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# Development of Biomarkers of Endocrine Disrupting Activity in Emerging Amphibian Model, *Silurana (Xenopus) tropicalis*

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Because amphibians show peculiar ecological features and interesting responses to some hormones, it is conceivable that amphibians are very useful animals for assessing the toxic effects of environmental contaminants, including endocrine disrupters. To develop methods of detecting endocrine toxicity of environmental chemicals in amphibians, we have started to assemble a biomarker tool kit for an emerging amphibian model, *Silurana* (*Xenopus*) *tropicalis*. We isolated full-length cDNAs encoding estrogen receptor α (ERα), ERβ, thyroid hormone receptor α (TRα), and TRβ of *S.* (*X.*) *tropicalis* to develop a reporter gene assay system, as an estimation tool for environmental chemicals. The amino acid sequences inferred from the four full-length cDNAs were highly homologous to those of ERα, TRα and TRβ of *X. laevis*, and ERβ of the Japanese quail. In particular, the *S.* (*X.*) *tropicalis* ERα shared a higher similarity of amino acid sequence with *X. laevis* ERα than the previously reported *S.* (*X.*) *tropicalis* ERα, as determined by Wu *et al.*[(1)] RT-PCR analysis showed that the two *ERα* and *ERβ* transcripts were expressed relatively abundantly in the brain, liver, and gonad/kidney complex of the *S.* (*X.*) *tropicalis* tadpole after gonadal sex differentiation occurring at developmental stages 54–59, suggesting that they are susceptible to estrogenic substances. A similar result was obtained in the two *TR* transcripts, although their expression levels were lower in the gonad/kidney complex than in the other tissues. Moreover, we identified *vitellogenin A* (*Vtg A*) and *Vtg B* as estrogen-responsive genes expressed in the female *S.* (*X.*) *tropicalis* liver using macroarray analysis and RT-PCR. In addition, *Rana japonica* Vtg was purified from serum using anion-exchange chromatography to produce anti-Vtg antibody as a protein marker. In the future, we are going to construct reporter gene assay systems using the full-length *ER* and *TR* cDNAs, analyze histologically the differentiation of gonads and thyroid glands in the *S.* (*X.*) *tropicalis* tadpole exposed to estrogenic chemicals, and produce sex-reversed male *S.* (*X.*) *tropicalis* to obtain all-male tadpoles. Using these tools, we hope to be able to identify endocrine disrupting compounds in laboratory experiments for hazard assessment purposes, and also detect endocrine toxicity in environmental samples as part of an integrated approach to assessing the impact of environmental contaminants on wild amphibian populations in Japan and the UK.

## 1. Introduction

Most anuran amphibians occupy both aquatic and terrestrial habitats, and are either carnivorous or herbivorous, depending upon their developmental phase, that is, frog or tadpole. In addition, amphibians show interesting responses to some hormones, such as sex reversal induced by sex steroidal hormones and metamorphosis induced by the thyroid hormone (TH). Thus, it is conceivable that amphibians are very useful animals as indicators of environmental changes.

Recent reports[2–4] have demonstrated that amphibian populations have declined around the world. The destruction of their habitat and environmental pollution are considered to be factors contributing to their population decrease, indicating the global destruction of the natural environment. A large number of studies have been available on the ecology and population ecology of amphibians;[2] however, it remains difficult to evaluate whether individual or chemical substances, and endocrine disrupters in particular, affect the wild population of amphibians. Thus, we aim to develop a ‘tool kit’ of biomarkers of endocrine toxicity in amphibians. Important reproductive and physiological functions in amphibians, such as gonadal differentiation and vitellogenin (Vtg) synthesis, are regulated primarily through nuclear ERs after the binding of estrogens. Bisphenol A (BPA) and nonylphenol (NP) have been shown to bind to estrogen receptor (ER) *in vitro*.[5] Endocrine disrupting compounds have also been reported to disrupt gonadal sex differentiation and induce elevated serum VTG concentrations in amphibians,[6,7] although the former toxic effect of BPA is still unclear.[8] Additionally, thyroid-disrupting chemicals may be detected in the amphibian model. Tetrabromobisphenol A (TBBPA) has been shown to bind to the human thyroid hormone receptor (TR), and it also suppresses tail shortening in tadpoles induced by TH.[9] Thus, information on the structures and expressions of ER and TR will be useful for understanding the mechanisms underlying the toxicological effects of endocrine disrupting compounds in amphibians. Moreover, a full-length receptor cDNA for these important genes can be used for the construction of reporter gene assays, which are powerful tools for the rapid evaluation of chemicals for hormonal activities.

*Xenopus laevis* has been commonly used in developmental biology because of the ease of laboratory husbandry and breeding by hormone treatment throughout the year. However, because of its relatively shorter generation time (about 5 months) and diploidy, in addition to its advantages shared with *X. laevis*, *Silurana* (*X.*) *tropicalis* now provides a more popular model system for genetic as well as developmental biology studies.[10,11] In this paper, we present our progress in assembling biomarkers of estrogenic and thyroidal activities in an amphibian model through the isolation and expression analysis of genes for estrogen and thyroid receptors, and estrogen-responsive *S.* (*X.*) *tropicalis*. Additionally, we report on the development of an anti-VTG antibody for a widely distributed Japanese Ranid amphibian (*Rana japonica*).

## 2. Development of Tools for Evaluating and Assessing Toxic Effects of Environmental Chemicals on Amphibians

### 2.1 *Cloning of hormone receptors and analyses of their expression during development*

Toxicological effects may be induced through nuclear hormone receptors after the binding of endocrine-disrupting compounds. Reporter-gene assay systems are very powerful tools for identifying functional ligands that bind to specific receptors. Full-length cDNA is indispensable for the construction of the reporter gene assay system. Moreover, the analyses of *ER* and *TR* expressions during development, and the tissue distribution of their transcripts will help us to establish methods of evaluating and assessing endocrine toxicity induced by environmental chemicals in

amphibians. Histological analysis of gonadal sex differentiation is also important to understand the profile of the expression of key genes during development. Therefore, we isolated two isoforms of the full-length *ER* and *TR* of *S.* (*X.*) *tropicalis*, and have characterized their expressions in different tissues throughout development, with particular emphasis on the period of gonadal differentiation.

#### 2.1.1 *Molecular cloning of* ERα *and* ERβ *cDNAs and amino acid comparison*

Although *ER* and *ER*-related protein cDNAs from many vertebrate species have been isolated so far, little information has been available on amphibian *ER* cDNAs. Weiler *et al.*(12) first succeeded in isolating an amphibian *ERα* cDNA from *X. laevis*, while more recently, *ERα* cDNA has been isolated from *S.* (*X.*) *tropicalis*.(1) We have also succeeded in isolating full-length *ERα* cDNA from *S.* (*X.*) *tropicalis* (Accession No. AB244211) using 5'/3' RACE and oligo-capping methods. The amino acid sequence of *S.* (*X.*) *tropicalis* ERα from our studies exhibit high homologies to those of *S.* (*X.*) *tropicalis* ERα,(1) *X. laevis* xer3, an ER-related protein(12) and an ER-related protein.(13) The two *S.* (*X.*) *tropicalis* ERα's, as in the two *X. laevis* ER-related proteins, lack amino acids localized within the D domain of the *X. laevis* xer3 (Fig. 1(a)). The

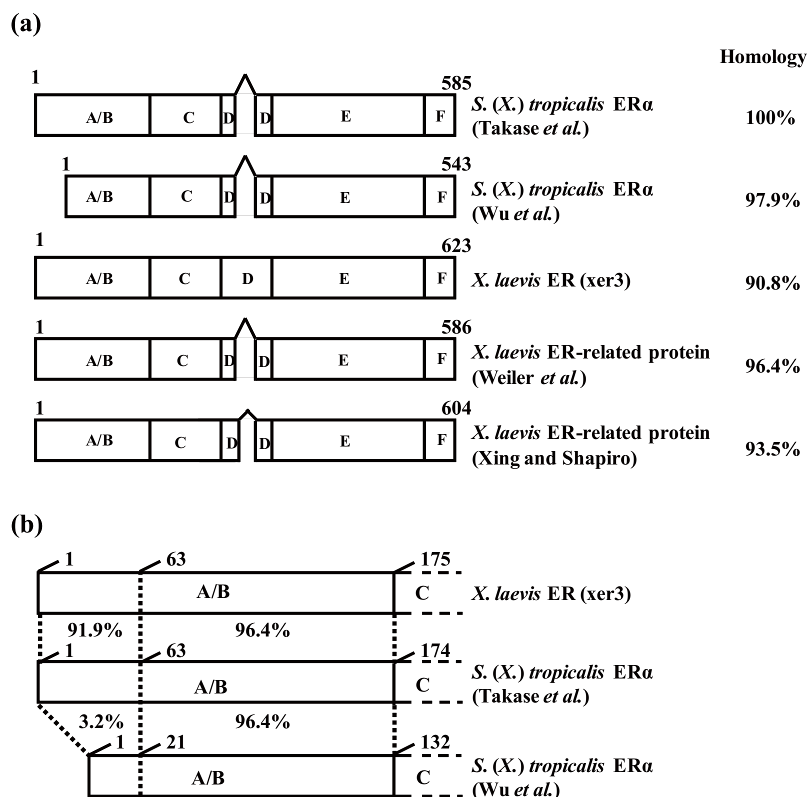



Fig. 1. Comparison of amino acid sequences of *S.* (*X.*) *tropicalis* ERα and its related proteins. The numbers on each illustration refer to the position of amino acid residues. (a) The amino acid sequence of *S.* (*X.*) *tropicalis* ERα determined by our group (Accession No. AB244211) is compared with those of *S.* (*X.*) *tropicalis* ERα determined by Wu *et al.* (Accession No. AY310902), *X. laevis* xer3 (Accession No. L20736), *X. laevis* ER-related protein determined by Weiler *et al.* (Accession No. L20735), and *X. laevis* ER-related protein determined by Xing and Shapiro (Accession No. L20738). Gap is observed in the D domain other than *X. laevis* xer3. (b) The amino acid sequences of the A/B domains of *S.* (*X.*) *tropicalis* ERα determined by our group, *X. laevis* xer3, and *S.* (*X.*) *tropicalis* ERα determined by Wu *et al.* are compared.

N-terminal amino acid sequence of *S.* (*X.*) *tropicalis* ERα is more highly homologous to that of *X. laevis* xer3, than to that of *S.* (*X.*) *tropicalis* ERα[1] (Fig. 1(b)).

In addition, Wu *et al.*[1] have isolated *ERβ* cDNA from *S.* (*X.*) *tropicalis*, which was the first cloning of *ERβ* cDNA from any amphibian species, although it was not full-length cDNA. We have succeeded in isolating full-length *ERβ* cDNA (Accession No. AB244212). The amino acid sequences of the C and E/F domains of the *S.* (*X.*) *tropicalis* ERβ that we have isolated show high similarities with those of medaka ERβ, roach ERβ, goldfish ERβ, zebrafish ERβ, quail ERβ, and human ERβ (Fig. 2). On the other hand, the E/F region of the frog ERβ is more closely related in terms of amino acid sequence to quail and human ERβs, than to teleost ERβs (Fig. 2). Thus, it is conceivable that the frog ERβ shares similar affinity for estrogen binding with higher vertebrate species. In contrast, few similarities were found in the A/B domain of the ERβ between *S.* (*X.*) *tropicalis* and other vertebrates (Fig. 2). Because it is well established that the A/B domain is involved in transcriptional activities,[14,15] the low similarity may be associated with differences in susceptibility to toxicological influences induced by endocrine disrupting compounds among vertebrate species.

#### 2.1.2 *Molecular cloning of* TRα *and* TRβ *cDNAs and amino acid comparison*

Two subtypes of TRα (TRαA and TRαB) mRNA and four subtypes of TRβ (TRβA1, TRβA5, TRβB1 and TRβB2) mRNA were isolated for the first time in amphibian species, specifically from *X. laevis*.[16] We isolated for the first time two full-length isoforms of *TR* mRNA from *S.* (*X.*) *tropicalis* using RT-PCR and 5'/3' RACE methods (Accession No. AB244213 for *TRα* cDNA and Accession No. AB244214 for *TRβ* cDNA). The inferred amino acid sequences of the *S.* (*X.*) *tropicalis* TRα and TRβ showed high similarities with those of the two TRα subtypes and the four TRβ subtypes of *X. laevis*, respectively (Figs. 3(a) and 3(b)). Because *X. laevis* is a pseu-

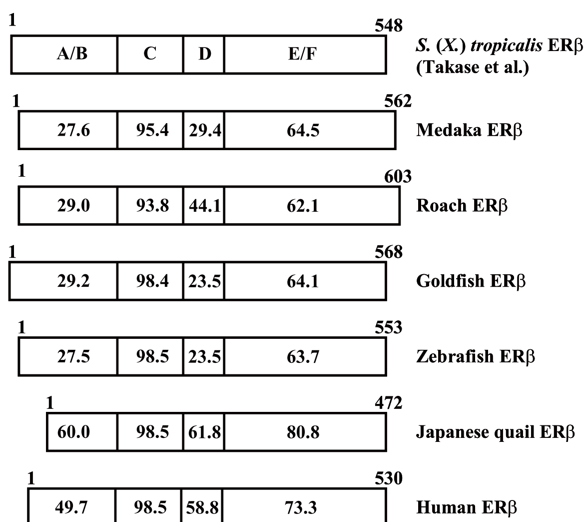



Fig. 2. Comparison of ERβ amino acid sequences from various vertebrates. The amino acid sequence of *S.* (*X.*) *tropicalis* ERβ determined by our group (Accession No. AB244211) is compared with those of medaka ERβ (Accession No. AB070901), roach ERβ (Accession No. AB190290), goldfish ERβ (Accession No. AF061269), zebrafish ERβ (Accession No. AF349414), quail ERβ (Accession No. AF045149) and human ERβ (Accession No. BC024181). The numbers on each illustration refer to the position of amino acid residues, and the numbers in each illustration are the values of % homology to the *S.* (*X.*) *tropicalis* ERβ sequence determined by our group.

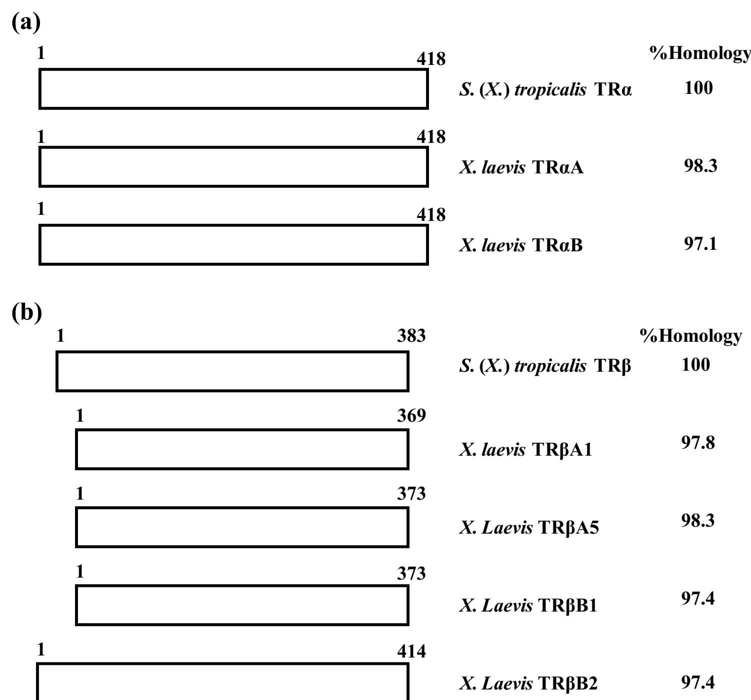



Fig. 3. Comparison of amino acid sequences of TRα (a) and TRβ (b) between frogs. The numbers on the right are the values of % homology to *S.* (*X.*) *tropicalis* TRα or TRβ. (a) The amino acid sequence of *S.* (*X.*) *tropicalis* TRα determined by our group (Accession No. AB244211) is compared with those of TRαA (Accession No. AB070901) and TRαB (Accession No. AB070901) of *X. laevis*. (b) The amino acid sequence of *S.* (*X.*) *tropicalis* TRβ determined by our group (Accession No. AB244211) is compared with those of TRβA1 (Accession No. AB070901), TRβA5 (Accession No. AB070901), TRβB1 (Accession No. AB190290) and TRβB2 (Accession No. AF061269) of *X. laevis*.

dotetraploid animal, it could be expected that the two subtypes of the *TRα* mRNA are expressed in this species, while the four subtypes of *TRβ* mRNA are expressed in this species. The isolation of the subtypes of the *S.* (*X.*) *tropicalis TRβ* mRNA should be further explored.

### 2.1.3 *Gonadal sex differentiation*

Before the analysis of receptor gene expression during development, we conducted a histological analysis of the differentiation and development of the gonads of *S.* (*X.*) *tropicalis* tadpole. Witschi[17] proposed that gonadal sex differentiation in amphibians could be classified into differentiated, undifferentiated and semidifferentiated types, according to his observations on the gonadal sex differentiation of *R. temporaria* as follows. In the differentiated type, gonads differentiate directly into testes in males, or ovaries in females. On the other hand, in the undifferentiated and semidifferentiated types, gonads differentiate into testes through an ovarian phase (for males), or directly into ovaries (for females), before or after metamorphosis, respectively. In *S.* (*X.*) *tropicalis*, undifferentiated gonads were observed in the tadpole at stage 54 (Nieuwkoop and Faber), and the histological features of the tadpole gonad at stage 60 showed testicular and ovarian differentiation (Fig. 4). In *X. laevis*, gonadal sex differentiation showed the differentiated type.[18] Thus, it seems that gonadal differentiation in the *S.* (*X.*) *tropicalis* tadpole can also be classified into the differentiated type, although a more detailed analysis of gonadal differentiation in *S.* (*X.*) *tropicalis* larvae is warranted.

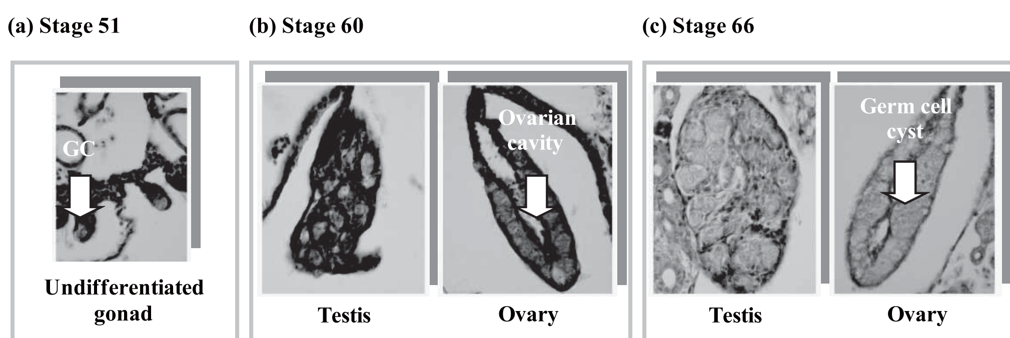



Fig. 4. Histological features of *S.* (*X.*) *tropicalis* gonads during development. (a) Undifferentiated gonad of tadpole at stage 51. A single germ cell is found in the genital ridge below the posterior cardiac vein. GC: germ cell. (b) Very immature testis (left panel) and ovary (right panel) of tadpole at stage 60. An ovarian cavity is present in the ovary. (c) Immature testis (left panel) and ovary (right panel) of froglet at stage 66. A germ cell cyst is present in the ovary.

### 2.1.4 *Expression of receptor genes*

The analysis of *ER* expression during development has been restricted to the tadpole around the hatching stage[19] and gonadal sex differentiation,[20] although several types of *Xenopus* and *Silurana ERs* have been isolated as mentioned above. *TR* expression has been analyzed in the head, hindlimb, and tail of the *X. laevis* tadpole before the completion of metamorphosis.[21] The expression of TR genes has also been analyzed in the TH- and TH-antagonist-primed *X. laevis* tadpole.[22] The characterization of the baseline *ER* and *TR* expression profiles in various tissues during a wide range of developmental stages before metamorphosis and after metamorphosis will be necessary before the utility of these genes as biomarkers of environmental exposure to endocrine disrupters could be validated.

We analyzed *ER* and *TR* expressions in various tissues including the brain, liver, heart, stomach, gonad/kidney complex, leg muscle, and back skin of male and female froglets 2 months after metamorphosis. RT-PCR analysis showed that both *ERα* and *ERβ* mRNAs were expressed abundantly in the brain, liver, and gonad/kidney complex without a clear-cut sex difference except for the *ERβ* expression in the gonad/kidney complex (Figs. 5(a) and 5(b)). On the other hand, both the *TRα* and *TRβ* mRNAs were also expressed in the brain, liver, and gonad/kidney complex, although their expressions were observed in the gonad/kidney complex at lower levels (Figs. 5(a) and 5(b)). Therefore, we analyzed the expressions of the *ER* and *TR* transcripts in the brain, liver and gonad/kidney complex during development before and after metamorphosis.

The *ERα* transcripts in the brain, liver and gonad/kidney complex, and the *ERβ* transcript in the brain were expressed at low levels in the tadpoles before gonadal sex differentiation, but became extremely abundant in the tadpoles after that (Figs. 6(a)–6(c)). In contrast, the *ERβ* transcript was expressed abundantly and consistently in the liver and gonad/kidney complex before and after gonadal sex differentiation (Figs. 6(b) and 6(c)). Next, *TR* expressions were analyzed in the developing brain, liver and gonad/kidney complex. Before gonadal sex differentiation, the *TRβ* mRNA was expressed slightly in the liver (Fig. 6(b)). However, after that, the three tissues expressed both *TRα* and *TRβ* mRNAs without significant alterations (Figs. 6(a)–6(c)). These results taken together suggest that the brain, liver, and gonad/kidney complex of the *S.* (*X.*) *tropicalis* tadpole may be more susceptible to toxic influences induced by endocrine disrupting compounds after gonadal sex differentiation. Mitsui *et al.*[11] have established a metamorphosis assay system using *S.* (*X.*) *tropicalis*. Because TH

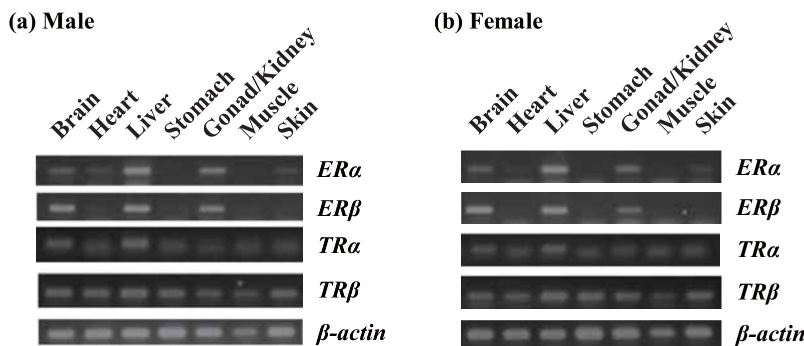



Fig. 5. RT-PCR analysis of receptor gene expressions in various tissues. Transcripts from the *ERα*, *ERβ*, *TRα*, and *TRβ* of *S.* (*X.*) *tropicalis* were analyzed in various tissues of male (a) and female (b) by RT-PCR. The amplified cDNAs were electrophoresed on 1% agar gel. *β-actin* was also amplified as an internal control.

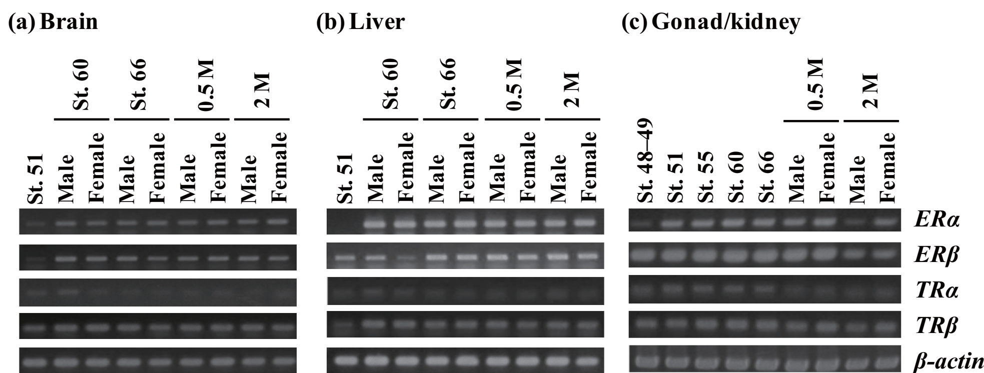



Fig. 6. RT-PCR analysis of receptor genes in brain (a), liver (b), and gonad/kidney complex (c) during development. The transcripts from the *ERα*, *ERβ*, *TRα*, and *TRβ* of *S.* (*X.*) *tropicalis* were analyzed in the developing brain, liver and gonad/kidney complex by RT-PCR. The amplified cDNAs were electrophoresed on 1% agar gel. *β-actin* was also amplified as an internal control.

induces the progressive transcription of *TRβ* in the *S.* (*X.*) *tropicalis* tadpole tail at a late developmental stage (Takase *et al.*, unpublished data), we expect the establishment of a more sensitive assay system combining the apical endpoints of larval development with the expression of *TR* genes in *S.* (*X.*) *tropicalis*.

## 2.2 *Isolation of genetic and protein markers*

Genetic and protein markers are useful for evaluating the responses of wildlife organisms to pollution. It is well accepted that VTG is a useful protein marker of the effects of estrogenic chemicals in lower vertebrate species. Thus, we endeavored to isolate estrogen-responsive genes, including VTG genes, in *S.* (*X.*) *tropicalis*, and have purified *R. japonica* VTG to produce an anti-*Rana* VTG antibody.

### 2.2.1 *Isolation of estrogen-responsive gene*

We isolated estrogen-responsive genes from a library of cDNAs from female *S.* (*X.*) *tropicalis* liver using a macroarray technique. Firstly, we constructed a cDNA library from mRNA extracted from female *S.* (*X.*) *tropicalis* liver, and then picked up 839 colonies with a cDNA insert from the cDNA library. The nucleotide sequences

of 809 out of the 839 cDNAs were analyzed, and 687 out of the 809 cDNAs shared high similarities in nucleotide sequence with genes on GenBank database (Table 1). Some of the 687 cDNAs shared the same nucleotide sequences with each other (Table 1). On the other hand, 122 out of the 809 cDNAs had no or low homology with genes on the database (Table 1). Finally, 516 cDNAs were identified as independent genes. For macroarray analysis, total RNA was extracted from the livers of male *S.* (*X.*) *tropicalis* exposed to E2, or a solvent, and female *S.* (*X.*) *tropicalis* exposed to a solvent. Biotin-labeled cDNA was prepared from the liver total RNA (Fig. 7). We then isolated seven cDNAs as candidate estrogen-responsive genes. Finally, two

Table 1
Blast analysis of cDNAs isolated from the liver cDNA library.

| Genes on GenBank database showing high similarity with the cDNAs | No. of cDNAs |
|---|---|
| Vitellogenins | 120 |
| 74kDa serum albumin | 23 |
| 68kDa serum albumin | 8 |
| Alpha globin | 9 |
| Beta globin | 15 |
| Transferrin | 7 |
| Ferritins | 6 |
| Ribosomal components | 45 |
| Mitochondrial components | 52 |
| etc. | 402 |
| No. of cDNAs showing low similarity or no similarity with the database genes | 122 |

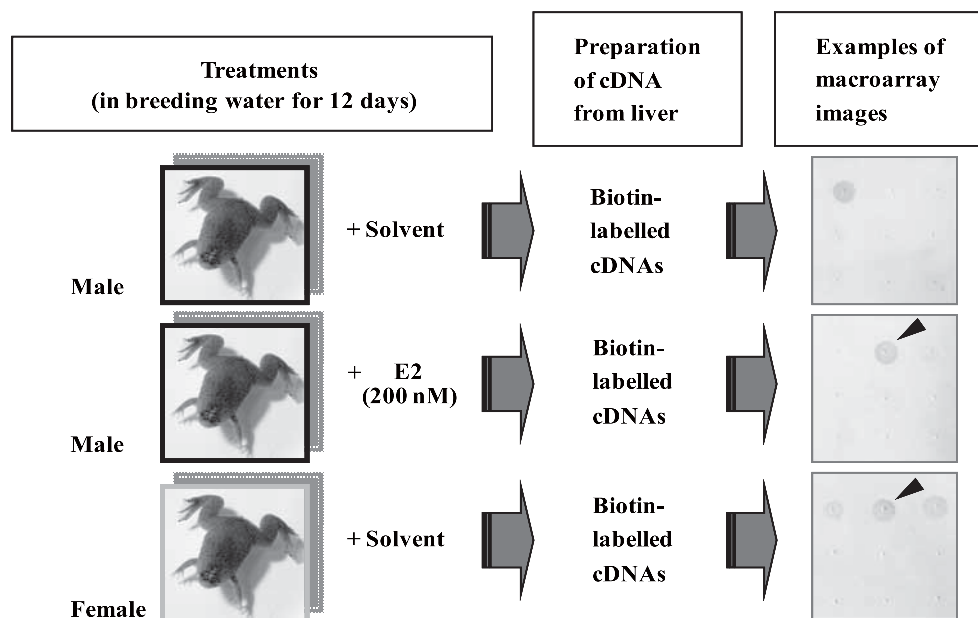



Fig. 7. Strategy for isolation of estrogen-responsive gene by macroarray method. Adult *S.* (*X.*) *tropicalis* was exposed to E2 or a solvent in breeding water for 12 days, after which total RNA was extracted from the liver. Complementary DNA was synthesized from the liver total RNA and labeled with biotin. Target cDNAs isolated from the library of cDNAs from a female *S.* (*X.*) *tropicalis* liver were blotted on a nylon membrane, and hybridized with the biotin-labeled cDNA. Finally, the hybridized cDNA was visualized by alkaline phosphatase reaction. Target cDNAs that hybridized with the biotin-labeled cDNA from both E2-treated male and solvent-treated female, but not solvent-treated male, were selected as candidate estrogen-responsive genes (arrow head in right panel).

cDNAs, representing *Vtg A* and *Vtg B* were identified as estrogen-responsive genes, which were verified by RT-PCR (Fig. 8). *Vtg A* and *Vtg B* expressions were induced in metamorphosing tadpoles exposed to 2 μM E2 (Fig. 9), suggesting that these two genes could be useful molecular markers of the estrogenic exposure of metamorphosing tadpoles and froglets after metamorphosis.

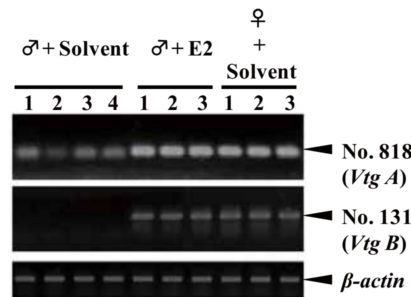



Fig. 8. RT-PCR analysis of *Vtg* expression in liver of adult frog. The estrogen-dependent expressions of no.131 (*Vtg B*) and no. 818 (*Vtg A*) genes, which were isolated as estrogen-responsive genes from the liver cDNA library using the macroarray method, were verified by RT-PCR analysis. Complementary DNA was synthesized from total RNA extracted from the liver of *S.* (*X.*) *tropicalis* exposed to 200 nM E2 or a solvent, and subjected to RT-PCR using specific primer pairs for no. 131 cDNA or no. 818 cDNA. The amplified cDNAs were electrophoresed on 1% agarose gel. The numbers on the electrophoresis image denote individual frogs.

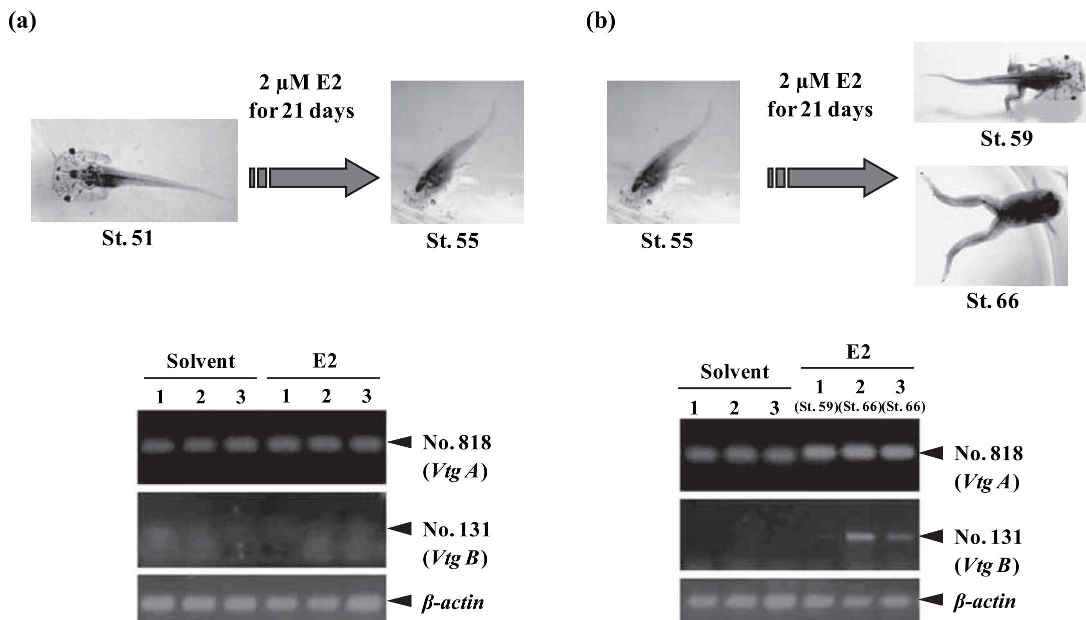



Fig. 9. RT-PCR analysis of *Vtg* expression in liver of tadpoles. *S.* (*X.*) *tropicalis* tadpoles at stage 51 (a) and stage 55 (b) were exposed to 2 μM E2 or a solvent in breeding water for 21 days. Complementary DNA was synthesized from total RNA extracted from the liver of the *S.* (*X.*) *tropicalis* tadpoles, and subjected to RT-PCR using specific primer pairs for no. 131 cDNA or no. 818 cDNA. The amplified cDNAs were electrophoresed on 1% agarose gel. The numbers on the electrophoresis image denote individual tadpoles.

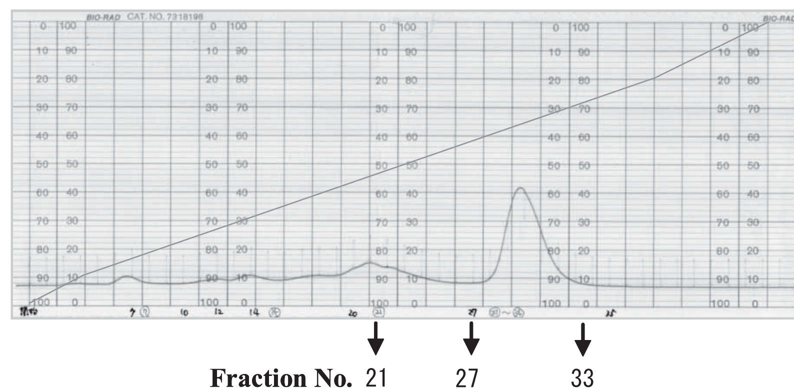
2.2.2 *Production of anti-*R. japonica *VTG antibody*

Serum of *R. japonica* exposed to E2 (50 µg/g body weight) by subcutaneous injection was fractionated by diethylaminoethyl (DEAE) column chromatography to purify VTG protein, which was verified by SDS-PAGE (Fig. 10).

## 3. Future Studies

To establish validated methods for evaluating the toxicity of environmental disrupting compounds and environmental samples in amphibians, we intend to construct a database describing the responses of *S.* (*X.*) *tropicalis* larvae exposed to endocrine disrupting compounds. This database will be complemented by the development of tools such as a reporter gene assay system and sex-reversed male *S.* (*X.*) *tropicalis* bioassay. Recently, Mitsui *et al.*[11] have established a metamorphosis assay system using *S.* (*X.*) *tropicalis* on the basis of the *Xenopus* Metamorphosis Assay (XEMA) selected by the OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA), as an *in vivo* assay.[23] As for the construction of the standard database, *S.* (*X.*) *tropicalis* tadpoles will be reared in breeding water with or without estrogenic chemicals, according to the method reported previously by Mitsui *et al.*,[11] and parameters such as total length, limb length, the histologies of the gonad and thyroid,

(a)




(b)

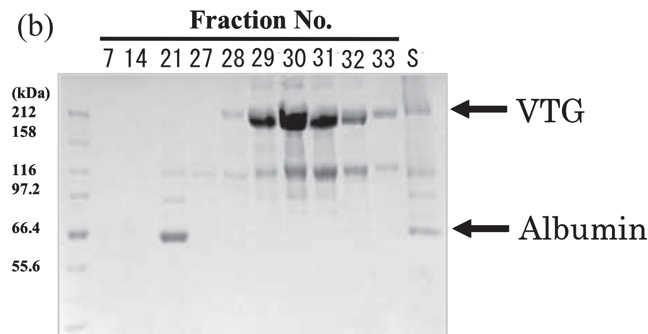



Fig. 10. Separation of *R. japonica* VTG by column chromatography. Serum of *R. japonica* given subcutaneously as a single injection of E2 was fractionated by anion-exchange chromatography. (a) A single large peak appeared on the chart of the elution pattern, corresponding to fraction nos. 27 through 33. (b) The protein in the fraction was separated and visualized by SDS-PAGE/CBB. Fraction nos. 28–33 contained 200 kDa protein, whose electrophoretic mobility was comparable to those of other known VTG molecules. In the lane S, the whole serum of *R. japonica* given subcutaneously as a single injection of E2 was electrophoresed.

and the expressions of the hormone-responsive genes *Vtg A* and *Vtg B* will be investigated throughout larval development. In addition, all-male tadpoles show promise as useful tools for the analysis of gonadal sex differentiation. Sex-reversed all-male *S.* (*X.*) *tropicalis* tadpoles can be produced by crossing sex-reversed adult males with normal adult males, on the assumption that the sex determination mechanism of *S.* (*X.*) *tropicalis* is of the ZZ/ZW type. We have succeeded in isolating full-length *ER* and *TR* cDNAs. To identify estrogenic and antiestrogenic chemicals or TH-like or anti-TH-like chemicals, we will develop in the near future reporter gene assay systems using full-length *ERα*, *ERβ*, *TRα* and *TRβ* cDNAs. Purified *R. japonica* VTG will be used in the near future to obtain polyclonal rabbit antisera against *R. japonica* VTG.

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