li, 2003). Since our yeast system was constructed by introducing not only XT TRs, but also XT SRC-1, the prominent species-specific ligand efficacies of XT and human TR strains may be caused by differences in the transcriptional activation potential of SRC-1s.

In the reporter assay using various phenolic and phenol compounds, neither BPA nor NP showed agonist activity in yeasts. Although these compounds are known to be xenoestrogens (Rudel et al., 1996), their adverse effects on TH homeostasis have also been reported (Moriyama et al., 2002; Ishihara et al., 2003; Yamauchi et al., 2003). On the other hand, all yeast strains for the TR assay responded to OBP, IPP, TBBPA, and TMBPA, which are chemicals suspected of exerting TH-disrupting effects (Fig. 5). These results suggest that our yeast reporter assay system is valuable for screening chemicals with TR agonist activities. The ligand potencies of TBBPA and TMBPA to

1 TRs were higher than those of OBP and IPP, which may be due to their structural
2
3
4 resemblance to THs. Moreover, TBBPA had higher potency to TR α than to TR β , as
5
6
7
8 was reported previously ([Veldhoen et al., 2006](#)). Although these compounds mainly
9
10
11 exhibit antagonist activities ([Kitamura et al., 2005](#); [Kudo et al., 2005](#)), TBBPA has been
12
13
14
15 shown to possess weak thyroid hormone activity as well as anti-thyroid hormone
16
17
18 activity in amphibians and humans ([Kitamura et al., 2005](#); [Jagnytsch et al., 2006](#);
19
20
21
22 [Hofmann et al., 2009](#)). Unlike the XT TR transactivation system established in human
23
24
25 embryonic kidney 293 (HEK293) cells ([Oka et al., 2012](#)), our XT TR strains clearly
26
27
28 exhibited their agonist activities of these chemicals, which were more sensitive than
29
30
31
32 those of human TR strains ([Fig. 5](#)). However, since XT TRs were intrinsically more
33
34
35 sensitive to endogenous THs, we were unable to clarify whether these chemicals had
36
37
38 more TH-disrupting effects on XT TRs than human TRs. In order to investigate this
39
40
41 issue in more detail, the order of the TH-disrupting activities of the chemicals to each
42
43
44 TR was compared by converting reporter activities to T₃ equivalent concentrations
45
46
47 (Table 4). The agonistic activities of these phenolic and phenol compounds
48
49
50 corresponded to higher concentrations of T₃ in XT TR strains than in human TR strains.
51
52
53
54
55
56
57
58
59 Our results suggest that these compounds may disturb TR-mediated gene signaling
60
61
62
63
64
65

1 more severely in XT than in humans.
2
3

4 The established yeast strains for the TR assay were also used to evaluate river water
5
6
7
8 samples (Fig. 6). All TR strains represented the highest sensitivity to water collected at
9
10
11 ST3, immediately downstream of the sewage plant (Fig. 6B, Table 5). A previous study
12
13
14 reported that effluent from domestic sewage treatment plants contained contaminants
15
16
17 with TH-like activity ([Murata and Yamauchi, 2008](#)); therefore, we assayed the fecal
18
19
20 and urinary samples of humans, which may be contained in sewage-treated water.
21
22
23
24
25 The results obtained showed that our TR strains successfully detected TH activities in
26
27
28 human body waste, particularly feces (Table 6). Although endogenous THs are mostly
29
30
31 metabolized to glucuronide- or sulfate-conjugated forms, small amounts of
32
33
34 conjugate-free THs have also been detected ([Faber et al., 1981](#); [Visser et al., 1990](#);
35
36
37 [Visser, 1996](#)). Previous studies showed that conjugated THs were hydrolyzed by
38
39
40
41 intestinal flora in humans ([de Herder WW et al., 1985](#); [Hazenberg et al., 1988](#)). Since
42
43
44
45 river water samples were prepared using a procedure without a deconjugation step,
46
47
48
49
50
51 THs originating from human (and animal) excrement may not have been fully
52
53
54
55 inactivated at the sewage plant. It is also possible that conjugated THs in human
56
57
58
59 excrement are deconjugated by bacteria in the environment, as is the case with
60
61
62
63
64
65

1 estrogens (Baronti et al., 2000). Therefore, sewage-treated water may exhibit
2
3
4 sufficient TH-like activity for detection by yeast reporter assays. The ligand potencies
5
6
7
8 contained in river water and human body waste were calculated as T₃-equivalent
9
10
11 concentrations (Tables 5 and 6). These results showed that the TH-like activities of
12
13
14
15 these samples corresponded to higher T₃-equivalent concentrations in XT TR strains
16
17
18 than human TR strains. Previous studies identified T₂ and T₄ as major iodothyronines
19
20
21 excreted in human urine and feces (Faber et al., 1981; Visser et al., 1990). Differences
22
23
24
25 in T₂ responses between XT and human TR strains (Fig. 4) may be attributable to the
26
27
28 detection sensitivities of TRs from both species. In conclusion, the results described
29
30
31 here imply that frogs are susceptible to various contaminants with TH-like activities,
32
33
34 such as industrial chemicals or even inactive human metabolites in the environment.
35
36
37
38
39
40

41 Amphibian metamorphosis is regarded as a suitable experimental model for directly
42
43
44 screening TH-disrupting chemicals (Degitz et al., 2005; Opitz et al., 2006). Animal
45
46
47 experiments require long periods of time and great expense in order to pursue their
48
49
50 goals, whereas yeast reporter assays are simpler, easier to handle, and are more
51
52
53 cost-effective. The relationship between our results and *in vivo* responses currently
54
55
56 remains unclear; however, when combined with animal experiments, the yeast
57
58
59
60
61
62
63
64
65

reporter assay system for frog TRs may be applied to screening systems for a wide variety of TH disruptors, including as yet unidentified substances.

ACKNOWLEDGMENTS

We thank Dr. Yoshio Yaoita, NBRP XenoBiores at Hiroshima University for providing *X. tropicalis* and his helpful suggestions on breeding techniques.

This work was supported by the Industrial Technology Research Grant Program from the New Energy and Industrial Technology Development Organization (NEDO) (#05A21501a) (M.K.) in 2005-2007, and by the Japan Science and Technology Agency (JST) (OSAKA H23-1) (T.Y. and M.K.) in 2008-2010, and was also supported in part by Grants-in-Aid for Scientific Research (KAKENHI #18651028 and #80182301) (T.Y. and M.K.) from the Japan Society for the Promotion of Science (JSPS).

REFERENCES

Ball SG, Sokolov J, Chin WW. 1997. 3, 5-Diiodo-L-thyronine (T₂) has selective thyromimetic effects *in vivo* and *in vitro*. J Mol Endocrinol 19:137-147.

Baronti C, Curini R, D'Ascenzo G, Di Corcia A, Gentili A, Samperi R. 2000. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environ Sci Technol 34:5059-5066.

Brent GA, Harney JW, Chen Y, Warne RL, Moore DD, Reed-Larsen P. 1989. Mutations of the rat growth hormone promoter which increase and decrease response to thyroid hormone define a consensus thyroid hormone response element. Mol Endocrinol 3: 1996-2004

Brown DD, Cai L. 2007. Amphibian metamorphosis. Dev Biol 306: 20-33

Bruke D, Dawson D, Stearns T. 2000. Methods in yeast genetics-A laboratory course manual. Cold Spring Harbor (2000 edition). Cold Spring Harbor: Cold Spring Harbor

Laboratory Press.

Burkhart JG, Helgen JC, Fort DJ, Gallagher K, Bowers D, Propst TL, Gernes M, Magnier J, Shelby MD, Lucier G. 1998. Induction of mortality and malformation in *Xenopus laevis* embryos by water sources associated with field frog deformities. Environ Health Perspect 106:841-848.

Cheek AO, Kow K, Chen J, McLaglan JA. 1999. Potential mechanisms of thyroid disruption in humans: interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. Environ Health Perspect 107:273-278.

Chu WL, Shiizaki K, Kawanishi M, Kondo M, Yagi T. 2009a. Validation of a new yeast-based reporter assay consisting of human estrogen receptors α/β and coactivator SRC-1: application for detection of estrogenic activity in environmental samples. Environ Toxicol 24:513-521.

Chu WL, Shiizaki K, Kawanishi M, Yagi T. 2009b. Differential responses of various

1 pharmaceuticals to human estrogen receptors α and β in newly-constructed yeast
2
3
4 reporter assays. Genes Environ, 31, 80-86.
5
6
7
8
9

10
11 Damm K, Thompson CC, Evans RM. 1989. Protein encoded by v-erbA functions as a
12
13 thyroid-hormone receptor antagonist. Nature 339:593-597.
14
15
16
17
18
19
20
21

22 Das B, Heimeier RA, Buchholz DR, Shi YB. 2009. Identification of direct
23
24 thyroid-hormone response genes reveals the earliest gene regulation programs during
25
26
27
28
29
30 frog metamorphosis. J Biol Chem, 284:34167-34178
31
32
33
34
35
36

37 Degitz SJ, Holcombe GW, Flynn KM, Kosian PA, Korte JJ, Tietge JE. 2005. Progress
38
39 towards development of an amphibian-based thyroid screening assay using *Xenopus*
40
41
42
43
44 *laevis*. Organismal and thyroidal responses to the model compounds 6-propylthiouracil,
45
46
47
48 methimazole, and thyroxine. Toxicol Sci 87:353-364.
49
50
51
52
53
54

55 de Herder WW, Hazenberg MP, Pennock-Schröder AM, Visser TJ. 1985.
56
57
58
59 Iodothyronine sulfate-hydrolyzing anaerobic bacteria isolated from human fecal flora.
60
61
62
63
64
65

1 FEMS Microbiol Lett 30: 347-351.
2
3
4
5
6
7

8 Dodd MHI, Dodd JM. 1976. The biology of metamorphosis: Physiology of the amphibia.
9

10 In: Lofts B, editor. New York: Academic Press. pp 467-599.
11
12
13
14
15
16
17
18

19 Faber J, Busch-Sørensen M, Rogowski P, Kirkegaard C, Siersbaek-Nielsen K, Friis T.
20

21 1981. Urinary excretion of free and conjugated 3',5'-diiodothyronine and
22
23
24

25 3',3'-diiodothyronine. J Clin Endocrinol Metab 53: 587-593.
26
27
28
29
30
31
32

33 Gietz RD, Schiestl RH, Willems AR, Woods RA. 1995. Studies on the transformation of
34
35

36 intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355-360.
37
38
39
40
41
42
43

44 Glass CK, Holloway JM, Devary OV, Rosenfeld MG. 1988. The thyroid hormone
45
46

47 receptor binds with opposite transcriptional effects to a common sequence motif in
48
49
50

51 thyroid hormone and estrogen response elements. Cell 54: 313-323.
52
53
54
55
56
57
58

59 Goffeau A, Aert R, Agostini-Carbone ML, Ahmed A, Aigle M, Alberghina L, Allen E,
60
61
62
63
64
65

Alt-Mörbe J, André B, Andrews S, *et al.* 1997. The yeast genome directory. Nature 387
(supp): 5-105

Hazenbergh MP, de Herder WW, Visser TJ. 1988. Hydrolysis of iodothyronine
conjugates by intestinal bacteria. FEMS Microbiol Lett 54: 9-16.

Hodin RA, Lazar MA, Chin WW. 1990. Differential and tissue-specific regulation of the
multiple rat c-erbA messenger RNA species by thyroid hormone. J Clin Invest
85:101-105.

Hofmann PJ, Schomburg L, Kohrle J. 2009. Interference of endocrine disrupters with
thyroid hormone receptor-dependent transactivation. Toxicol Sci 110: 125-137.

Houlahan JE, Findlay CS, Schmidt BR, Meyer AH, Kuzmin SL. 2000. Quantitative
evidence for global amphibian population declines. Nature 404:752-755.

Ishihara A, Nishiyama N, Sugiyama S, Yamauchi K. 2003. The effect of endocrine

1 disrupting chemicals on thyroid hormone binding to Japanese quail transthyretin and
2
3
4 thyroid hormone receptor. Gen Comp Endocrinol 134:36-43.
5
6
7
8
9

10
11 Ito-Harashima S, Shiizaki K, Kawanishi M, Kakiuchi K, Onishi K, Yamaji R, Yagi T.
12
13 2015. Construction of sensitive reporter assay yeasts for comprehensive detection of
14
15 ligand activities of human corticosteroid receptors through inactivation of *CWP* and
16
17
18
19
20
21
22 *PDR* genes. J Pharmacol Toxicol Methods 74:41-52.
23
24
25
26
27
28
29

30 Jagnytsch O, Opitz R, Lutz I, Kloas W. 2006. Effects of tetrabromobisphenol A on larval
31
32
33 development and thyroid hormone-regulated biomarkers of the amphibian *Xenopus*
34
35
36
37 *laevis*. Environ Res 101:340-348.
38
39
40
41
42
43

44 Katsu Y, Taniguchi E, Urushitani H, Miyagawa S, Takase M, Kubokawa K, Tooi O, Oka
45
46
47 T, Santo N, Myburgh J, Matsuno A, Iguchi T. 2010. Molecular cloning and
48
49
50
51
52 characterization of ligand- and species-specificity of amphibian estrogen receptors.
53
54
55 Gen Comp Endocrinol 168:220-230.
56
57
58
59
60
61
62
63
64
65

1 Kawanishi M, Sakamoto M, Ito A, Kishi K, Yagi T. 2003. Construction of reporter
2
3
4 yeasts for mouse aryl hydrocarbon receptor ligand activity. *Mutat Res* 540:99-105.
5
6
7
8
9

10
11 Kawanishi M, Takamura-Enya T, Ermawati R, Shimohara C, Sakamoto M,
12
13
14
15 Matsukawa K, Matsuda T, Murahashi T, Matsui S, Wakabayashi K, Watanabe T,
16
17
18
19 Tashiro Y, Yagi T. 2004. Detection of genistein as an estrogenic contaminant of river
20
21
22 water in Osaka. *Environ Sci Technol* 38:6424–6429.
23
24
25
26
27
28
29

30 Kawanishi M, Sakamoto M, Ito A, Shimohara C, Yagi T. 2006. Establishment of
31
32
33 reporter yeasts for guinea pig and Syrian hamster aryl hydrocarbon receptor ligand
34
35
36 activity. *Genes Environ* 28:167-172.
37
38
39
40
41
42
43

44 Kingsford MJ, Suthes IM, Gray CA. 1996. Exposure to sewage plumes and the
45
46
47 incidence of deformities in larval fishes. *Mar Poll Bull* 33:201-212.
48
49
50
51
52
53
54

55 Kitamura S, Kato T, Iida M, Jinno N, Suzuki T, Ohta S, Fujimoto N, Hanada H,
56
57
58
59 Kashiwagi K, Kashiwagi A. 2005. Anti-thyroid hormonal activity of
60
61
62
63
64
65

1 tetrabromobisphenol A, a flame retardant, and related compounds: affinity to the
2
3
4 mammalian thyroid hormone receptor, and effect on tadpole metamorphosis. Life Sci
5
6
7
8 76 :1589-1601.
9

10
11
12
13
14
15 Kogai T, Brent GA. 2005. Thyroid Hormones (T₄,T₃). In: Melmed S, Conn PM, editors.
16
17
18
19 Endocrinology. New York: Humana Press. pp 267-281.
20
21
22
23
24

25
26 Kudo Y, Yamauchi K. 2005. In vitro and in vivo analysis of the thyroid disrupting
27
28
29 activities of phenolic and phenol compounds in *Xenopus laevis*. Toxicol Sci 84:29-37.
30
31
32
33
34
35

36
37 Kulkarni SS, Buchholz DR. 2013. Developmental programs and endocrine disruption
38
39
40
41 in frog metamorphosis: the perspective from microarray analysis. Curr Top Dev Biol
42
43
44 103:329-364.
45
46
47
48
49
50

51
52 Lazar MA. 1993. Thyroid hormone receptors: multiple forms, multiple possibilities.
53
54
55 Endocr Rev 14:184-193.
56
57
58
59
60
61

1 Lechan RM, Fekete C, Toni R. 2010. Thyroid hormones in neural tissue. In: Etgen AM,
2
3
4 Pfaff DW, editors. Molecular mechanisms of hormone actions on behavior. California:
5
6
7
8 Academic Press. pp 197-226.
9

10
11
12
13
14
15 Leo C, Chen JD. 2000. The SRC family of nuclear receptor coactivators. Gene
16
17
18
19 245:1-11.
20
21
22
23
24

25
26 McKinney JD, Waller CL. 1994. Polychlorinated biphenyls as hormonally active
27
28
29 structural analogues. Environ Health Perspect 102:290-297.
30
31
32
33
34
35

36
37 Miller 3rd CA. 1998. A human aryl hydrocarbon receptor signaling pathway
38
39
40 constructed in yeast displays additive responses to ligand mixtures. Toxicol Appl
41
42
43
44 Pharmacol 160:297-303.
45
46
47
48
49
50

51 Miyagawa S, Lange A, Tohyama S, Ogino Y, Mizutani T, Kobayashi T, Tatarazako N,
52
53
54
55 Tyler CR, Iguch T. 2015. Characterization of *Oryzian latipes* glucocorticoid receptors
56
57
58
59 and their unique response to progestins. J Appl Toxicol 35:302-309
60
61
62
63
64
65

1
2
3
4 Moreno M, de Lange P, Lombardi A, Silvestri E, Lanni A, Goglia F. 2008. Metabolic
5
6
7
8 effects of thyroid hormone derivatives. *Thyroid* 18:5185-5190.
9

10
11
12
13
14
15 Moriyama K, Tagami T, Akamizu T, Usui T, Saijo M, Kanamoto N, Hataya Y, Shimatsu
16
17
18
19 A, Kuzuya H, Nakao K. 2002. Thyroid hormone action is disrupted by bisphenol A as
20
21
22 an antagonist. *J Clin Endocrinol Metab* 87:5185-5190.
23
24
25
26
27
28
29

30 Morvan-Dubois G, Demeneix BA, Sachs LM. 2008. *Xenopus laevis* as a model for
31
32
33
34 studying thyroid hormone signalling: from development to metamorphosis. *Mol Cell*
35
36
37 *Endocrinol* 293:71-79.
38
39
40
41
42
43

44 Murata T, Yamauchi K. 2008. 3,3',5-Triiodo-L-thyronine-like activity in effluents from
45
46
47
48 domestic sewage treatment plants detected by *in vitro* and *in vivo* bioassays. *Toxicol*
49
50
51 *Appl Pharmacol* 226:309-317.
52
53
54
55
56
57
58

59 Oka T, Mitsui-Watanabe N, Tatarazako N, Onishi Y, Katsu Y, Miyagawa S, Ogino Y,
60
61
62
63
64
65

1 Yatsu R, Kohno S, Takase M, Kawashima Y, Ohta Y, Aoki Y, Guillette LJ Jr, Igucg T.

2
3
4 2013. Establishment of transactivation assay systems using fish, amphibian, reptilian

5
6
7
8 and human thyroid hormone receptors. J Appl Toxicol 33:991-1000

9
10
11
12
13
14
15 Opitz R, Hartmann S, Blank T, Braunbeck T, Lutz I, Kloas W. 2006. Evaluation of

16
17
18
19 histological and molecular endpoints for enhanced detection of thyroid system

20
21
22
23 disruption in *Xenopus laevis* tadpoles. Toxicol Sci 90:337-348.

24
25
26
27
28
29
30 Paul BD, Buchholz DR, Fu L, Shi YB. 2007. SRC-p300 coactivator complex is

31
32
33
34 required for thyroid hormone-induced amphibian metamorphosis. J Biol Chem

35
36
37 282:7472-7481.

38
39
40
41
42
43
44 Rudel RA, Melly SJ, Geno PW, Sun G, Brody JG. 1996. Identification of alkylphenols

45
46
47
48 and other estrogenic compounds in wastewater, septage, and groundwater on cape

49
50
51
52 Cod, Massachusetts. Environ Sci Technol 32:861-869.

53
54
55
56
57
58
59 Sadow PM, Chassande O, Koo EK, Gauthier K, Samarut J, Xu J, O'Malley BW, Weiss

1 RE. 2003. Regulation of expression of thyroid hormone receptor isoforms and
2
3
4
5 coactivators in liver and heart by thyroid hormone. *Mol Cell Endocrinol* 203:65-75.
6
7
8
9

10
11 Sandler B, Webb P, Apriletti JW, Huber BR, Togashi M, Cunha Lima ST, Juric S,
12
13
14
15 Nilsson S, Wagner R, Fletterick RJ, Baxter JD. 2004. Thyroxine-thyroid hormone
16
17
18
19 receptor interactions. *J Biol Chem* 279:55801-55808.
20
21
22
23
24

25
26 Shiizaki K, Asai S, Ebata S, Kawanishi M, Yagi T. 2010. Establishment of yeast
27
28
29
30 reporter assay systems to detect ligands of thyroid hormone receptors alpha and beta.
31
32
33
34 *Toxicol In Vitro* 24:638-644.
35
36
37
38
39
40

41 Shiizaki K, Yoshikawa T, Takada E, Hirose S, Ito-Harashima S, Kawanishi M, Yagi T.
42
43
44
45 2014. Development of yeast reporter assay for screening specific ligands of retinoic
46
47
48
49 acid and retinoid X receptor subtypes. *J Pharmacol Toxicol Methods* 69:245-252.
50
51
52
53
54

55 Suen CS, Yen PM, Chin WW. 1994. In vitro transcriptional studies of the roles of the
56
57
58
59 thyroid hormone (T₃) response elements and minimal promoters in T₃-stimulated gene
60
61
62
63
64
65

transcription. J Biol Chem 269: 1314-1322.

Tata JR. 1998. Amphibian metamorphosis as a model for studying the developmental actions of thyroid hormone. Cell Res 8:259-272.

Vandenlangenberg SM, Canfield JT, Magner JA. 2003. A regional survey of malformed frogs in Minnesota (USA) (Minnesota malformed frogs). Environ Monit Assess 82:45-61.

Veldhoen N, Boggs A, Walzak K, Helbing CC. 2006. Exposure to tetrabromobisphenol-A alters TH-associated gene expression and tadpole metamorphosis in the Pacific tree frog *Pseudacris regilla*. Aquat Toxicol 78:292-302.

Visser TJ. 1996. Pathways of thyroid hormone metabolism. Acta Medica Austriaca 23:10-16.

Visser TJ, Buuren JCJ, Rutgers M, Rooda SJE, Herder WW. 1990. The role of

1 sulfation in thyroid hormone metabolism. Trends Endocrinol Metab 1:211-218.

2
3
4
5
6
7
8 Vos JG, Dybing E, Greim HA, Ladefoged O, Lambre C, Tarazona JV, Brandt I,
9
10
11 Vethaak AD. 2000. Health effects of endocrine-disrupting chemicals on wildlife, with
12
13
14
15 special reference to the European situation. Crit Rev Toxicol 30:71-133.
16
17
18
19
20
21

22
23 Warner A, Mittag J. 2012. Thyroid hormone and the central control of homeostasis. J
24
25
26 Mol Endocrinol 49:R29-R35.
27
28
29
30
31
32

33
34 Xu J, Li Q. 2003. Review of the *in vivo* functions of the p160 steroid receptor
35
36
37 coactivator family. Mol Endocrinol 17:1681-1692.
38
39
40
41
42
43

44
45 Yamauchi K, Ishihara A, Fukazawa H, Terao Y. 2003. Competitive interactions of
46
47
48 chlorinated phenol compounds with 3,3',5-triiodothyronine binding to transthyretin:
49
50
51 detection of possible thyroid-disrupting chemicals in environmental waste water.
52
53
54
55 Toxicol Appl Pharmacol 187:110-117.
56
57
58
59
60
61

1 Yaoita Y, Brown DD. 1990. A correlation of thyroid hormone receptor gene expression
2
3
4 with amphibian metamorphosis. Genes Dev 4:1917-1924.
5
6
7
8
9

10
11 Yen PM. 2001. Physiological and molecular basis of thyroid hormone action. Physiol
12
13
14
15 Rev 81:1097-1142.
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

FIGURE LEGENDS

Fig. 1. Maps of plasmids constructed in this study. Plasmid maps of the yeast receptor expression plasmid pUdp6-xTR (A), coactivator-expressing plasmid pESC-Leu-xSRC1 (B), and yeast reporter plasmid pYT-IR0x5 (C). In the pUdp6-xTR map, $P_{GAL1,10}$: *GAL1,10* dual directional promoter; T_{ADH1} : terminator sequence of yeast alcohol dehydrogenase gene 1 (*ADH1*); and T_{CYC1} : terminator sequence of yeast cytochrome C gene 1 (*CYC1*). The *Apa* I and *Eco*R V sites were indicated in (A).

Fig. 2. Optimization of yeast reporter assays for XT TR α and TR β . (A) Comparison of the response elements for XT TRs in yeast reporter assays. Reporter plasmids carrying IR-0 or DR-4-type TRE were introduced into yeasts expressing XT TR α or XT TR β . (B) Enhancement in the T_3 response by XT SRC-1 in XT TR strains carrying pYT-IR0x5. Yeast clones with or without the XT SRC-1 expression plasmid were exposed to T_3 (1 μ M). Data represent the average of duplicate experiments for a representative yeast transformant.

Fig. 3. XT TR β^{Asn179} as a highly active TR β ortholog of XT in the yeast reporter assay.

(A) Responses of XT TR β^{Ser179} (closed circle) and XT TR β^{Asn179} (open circle) strains against T₃. The yeast strains were exposed to various concentrations of T₃ and β -galactosidase activity was measured. Data represent the mean $\pm$ standard deviation (S. D.) of triplicate experiments (* p < 0.05, ** p < 0.01 vs. XT TR β^{Ser179} at the indicated ligand concentration). (B) Comparison of the partial amino acid sequences of the LBD of TR β orthologs across five amphibian species and humans. Position 179 of XT TR β and the corresponding positions in other species registered in the DDBJ/EMBL/GenBank were indicated in bold face letters.

Fig. 4. Responses to THs in yeast strains expressing TR α (square marks) and TR β (circle marks) of the frog (closed marks) and human (open marks). Data represent the mean $\pm$ S.D. of triplicate experiments (* p < 0.05, ** p < 0.01 vs. HS TR α ; # p < 0.05, ## p < 0.01 vs. HS TR β at the indicated ligand concentration).

Fig. 5. Responses to industrial chemicals in yeast strains expressing TR α (square marks) and TR β (circle marks) of the frog (closed marks) and human (open marks).

1 Data represent the mean $\pm$ S.D. of triplicate experiments.
2
3
4
5
6
7

8 **Fig. 6.** Assay of environmental samples. (A) A map showing the locations of water
9
10 sampling: Station 1 (ST1), Asakayama-Kouen, Asakayama 2-chome, Sakai-ku, Sakai
11
12 City, Osaka; Station 2 (ST2), Miyukiohashi, Kawai-cho, Kitakatsuragi-gun, Nara
13
14 (joined by Tomio River); Station 3 (ST3), Itayagase Bridge, Nukatabeminami-cho,
15
16 Yamatokooriyama City, Nara (joined by Saho River); Station 4 (ST4), Umedo Bridge,
17
18 Ooaza, Kawanishi-cho, Isobe-gun, Nara. (B) Responses of TR strains to river water.
19
20 The yeast strains expressing TR α and TR β of the frog and human were exposed to
21
22 organic substances extracted and concentrated from river water. Data represent the
23
24 results of samples with a concentration factor of 5,000.
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Table 1. Oligonucleotides used in the study for nuclear receptor and coactivator cDNA amplification

xTR α 1F	ATGAGCCTCGGGCAGCTGGG
xTR α 1R	ATGGCCAACCAAGGCAGGGA
xTR α 2F ^{a, b}	CCCC <u>CCCGGG</u> AACAAAATGGACCAGAATCTCAGCGGGCT
xTR α 2R ^a	CCCCGAATTCTCAGACTTCCTGGTCCTCAAAGA
xTR β F ^{a, b}	CCCC <u>CCCGGG</u> AACAAAATGCCAAGCAGTATGTCAGAGAC
xTR β R ^a	CCCCGAATTCCTAGTCCTCAAACACTTCCAAGA
xTR β fPCR ^c	TCCCATAGTTAGTGCGCCTGAGG
xTR β fPCR ^c	CCTCAGGCGCACTAACTATGGGA
xSRC1F ^{a, b}	CCCC <u>GCGGCCGC</u> AACAAAATGAGTGGCCTCGGGGACAGTT
xSRC1R ^a	CCCCTTAATTAATCATTCCGTCAGCAGTTGCTGT

^a Underlined sequences are additional nucleotides to introduce restriction enzyme recognition sites.

^b The sequences shown in italics are yeast ribosomal binding consensus sequences.

^c Underlined sequences are a mutation to create an Asn¹⁷⁹ to Ser¹⁷⁹ substitution in XT TR β .

Table 2. Detection limit and EC₅₀ of thyroid hormones in TR α and TR β assay yeasts of the frog (XT) and human (HS)

	T ₂		T ₃		T ₄	
	Detection limit (μ M)	EC ₅₀ (μ M)	Detection limit (μ M)	EC ₅₀ (μ M)	Detection limit (μ M)	EC ₅₀ (μ M)
XT TR α	0.1	2.1 $\pm$ 1.9	0.01	0.07 $\pm$ 0.006	0.01	0.05 $\pm$ 0.007*
XT TR β	0.1	0.54 $\pm$ 0.05**	0.01	0.06 $\pm$ 0.01	0.01	0.05 $\pm$ 0.008
HS TR α	1	6.1 $\pm$ 1.6	0.1	0.11 $\pm$ 0.04	0.1	0.09 $\pm$ 0.009*
HS TR β	0.1	0.83 $\pm$ 0.17**	0.01	0.08 $\pm$ 0.01	0.01	0.06 $\pm$ 0.01

T₂: 3,5-diiodo-L-thyronine; T₃: 3,5,3'-triiodo-L-thyronine; T₄: L-thyroxine; EC₅₀: 50% effective concentration.

EC₅₀ values were significantly different between XT and HS: *XT TR α vs. HS TR α ($p < 0.01$); **XT TR β vs. HS TR β ($p < 0.05$).

Table 3. Detection limit and EC₅₀ of chemicals in TR α and TR β assay yeasts of the frog (XT) and human (HS)

	OBP		IPP		TBBPA		TMBPA	
	Detection limit (μ M)	EC ₅₀ (μ M)	Detection limit (μ M)	EC ₅₀ (μ M)	Detection limit (μ M)	EC ₅₀ (μ M)	Detection limit (μ M)	EC ₅₀ (μ M)
XT TR α	3.2	60 $\pm$ 1.2	10	54 $\pm$ 7.3	0.010	0.57 $\pm$ 0.03	1.0	16 $\pm$ 3.0*
XT TR β	3.2	27 $\pm$ 1.8	3.2	63 $\pm$ 1.0**	1.0	0.77 $\pm$ 0.12	1.0	7.6 $\pm$ 0.38
HS TR α	10	n.d.	32	n.d.	0.032	0.56 $\pm$ 0.03	3.2	7.3 $\pm$ 0.26*
HS TR β	10	39 $\pm$ 13	32	55 $\pm$ 4.3**	0.32	0.66 $\pm$ 0.06	1.0	6.3 $\pm$ 0.49

OBP: *o*-*t*-Butylphenol; IPP: 2-isopropylphenol; TBBPA: tetrabromobisphenol A; TMBPA: tetramethylbisphenol A

n.d.: not determined.

EC₅₀ values were significantly different between XT and HS: *XT TR α vs. HS TR α ($p < 0.05$); **XT TR β vs. HS TR β ($p < 0.05$).

Table 4. T₃ equivalent concentrations of TH-like activities of chemicals detected in TR α and TR β assay yeasts of the frog (XT) and human (HS)

	T ₃ equivalent concentration (ng/mL)			
	OBP (100 μ M)	IPP (100 μ M)	TBBPA (1 μ M)	TMBPA (32 μ M)
XT TR α	14 $\pm$ 3.2	4.3 $\pm$ 0.4	36 $\pm$ 2.7	13 $\pm$ 0.3*
XT TR β	73 $\pm$ 16 ^{##}	9.6 $\pm$ 2.0 [#]	5.5 $\pm$ 0.4 [#]	46 $\pm$ 3.2 ^{##}
HS TR α	n.c.	n.c.	25 $\pm$ 1.2	19 $\pm$ 0.7*
HS TR β	13 $\pm$ 1.9 ^{##}	4.1 $\pm$ 1.0 [#]	3.8 $\pm$ 0.5 [#]	10 $\pm$ 1.0 ^{##}

n.c.: not calculable. Reporter activity was detectable, but not sufficiently high to calculate T₃ equivalent concentrations.

T₃ equivalent concentrations were significantly different between XT and HS: *XT TR α vs. HS TR α (p < 0.01); XT TR β vs. HS TR β ([#] p < 0.05, ^{##} p < 0.01).

Table 5. T₃ equivalent concentrations of TH-like activities of river water detected in TR α and TR β assay yeasts of the frog (XT) and human (HS)

	T ₃ equivalent concentration (ng/L)			
	ST 1	ST 2	ST 3	ST 4
XT TR α	94 $\pm$ 11	48 $\pm$ 6.4	294 $\pm$ 5.2	49 $\pm$ 4.0
XT TR β	113 $\pm$ 5.9	49 $\pm$ 0.8	1010 $\pm$ 68*	46 $\pm$ 1.0
HS TR α	n.c.	n.c.	291 $\pm$ 9.8	n.c.
HS TR β	101 $\pm$ 4.3	74 $\pm$ 14	830 $\pm$ 71*	n.c.

n.c.: not calculable.

T₃ equivalent concentrations were significantly different between XT and HS: *XT TR β vs. HS TR β ($p < 0.05$).

Table 6. T₃ equivalent concentrations of TH-like activities of human urine and feces detected in TR α and TR β assay yeasts of the frog (XT) and human (HS)

	T ₃ equivalent concentrations					
	Urine (ng/mL)			Feces (ng/g)		
	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
XT TR α	10 $\pm$ 0.5	22 $\pm$ 0.6**	38 $\pm$ 4.9**	76 $\pm$ 3.2	628 $\pm$ 85*	121 $\pm$ 58
XT TR β	23 $\pm$ 0.1	52 $\pm$ 0.9 [#]	77 $\pm$ 1.9 [#]	110 $\pm$ 1.4 [#]	1104 $\pm$ 37 [#]	609 $\pm$ 58 [#]
HS TR α	n.c.	2.3 $\pm$ 0.3**	15 $\pm$ 2.1**	n.c.	87 $\pm$ 1.0*	22 $\pm$ 14
HS TR β	20 $\pm$ 3.0	7.5 $\pm$ 0.5 [#]	21 $\pm$ 0.8 [#]	75 $\pm$ 5.7 [#]	226 $\pm$ 8.7 [#]	71 $\pm$ 0.9 [#]

n.c.: not calculable.

T₃ equivalent concentrations were significantly different between XT and HS: XT TR α vs. HS TR α (* p < 0.05, ** p < 0.01); XT TR β vs. HS TR β ([#] p < 0.01).

Fig. 1

Fig. 2

A

B

Fig. 3

A

B

<i>X. Tropicalis</i>	171	DIGQAPIV S APEGG	184
<i>X. Laevis</i>	161	DIGQAPIV N APEGG	174
<i>R. Catesbeiana</i>	161	DIGQAPIV N APEGG	174
<i>N. Maculosus</i>	161	DIGQAPIV N APESG	174
<i>A. Mexicanum</i>	161	DIGQAPI M NAPDGG	174
<i>H. sapiens</i>	249	DIGQAPIV N APEGG	262

Fig. 4

Fig. 5

Fig. 6