

Sensitivities of mRNA Expression of Vitellogenin, Choriogenin and Estrogen Receptor by Estrogenic Chemicals in Medaka, *Oryzias latipes*

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Gene expression levels of choriogenin, vitellogenin, and estrogen receptor were determined using a reverse transcription-polymerase chain reaction (RT-PCR) technique after exposure to estrogenic chemicals to compare the sensitivities of the biomarkers of endocrine disruption in medaka, *Oryzias latipes*. Mature male medaka were treated with a single dose of 100 μ l of 17 α -ethinylestradiol, nonylphenol, and bisphenol A for 6 days, then RNA was extracted from the livers of treated fish for RT-PCR. Primers of RT-PCR for choriogenin H and L, and estrogen receptor were synthesized based on previously known cDNA sequences, and primers for vitellogenin I and II were synthesized based on the partial cDNA which was sequenced in this study. When the five biomarker genes were amplified by RT-PCR under the same condition, the mRNA induction level of each gene was elevated with different sensitivities. Conclusively, choriogenin L, which is a precursor of zona radiata protein (ZI-3) with molecular weight of 49 kD, showed the most sensitive gene expression in all the treated groups.

Key words — medaka, vitellogenin, choriogenin, estrogen receptor

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INTRODUCTION

Endocrine disruptors are hormone-mimics that modify hormonal action in humans and animals.¹⁾ There is increasing concern about endocrine disruptors, especially environmental estrogens because of their ability to mimic natural hormones and to influence vital reproductive functions in humans and wildlife. Recently, Organization for Economic Cooperation and Development (OECD) has been working to revise the existing test guidelines and/or prepare new guidelines to screen and test endocrine disrupting chemicals. They agreed to define the endpoints of endocrine disruption in fish as gross morphology, gonad histology and biological markers such as vitellogenin in the 2nd OECD Expert Consultation Meeting on Endocrine Disruptors Testing in Fish, held in Tokyo, 15th–16th March, 2000.²⁾ Regarding the biomarkers of endocrine disruption in fish, recent research has focused on vitellogenin, choriogenin, estrogen receptor, and P450-aromatase,^{3–7)} but the screening and testing methods using the biomarkers have not yet been fully validated.

Recently, it was reported that choriogenin was more responsive to environmental estrogens than vitellogenin in Atlantic salmon, as determined by Enzyme-linked Immunosorbent Assay (ELISA).⁸⁾ However, no study attempted to compare responsiveness to estrogenic chemicals with the gene expression of possible biomarkers.

In the present study, mRNA induction levels of choriogenin H and L, vitellogenin I and II, and estrogen receptor in the medaka exposed to 17 α -ethinylestradiol, nonylphenol, and bisphenol A were compared using the reverse transcription-polymerase chain reaction (RT-PCR) method.

MATERIALS AND METHODS

Fish and Chemical Exposure — The orange-red variety of the adult medaka (*Oryzias latipes*) were maintained at the Environmental Toxicology Laboratory, National Institute of Environmental Research of Korea. They were raised under constant light/dark cycles of 18/6 hr and a temperature of 25 \pm 1°C. They were fed *ad libitum* three times, once with artemia and twice with commercial flake food. For the induction of mRNA expression of vitellogenin, choriogenin, and estrogen receptor, male medaka with a mean weight of 250 mg/fish

Table 1. Primer Sequences for RT-PCR of Choriogenin, Vitellogenin and Estrogen Receptor

Gene name	Primer sequences
Choriogenin L	F : 5'- GCCAAACCTGTAGTGCCATT -3'
	R : 5'- CTGCTCCACTGACCTCCTTC -3'
Choriogenin H	F : 5'- CGCCATCTACTACTTTCCCG -3'
	R : 5'- AATTTTGACCCATGATGAAA -3'
Vitellogenin I	F : 5'- CACTCATGGCTCTGAGGAA -3'
	R : 5'- GCAGAGTAAAGACTCAGTTC -3'
Vitellogenin II	F : 5'- GGGGCATTGATTGCAAGCAGT -3'
	R : 5'- CTCCTGAAGACCATGGTTAGG -3'
Estrogenin receptor	F : 5'- ACTCCCCTTACAGCCAGTCC -3'
	R : 5'- TGGACCAGCTCCTTGTCTGCC -3'
β -Actin	F : 5'- TTCAACAGCCCTGCCATGTACG -3'
	R : 5'- ATACCGCAGGACTCCATACCAA -3'

were exposed to 17 α -ethinylestradiol (Sigma Chemicals, St. Louis, MO, U.S.A.), bisphenol A (Sigma Chemicals) and nonylphenol (Sigma Chemicals), with single concentration of 100 μ l for 6 days, respectively. In case of juvenile study, 3 week-old juvenile medaka were exposed to 100 μ l 17 α -ethinylestradiol for 6 days. All the chemicals were dissolved in ethyl alcohol, and the control group was exposed to vehicle alone. A semi-static condition was applied for the exposure, and 50% of the test solution was changed every 24 hr.

RNA Extraction and RT-PCR — RNA was carefully extracted from the livers of treated males. Spawning female medaka were used as a control group. In the case of sexually immature juvenile fish, whole body homogenates were used for the RNA purification. For tissue disruption and preparation of total RNA, QIA-Shredder and RNeasy mini-kits (QIAGEN, Hilden, Germany) were used according to the manufacturer's instructions. Purified RNA samples were diluted at 1 μ g/ μ l for RT-PCR or stored at -80°C until further use. RT-PCR was performed using 1 μ g RNA, 1 μ l of avian myeloblastosis virus (AMV) reverse transcriptase (34 units/ μ l), 1 μ l of ribonuclease inhibitor (40 units/ μ l), 4 μ l of 10 mM deoxyribonucleoside-5'-triphosphate (dNTPs) and 1 μ l of oligo deoxythymidine (dT) primers in 20 μ l total volume at 42°C for 30 min and 99°C for 5 min, then 2 μ l aliquots from the reverse transcription reaction mixture were added to the 48 μ l PCR reaction mixture. Thirty cycles were then performed at 94°C for 30 sec, at 61°C for 30 sec, and at 72°C for 1 min. RT-PCR was carried out using a program temperature control system (Perkin Elmer 2400). The primers used for the RT-PCR of choriogenin (L: low

molecular weight and H: high molecular weight),^{9,10} estrogen receptor,¹¹ and β -actin,¹² were synthesized based on the previously known sequence. For the primers of vitellogenin I and II, partial sequencing of the cDNA was done in this study. The sets of primers for RT-PCR are shown in Table 1. The house-keeping gene β -actin of medaka was used as an internal standard. Aliquots of each amplified cDNA fragment from total 50 μ l PCR reaction volume were separated on 1.0% agarose gel.

Partial Sequence of Vitellogenin I and II — Reverse transcription was performed with an RNA template prepared from the livers of spawning females, AMV reverse transcriptase, RNase inhibitor, dNTPs and oligo dT primers in 20 μ l volume at 42°C for 30 min, and then PCR was performed in 50 μ l total mixture volume for 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. For the sequence analysis of vitellogenin (VTG) cDNA, amplification of cDNA specific for the VTG gene from the estrogen-treated medaka was performed using degenerated primers based on the published VTG sequences of other fish species; rainbow trout¹³ and mummichog.¹⁴ Amplified cDNA products were separated on 1.0% agarose gel by electrophoresis, and the cDNA fragments were cut out from the agarose gel and cloned into pGEM-T easy vector (Promega, Madison, WI, U.S.A.). Then the cDNA clones were applied to the automatic DNA sequencer (ABI3700, Applied Biosystems, Foster City, CA, U.S.A.). Comparison of the cloned cDNA and deduced amino acids with the vitellogenin of other fish species was carried out using the National Center for Biotechnology Information (NCBI) blast search program.

TCTGCCAATCAATCAGAGTCTATTGAAGCACTCATGGCTCTGAGGACAATTGCAAAAGAAACCAAGA
 ATGGTTCAGGAACTGGCTCTTCAAGCTCTACATGGACAAGGCTCTTGACCCAGAGCTCGTATGCTTTCAT
 GCATTGTGCTGTTGAGACCAATCCTCAATGGCGCTGATTTCCACCCCTTGCCAAATGCTGTTAAGTCAGA
 GGAGAATTTGCAGGTTGCCAGCTTCACATACTTCACATGAAGTCCCTGAGCAGGAGCGCTCCATGATT
 CATCCATCAGTAGCTGCAGCTTGAATGTTGCCATGAAGATCCTGAGTCCAAAGCTGGACAGACTGAGCT
 TACGTTACAGCAAAGCTGTCTATGGGGAAGCCTATAGCAGCTCTTAAATGCTTGGTGTGCTGTACCCG
 TTTCTACATCAACGATGCTGCTACCTTCCCTGCCAAGATCTGTTGTTGCCAAGACCAAGCCCTCTTTGTT
 GGAGCTGTGCTGATGTTCTGGAGATTGGATGAGAAGTGAAGTGAAGTGAAGTGAAGTGAAGTGAAGTGAAGT
 CTTCAATTTCTGACAGTGTGACAGGATAACAAAAATGAAACGTTGCATCAAGGCTCTTCTCAGTGGGA
 GATCCTTGCCCAACAACCCCTTAGTTCCCTGCTATGTCAAGCTTTTTGGACAAGGATTTGCCCTTTGCC
 AACATTGACAAACCCATGATTGAACGGGCTATTGAGCTTGGCAGTGGTCCCTCTGTTGAGGCTTATGGTT
 TAAAAGCTTTGAAAACCTGCTGCTCTTCTGGTGTCAATTTCCACCATGCAATGCTTGTGCTGTTTAGTGA
 GGTGCGACGCATATTGCCAATGCTGCTGGAATTCAGATGGAAGTCTTACTCTGCTGCTGTGGCT
 GTAGCCGCTGTAGAAATTAACCAACTCTGTCAACACGCT

(A)

AGATCATGACACGGGTCACATGGGGCATTGATTGCAAGCAGTACAACGCTTCAATCATCGCTGAAACTGG
 TCTGGTCGAAAAAGAACTGCAGCTCGATTGAAAAAGCCTGGGAAAGACTCCCTGAGGGCTGAAAAAGA
 TACGCCAAGTGGGCTTCTGAGTATCTTCCCGAATGCTGTTCCGAATGGAGTAAGTCAAGCAAAAGTCA
 AAAACTTCCAAAAACAGATCCAACTGACAGCTGTGTCATGAGACAACAACTAAATGTTACACTGAAGAC
 ACCTCAGAGTACTATACAAACTGGATTGGGTGTTCCAAATTTCTTACCCTTGGAGAAACTGCTGCT
 GAGTTGGAGGCTATCCTGAAAAGCTGGCCTGGCAAACTGTCTACATGCTCACCAAGCCATCGCTGCTC
 AGTGCTCTTGGTCAATGACAGCCTGATCAGTTCACAACAACAAAAATACAAAAATGAGATGCCCCATTC
 TTGCTACCAGGTCCTGGCTCAAGATTGCACATCAGAACTCAAATTCATGTTCTGCTGAAAGCAGACCAA
 ACTGCAGAACAGAGGATTAATGTCAAGATTGAAAAACATGGACATTGACATTTATTAAGGACAAATG
 CTACTGTGGTGAAGTCAACAGCCTGGAATCCTTCTGCCAACATGCCCTATCAGCATTCAACAGGCAA
 TATACAGATCAGAGAGAAGGCTGAGGGTGTCTCTGCATGCACTAACCATGGTCTTTCAGGAGGCTCTC
 TTCAACCATACTACAGTGAAGTTCAAGTTGCCAGCTGGATGAGAGGAAAGACTTGTGGACTATGTGGAA
 AGGCCAGCGTGAAGTCAAGGAAAGATGTCACACCCCAACGAGGAGTGTCTAAGAATGCAACCCACTT
 TGCTATTCTGGGTTCTGTCGGGACAAGAGTCCGCCATACCTCTGAGTGTCTACATGAAACTGAGTGT
 GTGAAGCTGGAGAAAGCAGGTCAACCCACTTAGGGGAAAGATTCTAAATGCTACTTCTGTGAGCCTGTGCT
 CGGTGCTTCCCGGCTGTATGCCTACAAGAAAAACCCTGTCAATGTGGATACCATTGTCTACCCATTG
 GTACAAATTAATCGTAAATGACGGCCTCAACAGCATCTTTGAGAAGAGCATTGATTTGAGGAGACCGC
 AGAGGCTCATCTTGCTGTGCTGCGCAGCTCAGTGTGCATAATTTATAACTTTGATCATGTTATACTC
 TGTTTTTAAACCAAGGTTGAAACTTAAATAAATAAAGGCATGTGAAAAA

(B)

Fig. 1. Partial cDNA Sequences of Medaka Vitellogenin I (A) and Vitellogenin II (B)

Underlined parts of the nucleotide sequences were used for the primers of RT-PCR for vitellogenin I and II, respectively.

RESULTS AND DISCUSSION

VTG, choriogenin (Chg), and estrogen receptor (ER) were compared in male medaka exposed to estrogenic chemicals 17 α -ethinylestradiol (EE2), bisphenol A, and nonylphenol. A comparison study was also done in sexually immature juvenile medaka. The synthetic oligo-primer sequences used for RT-PCR of medaka Chg and ER were designed according to previously reported cDNA sequences.⁹⁻¹¹ To design suitable oligo-primers for VTG RT-PCR, partial cDNA fragments were isolated from the estrogen-treated medaka, then two cDNA clones were acquired (Fig. 1). As a result of sequence comparison with VTG genes of other fish species, these two cDNA clones showed high homology with VTG of various fish species; therefore, they were confirmed as the parts of medaka VTG I and VTG II, respectively. 950 bp partial cDNA fragment of medaka VTG I showed a high degree of amino acid sequence similarity to mummichog VTG I (71%), rainbow trout VTG (61%), common carp VTG (53%), and zebrafish VTG (53%). A 1320 bp partial fragment

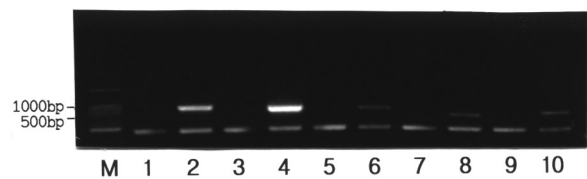


Fig. 2. Induction of mRNA of Choriogenin, Vitellogenin and Estrogen Receptor by 17 α -ethinylestradiol

Male medaka were treated with 100 μ g/l of 17 α -ethinylestradiol. RT-PCR was performed for 30 cycles. Upper bands are amplified cDNA fragments of biomarker genes, and lower bands are cDNA fragments of β -actin(440 bp) as an internal control. M: size markers, choriogenin H (1: control, 2: treated), choriogenin L (3: control, 4: treated), VTG I (5: control, 6: treated), VTG II (7: control, 8: treated), estrogen receptor (9: control, 10: treated).

of medaka VTG II had an amino acid sequence similar to mummichog VTG II (63%) and rainbow trout VTG (50%). Based on these cDNA sequence data of VTG, suitable oligo-primers of VTG I and VTG II RT-PCR were synthesized, respectively. All the primers of the biomarker genes used in this study are shown in Table 1.

Treatment of mature males with EE2 resulted in pronounced elevation of Chg and VTG mRNA levels. However, the ER mRNA level was relatively weak in comparison with Chg and VTG. The estimated size of the amplified RT-PCR products of the biomarker genes are as follows: 900 bp of choriogenin H, 860 bp of choriogenin L, 870 bp of vitellogenin I, 740 bp of vitellogenin II and 850 bp of estrogen receptor, respectively. Amplified cDNA fragments of these genes were not detected in the non-treated group, and β -actin cDNA fragments used as an internal standard were approximately equally expressed in the treated and control groups (Fig. 2). When we compared the sensitivity of VTG, Chg, and ER genes at the same concentration of EE2 by performing RT-PCR for 30 cycles, respectively, Chg L was shown to be the most responsive to EE2 in all the biomarkers tested in adult male medaka, and also in juvenile medaka (Fig. 3A and 3B). Choriogenin H and L were shown to be more sensitive than those of vitellogenin, and this result supported previous reports.¹⁵ This pattern of responsiveness was also shown in the nonylphenol- or bisphenol A- treated male medaka, although the estrogen receptor did not seem to be induced in the concentration of 100 μ g/l of the chemicals (Fig. 4A and 4B). mRNA of the estrogen receptor could be induced in a higher concentration of nonylphenol or bisphenol A than that used in this test. The sensitivity between vitellogenin I and II could not be assured because this study did

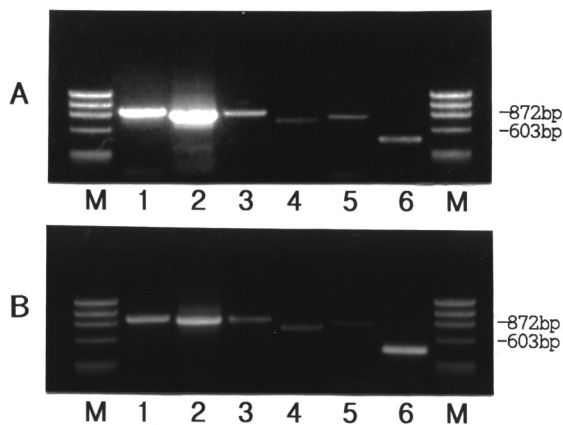


Fig. 3. RT-PCR of Choriogenin, Vitellogenin and Estrogen Receptor in Adult Male Medaka (A) and Juvenile Medaka (B) Exposed to 100 $\mu\text{g/l}$ of 17 α -ethinylestradiol. RT-PCR was performed for 30 cycles (M: size marker $\phi\times 173$ -HaeIII, 1: choriogenin H, 2: choriogenin L, 3: vitellogenin I, 4: vitellogenin II, 5: estrogen receptor, 6: β -actin).

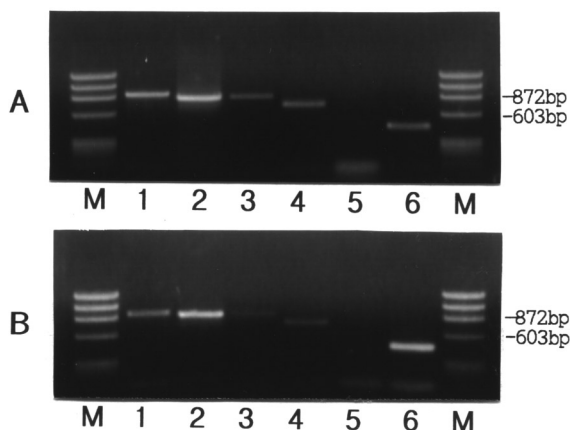


Fig. 4. RT-PCR of Choriogenin, Vitellogenin and Estrogen Receptor in Mature Male Medaka Exposed to 100 $\mu\text{g/l}$ of Nonylphenol (A) and Bisphenol A (B), Respectively. RT-PCR was performed for 30 cycles (M: size marker $\phi\times 173$ -HaeIII, lane 1: choriogenin H, 2: choriogenin L, 3: vitellogenin I, 4: vitellogenin II, 5: estrogen receptor, 6: β -actin).

not cover various concentrations. The sensitivity patterns of the five biomarkers tested in this study were almost the same in sexually immature juvenile medaka as in sexually mature male fish.

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