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Development and Validation of Endogenous Reference Genes for Expression Profiling of Medaka (*Oryzias latipes*) Exposed to Endocrine Disrupting Chemicals by Quantitative Real-Time RT-PCR

Zhaobin Zhang and Jianying Hu[1]

College of Environmental Science, Peking University, Beijing 100871, China

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The quantitative real-time reverse transcription polymerase chain reaction (Q-RT-PCR) technique has been increasingly used in endocrine disrupting chemicals (EDCs) research. Usually, an appropriate endogenous control gene is critical for Q-RT-PCR to normalize the errors and sample-to-sample variations that occur in the course of tissue collection, RNA isolation, and RT-PCR. In this study, we cloned ribosomal protein L7 (RPL-7) from medaka (*Oryzias latipes*), and then used Q-RT-PCR to study its transcription characteristics and those of glyceraldehyde-3-phosphate dehydrogenase, β-actin, mitochondrial 16S ribosomal RNA (16S rRNA), and 18S rRNA. Of the five genes, RPL-7 and 18S rRNA were expressed with the less variance among the same tissue samples, in different tissues, and stages of development and were unaffected by EDCs exposure. The expression levels of RPL-7 among different tissues were between $9.76 \times 10^6 \pm 9.49 \times 10^5$ and $1.39 \times 10^7 \pm 1.69 \times 10^6$ copies/μg RNA but those of 18S rRNA were as high as $4.48 \times 10^{11} \pm 5.95 \times 10^{10}$ to $5.90 \times 10^{11} \pm 1.21 \times 10^{10}$ copies/μg RNA, which is above the usual detection scope of Q-RT-PCR if no complementary DNA reaction dilution is performed. As a result, RPL-7 is the single suitable endogenous control gene for expression profiling in future studies, especially in studies on the EDCs issue using medaka.

Key Words: **quantitative real-time RT-PCR; endocrine disruption; endogenous control; *Oryzias latipes*.**

Endocrine disruption, caused by a variety of compounds with the potential to disturb the endocrine systems in wildlife and humans, has been of great concern to public and toxicologists (Daston *et al.*, 2003; Sumpter and Johnson, 2005). It has been found that these endocrine disrupting chemicals (EDCs) can mimic, block, or interfere with hormones in the body and subsequently affect development and reproduction (Aravindakshan *et al.*, 2004; Guillette *et al.*, 1995). In the toxicological processes, alterations in gene expression are responsible for the conventional physiological and histological responses (Fielden and Zacharewski, 2001; Moggs, 2005). And some genes, such as vitellogenin (VTG), have been widely used as a biomarker to determine EDCs (Daston *et al.*, 2003; Rankouhi *et al.*, 2004; Xie *et al.*, 2005; Zhang *et al.*, 2005b).

With the development of reagents and hardware, quantitative real-time reverse transcription polymerase chain reaction (Q-RT-PCR) has become a more accurate, specific, sensitive, and high-throughput technique for the quantification of gene expression than traditional methods such as Northern blot analysis, RNase protection assay, and traditional RT-PCR (Bustin, 2000; Zhang *et al.*, 2005a). It is easy to perform and can be used for the detection of low-abundance messenger RNA (mRNA), often obtained from limited tissue samples. Such performances are perfectly suited to studying the low expression of genes involved in the toxicological processes and to measuring the gene expression in endocrine-gland cells dispersed among other tissues (Hilscherova *et al.*, 2004; Kazeto *et al.*, 2004; Lyssimachou *et al.*, 2006). Research using Q-RT-PCR will certainly promote the understanding of the toxicological processes of EDCs, prediction of endocrine disruption properties of new chemicals, and assessment of bioactivity of EDCs in the environment (Lyssimachou *et al.*, 2006; Moggs, 2005; Zhang *et al.*, 2005a). In the course of gene expression measurement, however, the inconsistencies in the procedures of tissue collection, RNA isolation, and complementary DNA (cDNA) synthesis may lessen the reliability of Q-RT-PCR (Thellin *et al.*, 1999). To correct the sample-to-sample variation when determining the gene expression, an accepted method is to select a cellular housekeeping gene that serves as an endogenous control, against which the target gene expression levels can be normalized (Bustin, 2000; de Kok *et al.*, 2005). According to previous papers (Bustin, 2000; de Kok *et al.*, 2005; Radonic *et al.*, 2004) and our understanding, an acceptable endogenous control for EDCs study should satisfy several conditions. Firstly, it should be expressed with the less-variance among same tissue samples, and constantly expressed among different tissues, at various stages of development. Secondly, the endogenous control should be unaffected by EDCs treatments under the doses of which is sufficient to elicit overt toxicity. Lastly, the endogenous control should be expressed at roughly the same level as the RNA under study.

[1] To whom correspondence should be addressed. Fax: +86 10-62765520. E-mail: hujy@urban.pku.edu.cn.

To the best of our knowledge, however, there have not been any housekeeping genes which have been strictly validated as endogenous control for EDCs studies.

As traditional endogenous controls, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and 18S ribosomal RNA (18S rRNA) are often used for gene transcription normalization (Bustin, 2000; Radonic *et al.*, 2004). However, it has been reported that these genes were unstably expressed by EDCs exposure (Farnell and Ing, 2003; Hsu and Frankel, 1987; Kamata *et al.*, 2005; Larkin *et al.*, 2003). Ribosomal protein L7 (RPL-7) is a housekeeping gene that has been recently used to normalize gene expression in mammals (Huot *et al.*, 2001; Muroya *et al.*, 2005; Sun *et al.*, 2004), and has been adopted as an internal control in studying mature pig sertoli cells and mouse uterus treated with EDCs (Das *et al.*, 1998; Kanematsu *et al.*, 2005). However, RPL-7 has never been used as endogenous reference in lower vertebrate, such as in fish which are widely used in EDCs study.

In this study, the complete mRNA sequence of RPL-7 of medaka (*Oryzias latipes*), one of the most popular model fish species in EDCs (El-Alfy and Schlenk, 2002; Foran *et al.*, 2002; Shima and Mitani, 2004), was cloned and sequenced, and the basic genomic information regarding the RPL-7 was analyzed. We then investigated whether RPL-7, as well as the traditional endogenous controls, could be used as an endogenous reference gene for Q-RT-PCR, with particular emphasis on studying its suitability for EDCs issue using medaka.

MATERIALS AND METHODS

Chemicals. The 17α-ethynylestradiol (EE_2) was purchased from Fluka Chemie AG (Buchs, Switzerland); 17β-estradiol (E_2), and dimethyl sulfoxide (DMSO) from Sigma (St Louis, MO); bisphenol A (BPA) and 4-nonylphenol (4-NP) from Tokyo Chemical Industry (Tokyo, Japan); *o,p′*-dichlorodiphenyldichloroethylene (DDE) and *p,p′*-DDE from Chem Service (West Chester, PA); tributyltin chloride (TBT-Cl) from Hayashi Pure Chemical Industries (Tokyo, Japan); and testosterone (T) from Wako Pure Chemical Industries (Osaka, Japan).

Animals. Orange red strain medaka (*O. latipes*) were maintained in active carbon-treated tap water with hardness 81.1 ± 1.2 mg/l calcium carbonate, pH 7.9 ± 0.1, dissolved oxygen 7.8 ± 0.3 mg/l, and unionized ammonia 0.012–0.002 mg/l, at 25 ± 1°C, on a 16:8-h light:dark cycle. Fish were fed live brine shrimp (*Artemia salina*) twice a day.

Collection of different tissues. Gill, muscle, ovary, testis, liver, brain, spleen, eye, and intestine tissue from 10-week-old adult fish ($n = 6$–9) were collected and frozen in liquid nitrogen until RNA isolation.

Egg and fry sampling. Fertilized eggs were harvested at 1, 4, and 8 days postfertilization (dpf), and fries were sampled at 2, 6, 12, and 20 days posthatch (dph). All samples were immediately frozen in liquid nitrogen until RNA isolation. More than 10 eggs or fries were used in each stage collectively except that 12 and 20 dph were isolated from six fries separately.

Animal treatment. Adult male medaka (10 weeks old) was used in the experiment. Each of 10 fish was separately exposed to 200 ng/l EE2, 200 ng/l E2, 100 μg/l BPA, 100 μg/l 4-NP, 100 μg/l *o,p′*-DDE, 100 μg/l *p,p′*-DDE, 1 μg/l TBT-Cl, and 1 μg/l T, with a final volume of 2 l in glass tanks, with static renewal of the water every 24 h, respectively. Chemical-stock solutions were prepared in DMSO and the ratio of chemical-stock solution/water was 0.005% (vol/vol). A 0.005% (vol/vol) DMSO group was used as control in the experiment. After 3 weeks of exposure, the fish were sampled, and then the livers and testes were isolated and frozen in liquid nitrogen until RNA isolation.

RNA preparation. Total RNA from frozen samples was isolated by 1 ml of Trizol reagent (Gibco BRL, Life Technologies, Gaithersburg, MA), according to the manufacturer's instructions. To remove genomic DNA contamination, total RNA was digested by RNase-free DNase I (TaKaRa Biotechnology, Dalian, China) and then purified. The total RNA recovered from DNase I digestion was measured at 260 and 280 nm using a spectrophotometer. The 260 nm reading was used to estimate the concentration of total RNA. The 260/280-nm ratios, as well as a 1% agarose-formaldehyde gel stained with ethidium bromide, were used to verify the RNA quality in each sample. The RNA concentrations were adjusted to 100 ng/μl for first-strand cDNA synthesis.

First-strand cDNA synthesis. Synthesis of first-strand cDNA was performed using TaqMan Reverse Transcription Reagents from Applied Biosystems (Foster City, CA), soon after the RNA had been prepared. Total RNA of 100 ng was combined with 4 μl of deoxy-nucleotide triphosphate mixture (containing 2.5mM each of deoxy-adenosine triphosphate, deoxy-guanosine triphosphate, deoxy-cytidine triphosphate, and deoxy-thymidine triphosphate) and 1 μl of 50μM random hexamers. Mixes were denatured at 70°C for 10 min and then quickly cooled on ice for more than 1 min. Reverse transcription was carried out in 1× TaqMan RT buffer, 5.5mM magnesium chloride, 0.4 U/μl of RNase inhibitor, 3.5 U/μl of MultiScribe Reverse Transcriptase, and RNase-free water to a final volume of 20 μl. The reaction was incubated at 37°C for 1 h, and then the reverse transcriptase was inactivated (95°C, 5 min). All samples were reverse transcribed in duplicate for further study by real-time PCR. A control without reverse transcriptase was performed in each RNA sample to confirm that no DNA contamination had occurred.

RPL-7 cDNA clone and sequence analysis. To amplify a partial RPL-7 gene of medaka, degenerate primers (Table 1) were designed from alignment of available RPL-7 mRNA sequences from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Total RNA from liver was used for RT-PCR, and the purified RT-PCR product was cloned into pMD 18-T Vector and sequenced by a M13 primer. A 355-bp conserved region was obtained. To obtain the complete 3′ end, Oligo dT-3sites Adaptor Primer was used for synthesizing first-strand cDNA by SuperScript III (Invitrogen, Carlsbad, CA). The first-strand cDNA was then amplified by PCR with the primers for 3′ RACE (rapid amplification of cDNA ends) (Table 1). The anticipated amplicon was purified from PCR reaction and sequenced with corresponding PCR primers. To obtain the complete 5′ ends, a phosphorylated primer (reverse) was used for synthesizing first-strand cDNA. The hybrid RNA was digested from cDNA by RNase H (TaKaRa Biotechnology, Dalian, China), and then circular single-strand cDNA was generated by self-ligating with T4 RNA Ligase (TaKaRa Biotechnology, Dalian, China). Primers for 5′ RACE (Table 1) was used for amplifying the 5′-end of RPL-7, and the purified amplicon was cloned into pMD 18-T Vector and sequenced. Genomic DNA sequence and chromosomal location were determined by using the BLAST algorithm at the Medaka Genome Project Working Draft (http://pre.ensembl.org/Oryzias_latipes). Gene organization (exon-intron boundaries) was examined by comparison with cDNA, and confirmed that the nucleotide sequences of all introns adjoining the splicing junctions were consistent with the recognized consensus sequence "gt-ag." The gene organization was then compared with those of green-spotted pufferfish (CDMT00066692001/FD0ABB1CD06.contig), zebrafish (NC_007113), chicken (NC_006089), house mouse (NC_000067), Norway rat (XM_216318), and human (NM_000971). Microsyntenic relationship was established by comparing the flanking genes to zebrafish RPL-7 gene and medaka homolog by BLAST search (http://pre.ensembl.org/Oryzias_latipes/blastview). The deduced amino acid sequence of medaka RPL-7 was aligned with other sequences (Table 2) obtained from GenBank, using the ClustalX program (Thompson *et al.*, 1997), and adjusted manually using BioEdit 7.0.1 (Hall, 1999). Based on the alignment, the protein sequence similarities were calculated by using the identity matrix in BioEdit.

TABLE 1
Primers Used for Gene Clone, 3′ RACE and 5′ RACE

Primers		Sequences
Degenerate primers	Forward primer	5′-AT[T/C][A/C]G[A/T/G]AT[A/T/C][A/C]G[A/T/C/G]GGTAT[A/T/C]AA-3′
	Reverse primer	5′-TT[G/A]AA[A/T/G/C]GGCCA[A/T/G/C]A[G/A] [G/A]AA[A/T/G]TT-3′
	Adaptor primer	5′-CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTTTTT-3′
Primers for 3′ RACE	Forward primer	5′-GAACTTCAAGCCTGCCAACA-3′
	Reverse primer	5′-CTGATCTAGAGGTACCGGATCC-3′
	Phosphorylated primer	5′-(P) CGTAGCCACGTTTGTA-3′
Primers for 5′ RACE	Forward primer	5′-CAAGGCTTCAATCAACATGCTG-3′
	Reverse primer	5′-TGAAGATCTGGCGCAGACG-3′

Synthesis of RNA standards. For quantification of gene expression, RNA standards representing the gene amplicon sequences of GAPDH, RPL-7, β-actin, 16S rRNA, and 18S rRNA were synthesized by *in vitro* transcription. RT-PCR amplicons were first cloned into the pGEM-T vector (Promega, Madison, WI), and the sequence authenticity of the cloned amplicons was verified by automatic DNA sequencing and followed by an alignment with their corresponding sequences in Genbank. Linear DNA templates for *in vitro* transcription were obtained according to the method reported in previous paper (Zhang *et al.*, 2005a). *In vitro* transcription was performed using 400 ng of purified DNA template with T7 RNA polymerase or SP6 RNA polymerase (TaKaRa Biotechnology) to produce sense RNA. Residual DNA was removed by incubation with RNase-free DNase I, and RNA were extracted with Trizol reagent. RNA quality was assessed by formaldehyde gel-electrophoresis, and RNA concentration was accurately determined using the Quant-iT RNA Assay Kit (Invitrogen) and converted to copy number per microliter according to the formula described by Zhang *et al.* (2005a). The quantified RNA was serially diluted to prepare the RNA standards (1×10^3 to 1×10^8 copies/μl).

Real-time PCR assay. Real-time PCR with SYBR green detection was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to protocols established by the manufacturer. Primers for β-actin and VTGi were according to Inui *et al.* (2003). Primers for GAPDH, RPL-7, 16S rRNA, steroidogenic acute regulatory protein (StAR), and 18S rRNA (Table 3) were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA) and validated by Premier Primer 5.0 (Premier Biosoft International, Palo Alto, CA). The PCR reaction mixture contained 12.5 μl of 2× SYBR Green PCR master mix (Applied Biosystems), 250nM each of forward and reverse primers, 3 μl of cDNA template and nuclease-free water in a total volume of 25 μl. The reactions were incubated at 50°C for 2 min to activate the uracil N9-glycosylase (in SYBR Green PCR master mix) and then for 10 min at 95°C to inactivate the uracil N9-glycosylase

TABLE 2
Full-length RPL-7 Genes Used in Alignment and their Amino Acid Identity (%) with that of Medaka

Source	Organism	Accession number	Amino acid identity (%)
Vertebrates			
Human	*Homo sapiens*	BC071895	79.8
Orangutan	*Pongo pygmaeus*	CR859318	80.1
Macaque	*Macaca fascicularis*	AB169738	80.1
House mouse	*Mus musculus*	BC096452	72.5
Norway rat	*Rattus norvegicus*	XM_216318	75.3
Cattle	*Bos taurus*	BT021516	80.6
Dog	*Canis familiaris*	XM_535102	79.4
Chicken	*Gallus gallus*	AJ720578	82.5
Clawed frog	*Xenopus laevis*	BC106361	82.5
Medaka	*Oryzias latipes*	DQ118296	—
Pufferfish	*Tetraodon nigroviridis*	CAAE01014544	93.8
Zebrafish	*Danio rerio*	BC107515	84.5
Invertebrates			
Domestic silkworm	*Bombyx mori*	AY769274	60.8
Fall armyworm	*Spodoptera frugiperda*	AY072288	62.2
Fruit fly	*Drosophila melanogaster*	NM_078807	60.0
Greenbugs aphid	*Lysiphlebus testaceipes*	AY961554	60.7
Honey bee	*Apis mellifera*	XM_393614	61.9
Beetle	*Biphyllus lunatus*	AM049000	63.3
Black-legged tick	*Ixodes scapularis*	DQ066291	66.7
Marine sponge	*Suberites domuncula*	AY857418	63.8
Social amoeba	*Dictyostelium discoideum*	XM_63805	58.2
Plant			
Rice	*Oryza sativa*	XP_480842	62.5
Bread wheat	*Triticum aestivum*	AY846828	58.0
Thale cress	*Arabidopsis thaliana*	AP002038	61.0
Potato	*Solanum tuberosum*	DQ228335	61.0
Fungi			
M. grisea	*Magnaporthe grisea*	XM_359540	57.7
C. elegans	*Caenorhabditis elegans*	NM_058275	57.2
D. hansenii	*Debaryomyces hansenii*	CAG86698	55.0
C. globosum	*Chaetomium globosum*	DQ084481	60.4
N. crassa N150	*Neurospora crassa* N150	XM_957857	59.6

TABLE 3
Primers Used for Q-RT-PCR

Genes	Accession no.	Sequences (first row, forward primers; second row, reverse primers)	Amplicon size (bp)
GAPDH	AV671008	5′-TGTGGAAAAGGCCTCACTTCA-3′	56
		5′-CAGACACGACCACACGCTGT-3′	
RPL-7	DQ118296	5′-CGCCAGATCTTCAACGGTGTAT-3′	72
		5′-AGGCTCAGCAATCCTCAGCAT-3′	
β-actin	S74868	5′-TCCACCTTCCAGCAGATGTG-3′	76
		5′-AGCATTTGCGGTGGACGAT-3′	
16S rRNA	AP008946	5′-ACGACCTCGATGTTGGATCAG-3′	51
		5′-CCTTAATAGCGGCTGCACCA-3′	
18S rRNA	AB105163	5′-CGTTCAGCCACACGAGATTG-3′	56
		5′-CCGGACATCTAAGGGCATCA-3′	
VTGi	AB064320	5′-CTCCAGCTTTGAGGCCATTTAC-3′	81
		5′-ACAGCACGGACAGTGACAACA-3′	
StAR	DQ988930	5′-GAAGCAAGGCGAAGATGCAC-3′	71
		5′-TTTCAACAGTCCAGCCGTCC-3′	

and activate the Amplitaq Gold DNA polymerase (in SYBR Green PCR master mix), followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Following the final cycle of the PCR, the reactions were denatured over a 35°C temperature gradient at 0.03°C/s from 60°C to 95°C to determine the quality of PCR products. The quantification of GAPDH, RPL-7, β-actin, 16S rRNA, and 18S rRNA expression was based on their absolute standard curves, respectively. Relative quantifications of VTGi and StAR expression in liver of adult medaka (Oryzias latipes, orange red strain) exposed to EDCs were analyzed using internal controls (GAPDH, RPL-7, β-actin, and 18S rRNA) by the comparative cycle threshold (Ct) method (Zhang *et al.*, 2005a).

Statistical analysis. The statistical program SPSS (Ver 11.5; Chicago, IL) was used to collate and analyze all the collected data. The relative standard deviation (RSD), the ratio of SD to the mean, was used to describe the divergences of transcription levels among tissues, stages, and groups exposed to EDCs. Differences were evaluated by ANOVA followed by Tukey's test. Differences with $p < 0.05$ were considered to be significant.

RESULTS

Clone and Sequence Analysis of Medaka RPL-7

The complete mRNA sequence of medaka RPL-7 was 822 bp in length including 19-bp 5′ untranslated region (UTR), 66-bp 3′ UTR (including poly A tail), and an open reading frame of 738 bp from nucleotides 20 to 757 (including the stop codon) that code for a polypeptide of 245 amino acids (Fig. 1). This gene was found to be located on chromosome 20 and on region HdrR_200510_scaffold149_contig60563 with seven exons and six introns. The exon-intron boundaries of the RPL-7 gene have conserved the classical ''gt-ag'' intron splice motifs (Fig. 2). Comparing the gene organizations of RPL-7 genes from green-spotted pufferfish, zebrafish, chicken, house mouse, Norway

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                10         20         30         40         50         60         70         80         90
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTCTTTCTCGGTGGTCGCAATGGCGGACGCAGAAAAAAAAGTTCCGGCGGTCCCTGAGAGCCTTTTGAAAAGGCGAAAGGCCTATGCAGC
                      M  A  D  A  E  K  K  V  P  A  V  P  E  S  L  L  K  R  R  K  A  Y  A  A                 24

               100        110        120        130        140        150        160        170        180
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AATGAAGGCCATGCGTGTCAAGAAGTTGCTGGCTGAGAAAAAGGCCCGTAAGGTGACCAGGAAACTCATATACAAGAGGGCTGAGAAGTA
  M  K  A  M  R  V  K  K  L  L  A  E  K  K  A  R  K  V  T  R  K  L  I  Y  K  R  A  E  K  Y                 54

               190        200        210        220        230        240        250        260        270
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCACAAGGAGTACAGGCAGATGGAGAGGCGTGAGATCCGCCTGGCTCGTATGGCTCGCAAAGTCGGGAACTACTATGTGCCAGCTGAACC
  H  K  E  Y  R  Q  M  E  R  R  E  I  R  L  A  R  M  A  R  K  V  G  N  Y  Y  V  P  A  E  P                 84

               280        290        300        310        320        330        340        350        360
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAAACTGGCCTTTGTCATCAGGATCCGAGGTATCAACGGAGTCAGCCCCAAGGTCCGCAAAGTTCTGCAGCTTCTCCGTCTGCGCCAGAT
  K  L  A  F  V  I  R  I  R  G  I  N  G  V  S  P  K  V  R  K  V  L  Q  L  L  R  L  R  Q  I                114

               370        380        390        400        410        420        430        440        450
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTTCAACGGTGTATTTGTCAAGCTGAACAAGGCTTCAATCAACATGCTGAGGATTGCTGAGCCTTACATTGCTTGGGGATACCCCAACTT
  F  N  G  V  F  V  K  L  N  K  A  S  I  N  M  L  R  I  A  E  P  Y  I  A  W  G  Y  P  N  L                144

               460        470        480        490        500        510        520        530        540
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAAGTCTGTGCGAGAGCTCATCTACAAACGTGGCTACGGCAGGATGAGGAAGCAGCGCATCGCCCTCACAGACAATGCTTTGGTGGAGAA
  K  S  V  R  E  L  I  Y  K  R  G  Y  G  R  M  R  K  Q  R  I  A  L  T  D  N  A  L  V  E  K                174

               550        560        570        580        590        600        610        620        630
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGCTCTTGGCAAATATGGCATCATCTGTGTTGAGGACCTCATCCATGAAATCTACACTGTTGGAAAGAACTTCAAGCCTGCCAACAACTT
  A  L  G  K  Y  G  I  I  C  V  E  D  L  I  H  E  I  Y  T  V  G  K  N  F  K  P  A  N  N  F                204

               640        650        660        670        680        690        700        710        720
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCTGTGGCCCTTCAAACTGTCGTCACCTCGTGGAGGAATGAACAAGAAGACCACACACTTTGTGGAGGGAGGCGACGCCGGCAACAGGGA
  L  W  P  F  K  L  S  S  P  R  G  G  M  N  K  K  T  T  H  F  V  E  G  G  D  A  G  N  R  E                234

               730        740        750        760        770        780        790        800        810
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGACCAGATCAACAGAATGATCCGAAGGATGAACTAAACTTAAGGTTTGCAACGGACTTAAAATGTACAATAAAAAAACCTTTGGAAGAA
  D  Q  I  N  R  M  I  R  R  M  N  *                                                                      245

               820
....|....|..
AAAAAAAAAAA
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FIG. 1. Nucleotide and deduced amino acid sequence of medaka (orange red strain, *Oryzias latipes*; GenBank accession No. DQ118296). The numbers above the sequence indicate the nucleotide positions and the values on the right and left correspond to the amino acid residues.

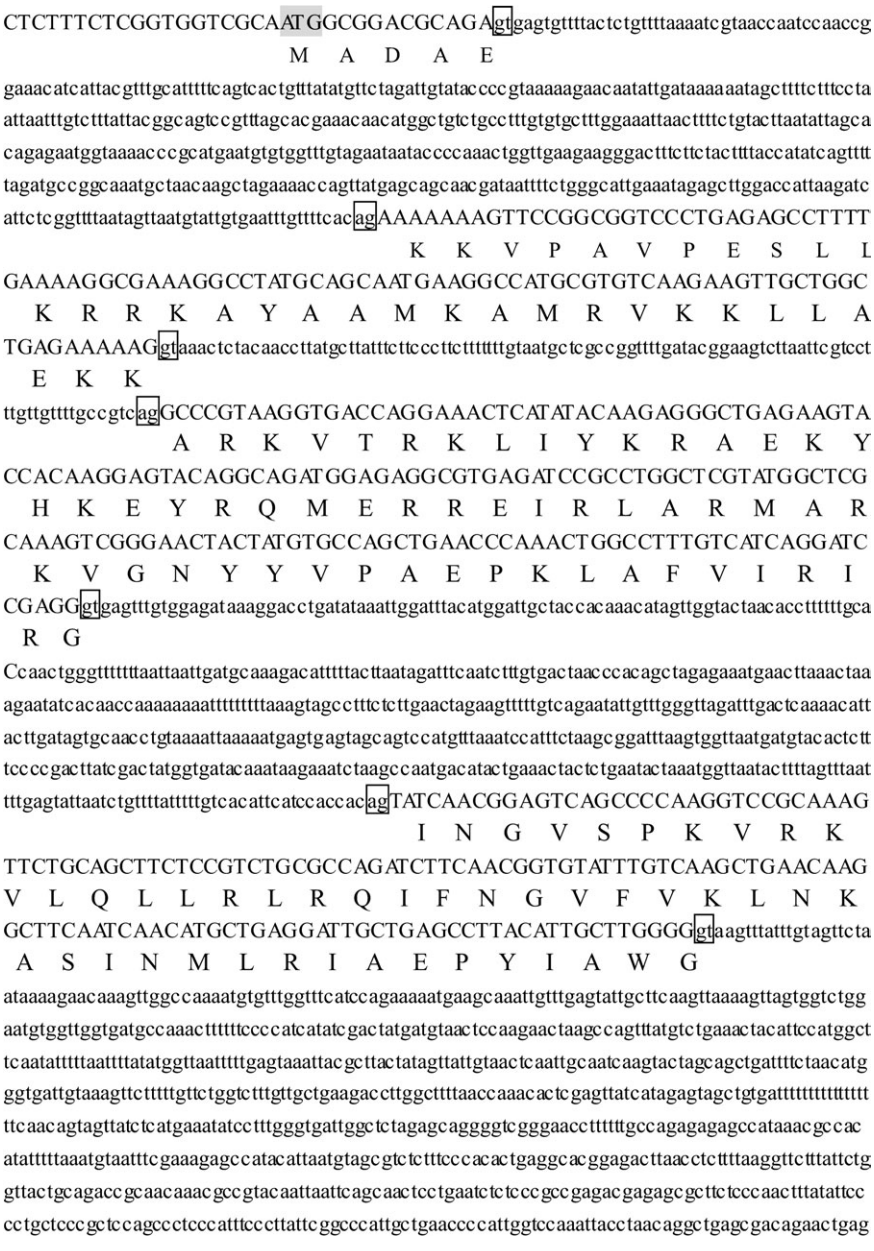


FIG. 2. The medaka RPL-7 gene sequence and deduced amino acid sequence. The intron sequences are in lower case and the intron splice sites are boxed. Transcription start site and the stop codon are indicated with gray background. The translation of the exon-coding regions is given by one-letter translation.

rat, and human, it was found that the seven exons and six introns are conserved from fish RPL-7 to human RPL-7, except for that of Norway rat with only six exons and five introns (Fig. 3). The genetic map positions of medaka RPL-7 and flanking genes, from the comparison with zebrafish RPL-7 region, are show in Figure 4. It was found that the genomic region harboring medaka LOC555921, RPL-7, rdh10, and im:6905459 is syntenic with that of zebrafish. By multiple-sequence alignment (Fig. 5), it was found that RPL-7 was highly conserved across eukaryotes and the C-terminal nucleic acid–binding domain (NBD), which is reported to bind to 28S rRNA and to be involved in the attachment of protein L7 to the large ribosomal subunit (von Mikecz *et al.*, 1999), was conserved during evolution. The protein sequence of medaka showed identities of 72.5–93.8% with those of other vertebrates, 58.2–66.7% with those of invertebrates, 58.0–62.5% with those of plants, and 55.0–60.4% with those of fungi (Table 2).

Gene Expression in Various Tissues

RNA levels of candidate genes (GAPDH, β-actin, 16S rRNA, 18S rRNA, and RPL-7) in brain, eye, gill, muscle, ovary, testis,

FIG. 2. (Continued)

liver, intestine, and spleen of adult medaka were quantified by Q-RT-PCR (Fig. 6). It was found that GAPDH was expressed at the lowest level among the five housekeeping genes with a large variation among different tissues (RSD = 54.6%). The highest expression level of GAPDH was $7.14 \times 10^6 \pm 2.89 \times 10^6$ copies/μg RNA in intestine, which was significantly higher than those in eye, gill, liver, and muscle ($p < 0.05$). The lowest level of GAPDH was $1.31 \times 10^6 \pm 6.69 \times 10^5$ copies/μg RNA in muscle, which was significantly lower than those in ovary, testis, and intestine ($p < 0.05$). Levels of β-actin mRNA were about 6.61- to 47.58-folds higher than those of GAPDH. It was expressed constantly in each of the tissues but varied widely among different tissues, with an RSD of 38.5%. The highest level of β-actin was $8.57 \times 10^7 \pm 2.11 \times 10^7$ copies/μg RNA

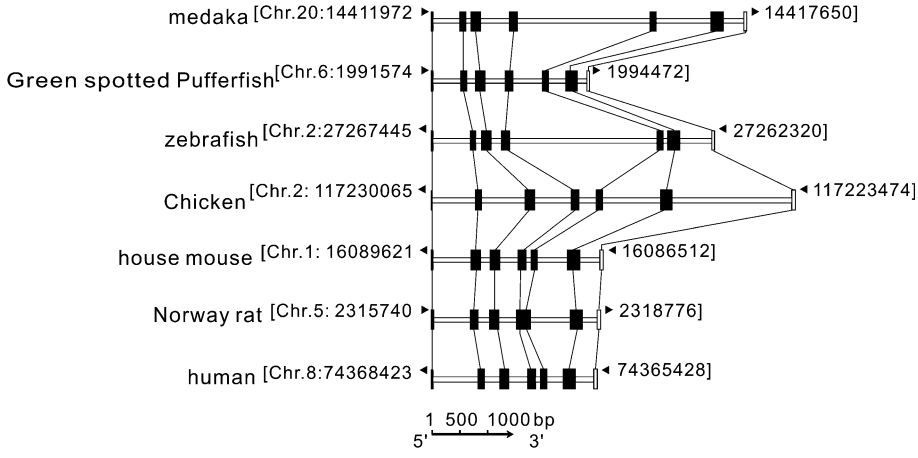


FIG. 3. Schematic diagram of intron-exon structures of vertebrate RPL-7 genes. Filled boxes represent coding sequences; open boxes represent noncoding UTR regions; and the double-line represents the introns. Characters and numbers in the square brackets indicate the genomic location on chromosomes and arrowheads indicate the directions of gene transcription.

found in ovary, which was significantly higher than those in brain, liver, intestine, and eye ($p < 0.05$). The lowest level of β-actin was $2.31 \times 10^7 \pm 4.34 \times 10^6$ copies/μg RNA in eye, which was significantly lower than those in brain, gill, muscle, ovary, testis, and intestine ($p < 0.05$). Levels of 16S rRNA were even higher; they were 4.33- to 27.81-folds higher than those of β-actin. The RSD of 16S rRNA was 41.3%; the highest level was found in spleen (8.89×10^8 copies/μg RNA); and the lowest level was $2.88 \times 10^8 \pm 7.21 \times 10^7$ copies/μg RNA in eye, which was significantly lower than that in intestine ($p < 0.05$). 18S rRNA level was extremely high which was more than thousands times those of others candidate genes. But the level of 18S rRNA was constant in different tissues (RSD = 8.2%), and no significant difference was found in 18S rRNA levels among the tissues. The highest level of 18S rRNA, $5.99 \times 10^{11} \pm 1.27 \times 10^{11}$ copies/μg RNA, was found in ovary and the level in testis was the lowest ($4.32 \times 10^{11} \pm 6.23 \times 10^{10}$ copies/μg RNA). The transcript levels of RPL-7 were about 1.61- to 8.78-fold higher than those of GAPDH, and 3.5×10^4- to 5.5×10^4-folds lower than those of 18S rRNA. RPL-7 was constantly expressed in each of the tissues (RSD = 11.3%), and no significant difference was found among levels in different tissues. The highest level of RPL-7, $1.37 \times 10^7 \pm 1.56 \times 10^6$ copies/μg RNA, was found in ovary and the lowest $9.79 \times 10^6 \pm 2.04 \times 10^6$ copies/μg RNA in brain.

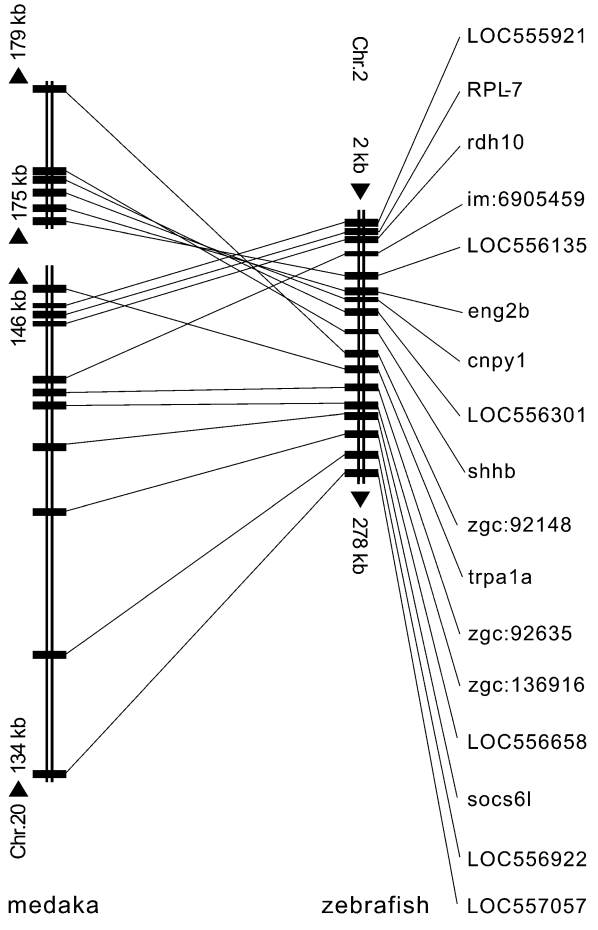


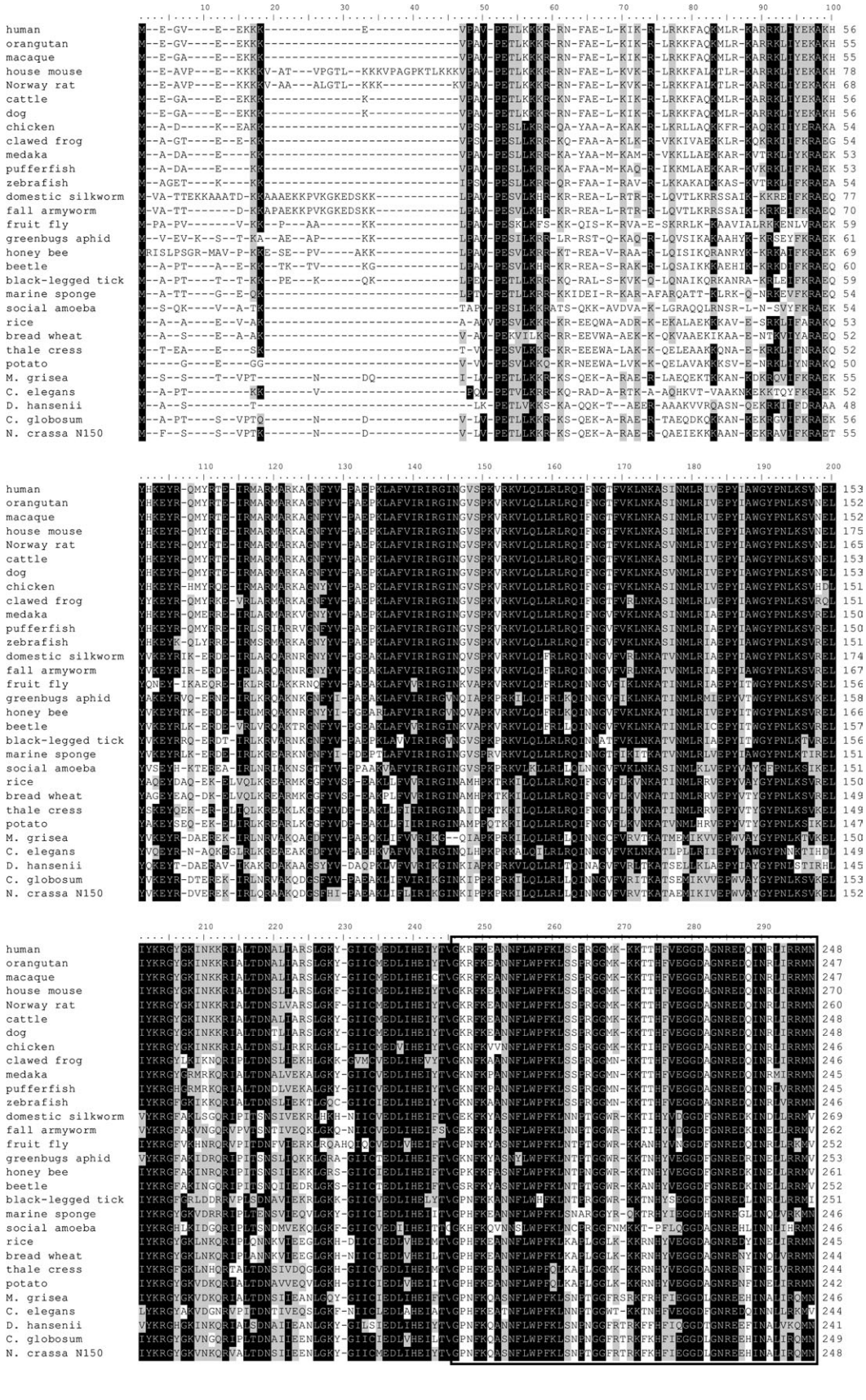
FIG. 4. The genetic map positions of medaka RPL-7 and flanking genes, compared with those of zebrafish.

Gene Expression in Early Stages

The expressions of the five candidate genes (GAPDH, β-actin, 16S rRNA, 18S rRNA, and RPL-7) in 1, 4, and 8 dpf eggs and 2, 6, 12, and 20 dph fry of medaka were also analyzed in this study (Fig. 7). It was found that GAPDH was expressed variably at different development stages with an RSD of 43.0%, while β-actin, 16S rRNA, 18S rRNA, and RPL-7 were relatively constantly expressed, and their RSD values were 12.6%, 13.8%, 4.7%, and 6.7%, respectively.

Gene Expression in Livers and Testes of Medaka Exposed to EDCs

Figure 8 shows the expression characteristics of the GAPDH, β-actin, 16S rRNA, 18S rRNA, and RPL-7 in livers


human
orangutan
macaque
house mouse
Norway rat
cattle
dog
chicken
clawed frog
medaka
pufferfish
zebrafish
domestic silkworm
fall armyworm
fruit fly
greenbugs aphid
honey bee
beetle
black-legged tick
marine sponge
social amoeba
rice
bread wheat
thale cress
potato
M. grisea
C. elegans
D. hansenii
C. globosum
N. crassa N150

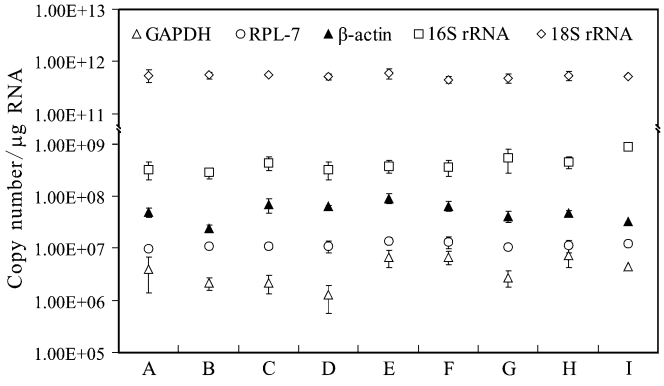


FIG. 6. Expression of GAPDH, RPL-7, β-actin, mitochondrion 16S rRNA and 18S rRNA in various tissues of adult medaka (*Oryzias latipes*, orange red strain) analyzed by Q-RT-PCR. (A) Brain; (B) eye; (C) gill; (D) muscle; (E) ovary; (F) testis; (G) liver; (H) intestine; and (I) spleen. Data of RNA copy number per microgram total RNA are represented as mean ± SD ($n \geq 6$; in particular, total RNA was isolated from spleens of nine fish together).

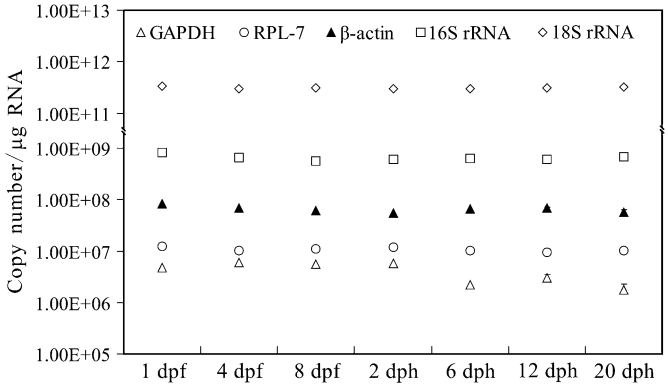


FIG. 7. Expression of GAPDH, RPL-7, β-actin, mitochondrion 16S rRNA and 18S rRNA in 1, 4, and 8 dpf eggs and 2, 6, 12, and 20 dph fries of medaka (*Oryzias latipes*, orange red strain) analyzed by real-time RT-PCR. Data of RNA copy number per microgram RNA are represented mean ± SD (total RNA of each stage was isolated together from more than 10 eggs or fries, except that 12 and 20 dph were isolated from six fries separately).

of the fish which were separately exposed to 200 ng/l EE2, 200 ng/l E2, 100 μg/l BPA, 100 μg/l NP, 100 μg/l *o,p′*-DDE, 100 μg/l *p,p′*-DDE, 1 μg/l TBT-Cl, and 1 μg/l T for 3 weeks. We found that expression of GAPDH was varied largely (RSD = 24.8%). It was significantly upregulated by 200 ng/l EE2, 200 ng/l E2, 100 μg/l *o,p′*-DDE, and 1 μg/l T ($p < 0.05$) but downregulated by 1 μg/l TBT-Cl ($p < 0.05$). The expression of β-actin was also affected (RSD = 27.4%). It was significantly downregulated by 200 ng/l EE2, 200 ng/l E2, 100 μg/l BPA, 100 μg/l NP, and 100 μg/l *o,p′*-DDE ($p < 0.05$). The expression of 16S rRNA was downregulated significantly by 200 ng/l EE2, 200 ng/l E2, and 100 μg/l *o,p′*-DDE ($p < 0.05$), and RSD = 16.5%. In comparison, RPL-7 and 18S rRNA were expressed much more constantly with RSD of 7.6% and 4.1%, respectively, and no significant variation was observed from their transcript levels in the groups exposed to EDCs.

The expression of GAPDH, β-actin, 16S rRNA, 18S rRNA, and RPL-7 in the testes of the EDCs-exposed adult medaka was also analyzed (Fig. 9). It was found that RPL-7, β-actin, 16S rRNA, and 18S rRNA were expressed in consistent levels across different groups with RSD of 4.2%, 5.1%, 5.3%, and 2.6%, except for GAPDH which was expressed with large fluctuation in most of the groups (RSD = 15.5%) but no significant variation was observed.

Relative Quantification of VTGi and StAR Expression in Livers of Male Medaka Exposed to EDCs

The variation of VTGi and StAR expression in livers of male medaka separately exposed to 200 ng/l EE2, 200 ng/l E2, 100 μg/l BPA, 100 μg/l NP, 100 μg/l *o,p′*-DDE, 100 μg/l *p,p′*-DDE, 1 μg/l TBT-Cl, and 1 μg/l T was investigated when RPL-7 GAPDH, β-actin, 16S rRNA, and 18S rRNA were used as internal control genes, respectively (Fig. 10). VTGi was significantly induced by estrogenic chemicals (EE2, E2, BPA, 4-NP, and *o,p′*-DDE), anti-androgenic chemical (*p,p′*-DDE), and androgen (T), and was significantly repressed by TBT ($p < 0.05$) using 18S rRNA as the internal control. It was found that the VTGi expression using RPL-7 as the internal control was similar to that using 18S rRNA, while the VTGi expression using GAPDH or β-actin as the internal control was underestimated or overestimated in some of the groups. On the other hand, there was no significant variation of StAR expression for the exposure groups of 200 ng/l EE2, 200 ng/l E2, 100 μg/l BPA, 100 μg/l NP, 100 μg/l *o,p′*-DDE, 100 μg/l *p,p′*-DDE, 1 μg/l TBT-Cl, and 1 μg/l T when using 18S rRNA and RPL-7 as internal control, however, when GAPDH was used as the internal control, the StAR expression was significantly underestimated in the groups of 200 ng/l E2 and 100 μg/l *o,p′*-DDE ($p < 0.05$), and the StAR expression using β-actin, was significantly overestimated in the groups of 200 ng/l EE2, 200 ng/l E2, 100 μg/l BPA, and 100 μg/l NP ($p < 0.05$).

DISCUSSION

While more and more papers are publishing on the issue of the differential gene expression in response to EDCs measured by Q-RT-PCR and some traditional endogenous controls have been found unsuitable for EDCs study, there has been little

FIG. 5. Alignment of the amino acid sequence of RPL-L7 from 30 eukaryote species including vertebrate, invertebrate, plant, and fungus species (Table 2). The framed region is a NBD. Gaps are represented by dashes. Similar residues are shaded in gray, and identical residues are in white lettering with a dark background. A PAM250 similarity matrix and a threshold of 70% for shading were used.

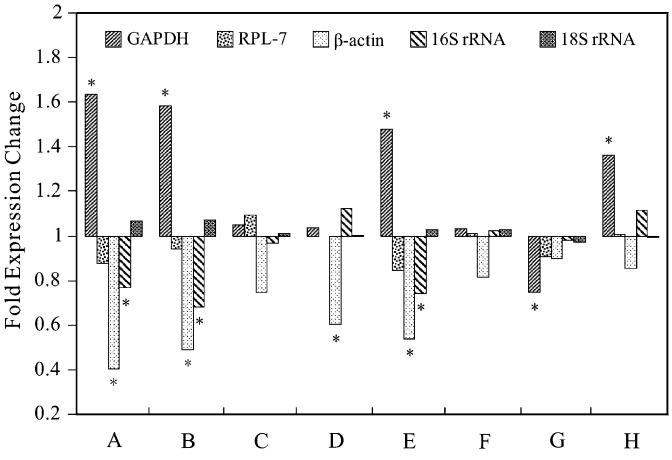


FIG. 8. Expression characteristics of GAPDH, RPL-7, β-actin, mitochondrion 16S rRNA and 18S rRNA in liver of adult male medaka (*Oryzias latipes*, orange red strain) exposed to EDCs: (A) 200 ng/l EE2; (B) 200 ng/l E2; (C) 100 μg/l BPA; (D) 100 μg/l NP; (E) 100 μg/l *o,p′*-DDE; (F) 100 μg/l *p,p′*-DDE; (G) 1 μg/l TBT-Cl; and (H) 1 μg/l T. Data represent the fold of the mean of the mRNA copy number to that of the control. Asterisks (*) indicate statistically significant difference from the control ($p < 0.05$), $n = 6$.

concern given to the development and validation of endogenous control for EDCs studies.

Of traditional endogenous controls, we found that GAPDH mRNA levels were not constant among different tissues and at the early developmental stages (Figs. 6 and 7), which were similar to the results that reported previously (Al-Bader and Al-Sarraf, 2005; Bustin, 2000; Glare *et al.*, 2002). It was also found that GAPDH was significantly affected by some EDCs exposure in livers and testes (Figs. 8 and 9). In fact, this phenomenon was also found in immortalized sheep endometrial stromal cells and human endometrial adenocarcinoma cells under E2 exposure (Farnell and Ing, 2003). β-Actin is frequently used as a quantitative reference for RT-PCR assays (Inui *et al.*, 2003; Zhang *et al.*, 2005b). It has been reported that β-actin mRNA levels were not constant among different tissues in porcine (Foss *et al.*, 1998; Glare *et al.*, 2002), and that β-actin mRNA levels were downregulated in liver of sheephead minnow (Larkin *et al.*, 2003) and upregulated in immature rat uterus by estrogen treatment (Hsu and Frankel, 1987). Our results demonstrated that the transcription of β-actin varied widely in different tissues (Fig. 6) and was significantly downregulated by the estrogenic chemicals in livers (Fig. 8). 18S rRNA is another commonly used internal control for gene expression normalization, and was advocated as a quantitative reference for Q-RT-PCR assays (Schmittgen and Zakrajsek, 2000; Thellin *et al.*, 1999). In this study, we found its expression level was extremely stable compared with other housekeeping genes (Figs. 6–9). However, the transcript level of 18S rRNA is dramatically high, which is above the detection maximum of Q-RT-PCR if no cDNA-reaction dilution was performed. Furthermore, there are arguments against the use of 18S rRNA as internal control (Nicot *et al.*, 2005) because 18S rRNA cannot be used to normalize targets that have been enriched for mRNA and cannot be reverse transcribed in oligo (dT) primed cDNA synthesis. Thus, these adverse aspects would limit its use in Q-RT-PCR.

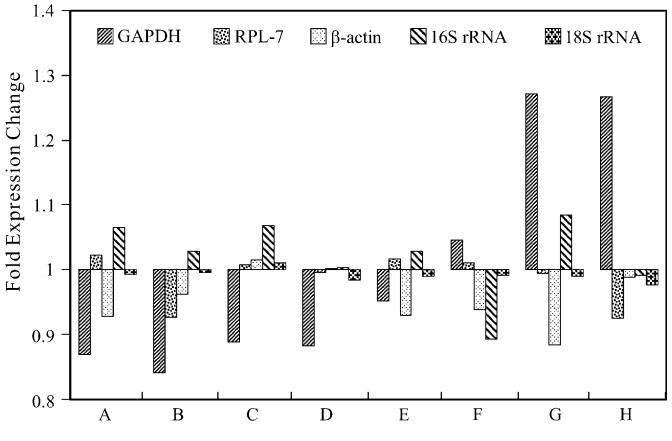


FIG. 9. Expression characteristics of GAPDH, RPL-7, β-actin, mitochondrion 16S rRNA and 18S rRNA in testes of adult medaka (*Oryzias latipes*, orange red strain) exposed to EDCs: (A) 200 ng/l EE2; (B) 200 ng/l E2; (C) 100 μg/l BPA; (D) 100 μg/l NP; (E) 100 μg/l *o,p′*-DDE; (F) 100 μg/l *p,p′*-DDE; (G) 1 μg/l TBT-Cl; and (H) 1 μg/l T. Data represent the fold of the mean of the mRNA copy number to that of the control, $n = 6$.

RPL-7 is an integral part of the large subunit of ribosome (von Mikecz *et al.*, 1999). It has been found constantly expressed in some mammalian tissues and cells at stages of development, and has been used to normalize mammalian gene expression in many studies (Huot *et al.*, 2001; Kanematsu *et al.*, 2005; Muroya *et al.*, 2005; Sun *et al.*, 2004). We cloned the complete mRNA sequence of the RPL-7 from medaka (Fig. 1), and we found RPL-7 was highly conserved not only among vertebrate species but also across eukaryotes, and the C-terminal NBD was conserved during evolution. From results of gene expression measurement, RPL-7 was constantly expressed in differential tissues and stages (Figs. 6 and 7) of medaka, and its mRNA level was unaffected in livers and testes (Figs. 8 and 9) of adult male medaka exposed to EDCs. From Figure 10, it was found that the expression of target gene did not significantly change when using RPL-7 as the internal control, compared with the use of 18S rRNA, and RPL-7 was expressed at moderately abundant levels (Figs. 6 and 7). These results demonstrated that RPL-7 is with the character of stability of 18S rRNA but the adverse aspects. In addition, RPL-8 gene has been used as an internal control for normalizing gene expression (Larkin *et al.*, 2003; Sasaki *et al.*, 2006). We also analyzed the expression of RPL-8 in different tissues of adult medaka and stages of the early life (shown in Supplementary Data), and found it is expressed a similar level and profile with that of RPL-7.

As shown in Figure 10, the relative quantification of VTGi and StAR expressions is basically influenced by the internal controls. When using 18S rRNA or RPL-7 as an internal control, which are relatively stably expressed, VTGi was found

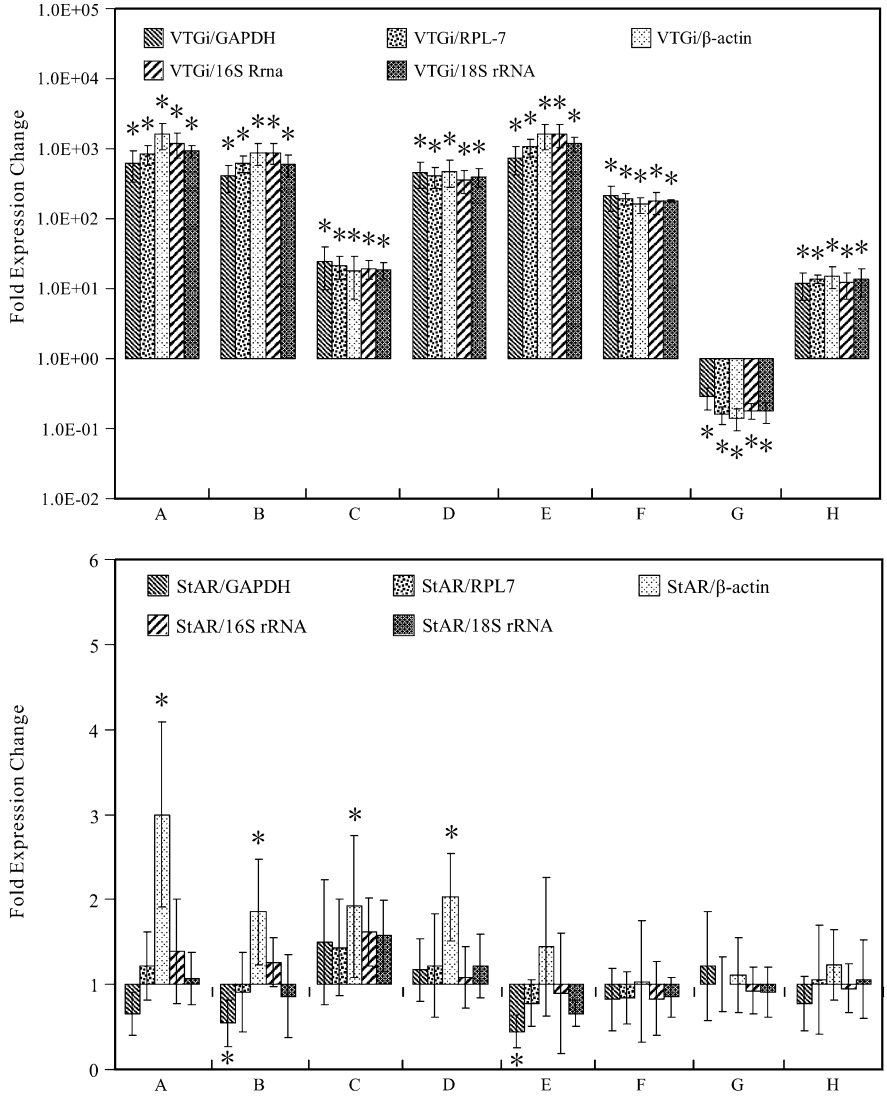


FIG. 10. Relative quantification of VTGi and StAR expression using internal controls (GAPDH, RPL-7, β-actin, 16S rRNA, and 18S rRNA) in liver of adult medaka (*Oryzias latipes*, orange red strain) exposed to EDCs: (A) 200 ng/l EE2; (B) 200 ng/l E2; (C) 100 μg/l BPA; (D) 100 μg/l NP; (E) 100 μg/l *o*,*p*′-DDE; (F) 100 μg/l *p*,*p*′-DDE; (G) 1 μg/l TBT-Cl; and (H) 1 μg/l T. Data represent the fold of the mean of the mRNA copy number to that of the control. Asterisks (*) indicate statistically significant difference from the control ($p < 0.05$), $n = 6$.

to be significantly induced by EE2, E2, BPA, 4-NP, *o*,*p*′-DDE, *p*,*p*′-DDE, and T but significantly repressed by TBT ($p < 0.05$). And StAR, a gene regulated by cyclic adenosine monophosphate–mediated signaling, was found to be not affected by E2, T, and other EDCs, which was similar to the result reported in mouse Leydig tumor cells (Schwarzenbach *et al.*, 2003). On the other hand, when β-actin or GAPDH was used as internal control, VTGi expression was found to be overestimated or underestimated in most of the EDCs exposing groups, while StAR was found to be pseudoinduced or pseudorepressed due to the variation of β-actin or GAPDH expression.

In conclusions, among five candidate genes (GAPDH, β-actin, 16S rRNA, 18S rRNA, and RPL-7), RPL-7, of which the complete mRNA sequence was cloned from medaka in this study, was found to be the most acceptable and appropriate endogenous control for gene expression profiling, especially for EDCs studies, due to its constant expression in different tissues and stages of development, and because it is unaffected by EDCs exposure and has a moderate transcript level.

SUPPLEMENTARY DATA

In this supplementary data, expression of RPL-8 in various tissues of adult medaka and stages of the early life are illustrated in Figures I and II, respectively. It was found that level of RPL-8 was relatively constant in different tissues and stages of medaka, and the RSD values were 11.4% and 6.5%,

respectively. There is no significant difference was found in RPL-8 expression among the tissues.

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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