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The Establishment of a Quantitative RT-PCR Assay for Estrogen Receptor mRNA in Japanese Eel, *Anguilla japonica*

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ABSTRACT—A sensitive quantitation system using reverse transcription-polymerase chain reaction (RT-PCR) was developed to measure the low estrogen receptor (ER) mRNA levels in Japanese eel (*Anguilla japonica*). Two types of cytoskeletal actins, β- and γ-actins, were distinguished in Japanese eel and used as the internal control for exact quantitation. Actin and ER primers of this study annealed to different exons, allowing for the skipping of DNase treatment. Accordingly, ER and actin RT-PCR products showed a single band and were amplified with the same efficiency during PCR. The ER mRNA amount was calculated as a relative value, normalized over actin (β and γ) mRNA. The results thus obtained by RT-PCR agreed with the results from Northern blot analysis of liver from pre-vitellogenic and hormone-treated early vitellogenic eel. Using this system, the ER mRNA levels were further measured in coelomic epithelium, pituitary, brain and ovary. In the liver, ER mRNA levels of the early vitellogenic eel increased about by 2.7 folds compared to those of the immature eel. In contrast, changes in levels of ER transcripts were not observed in other tissues. This system can be used to detect relative ER mRNA levels around 100-fold lower than those of actin mRNA in all tissues in which it has been difficult to measure ER mRNA by Northern blot analysis.

INTRODUCTION

In all vertebrate species, estrogen, mainly estradiol-17β (E_2), plays a central role in cell growth and differentiation in female reproductive organs. Moreover, in teleosts, as in other oviparous species, E_2 mainly controls vitellogenin (VTG) synthesis in the liver (Wallace, 1985) and acts through the estrogen receptor (ER). The ER is a ligand-dependent transcription factor that directly binds to target DNA sequences and that regulates the transcriptional activity of target genes (Mangelsdorf *et al*., 1995).

Estrogen receptor cDNA has now been isolated from human (Green *et al*., 1986), rat (Koike *et al*., 1987), mouse (White *et al*., 1987), chicken (Krust *et al*., 1986; Maxwell *et al*., 1987), and *Xenopus* (Weiler *et al*., 1987). In fish, rainbow trout, *Oncorhynchus mykiss*, (Pakdel *et al*., 1990) and Japanese eel, *Anguilla japonica*, (Todo *et al*., 1996) ER cDNAs have been cloned. Subsequent molecular analyses were aimed at investigating regulation of ER gene expression in various tissues. Thus, the differential regulation of ER was demonstrated in the rat uterus, liver and pituitary (Shupnik *et al*., 1989), and in the chicken oviduct and liver (Ninomiya *et al*., 1992). In fish, Salbert *et al*. (1993) reported differential regulation of ER in the rainbow trout liver, pituitary and hypothalamus.

The Northern blot technique has generally been used for quantitating ER mRNA. However, this techniques is not very sensitive and requires large amounts of poly(A)$^+$ RNA when the target mRNA amount is low. In Japanese eel, ER mRNA levels were especially low compared with other species. Thus, Northern hybridization required about 5 μg poly(A)$^+$ RNA for each sample analyzed, even from liver tissue in which ER is mainly expressed in teleosts (Todo *et al*., 1996). The accurate quantification of such low levels of Japanese eel ER mRNA is often difficult, requiring the use of more sensitive techniques for measuring ER mRNA. Recently, a sensitive assay system using RT-PCR was developed to determine the ER mRNA level in chicken (Ninomiya *et al*., 1992). However, there are no reports of such sensitive ER mRNA assays using RT-PCR in fish.

In the present study, we established a sensitive quantitation system using RT-PCR, and analyzed ER expression in various tissues related to sexual maturation in the Japanese eel.

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MATERIALS AND METHODS

Animals and treatments

Glass eels were feminized by administering estradiol-17β mixed in the feed (20 mg/kg-pellet) for one month (Tachiki and Nakagawa, 1993) and then were cultivated for two and a half years. Adult female eels (0.5-1 kg in body weight) were kept in recirculating seawater tanks with a capacity of 1000 l at 20°C and not fed throughout the experimental period. Cultivated Japanese eel has immature ovaries in the pre-vitellogenic stage and vitellogenesis can be induced by treatment with chum salmon pituitary homogenate (SPH) (Yamamoto *et al.*, 1974). Five eels each were used as initial and SPH-injected groups. Fish received weekly injections of SPH over a two-week period at a dose of 40 μg per g body weight, which resulted in advancing the ovaries to the early vitellogenic stage. Three days after the last injection, fish were anesthetized in 0.1% ethyl aminobenzoate, sacrificed, tissues (liver, coelomic epithelium, pituitary, brain and ovary) removed, then quickly frozen in liquid nitrogen and kept at −80°C until use.

cDNA synthesis

Total RNA was extracted from tissue by the single-step acid guanidinium isothiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) using ISOGEN (Wako Pure Chemical Industries). Reverse transcription and PCR were carried out according to the method described by Nagae *et al.* (1997). cDNAs were synthesized from total RNA (1 μg) with random hexamer primers using M-MLV reverse transcriptase (GIBCO BRL) in a 20 μl reaction. Reverse transcriptions were performed for 60 min at 37°C, then stopped by incubation for 5 min at 99°C.

Synthesis of ER and β-actin specific primers

To generate cDNA products from the mRNAs of ER and β-actin, two primer sets were produced with an Applied Biosystems DNA Synthesizer model 391. ER primers were synthesized on the basis of the cDNA nucleotide sequences for Japanese eel ER (Todo *et al.*, 1996). Sequences of ER primers were:

sense, 5'-AACCTGTGCACAACGTCTAG-3' (nucleotides 1309-1328, C domain);

antisense, 5'-CTGCTGTGCATGGTGTTG-3' (nucleotides 1578-1595, E domain).

β-actin primers were designed based on completely conserved cDNA nucleotide sequences of β-actin of common carp (Liu *et al.*, 1990). Unlike a previous study (Nagae *et al.*, 1997), in which a pair of β-actin primers annealed to sequences located on the same exon, we selected primers forecasted to anneal to separate exons. Sequences of actin primers were:

sense, 5'-GACATGGAGAAGATCTGGCA-3' (exon 3);

antisense, 5'-GTCAGGATCTTCATGAGGTAGTC-3' (exon 4).

Sequence determination of PCR products by β-actin primers

cDNA fragments were amplified by PCR using β-actin primers. The cDNA was then subcloned into the pCR 2.1 vector using the TA cloning kit (Invitrogen) and transformed into *E. coli* strain XL-1. The DNA sequence was determined from both strands using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer).

RT-PCR

Five μl of reverse-transcribed total RNA were added to 45 μl of reaction mixture. PCR was performed at the following reagent concentrations: 18.5 μl sterile distilled H_2O, 5 μl 1st-stranded cDNA (250 ng), 5 μl 10 × buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl), 5 μl 25 mM $MgCl_2$, 5 μl 2.5 mM each of dNTP mixture, 2.5 μl (10 mM respectively) of 5' and 3' synthetic oligonucleotide primers (described above), 1 μl [α-^{32}P]dCTP (3.3 mM, 370 MBq/ml; ICN Pharmaceuticals Inc.) and 0.5 μl recombinant Taq DNA polymerase (5 U/μl; Takara). The tubes were placed in a thermal cycler (Gene Amp PCR System 2400, Perkin Elmer) and incubated at 94°C for 2 min, then cycled 22 times. One cycle of PCR was carried out at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec. Ten μl of PCR product was collected after 16, 18, 20 and 22 cycles and electrophoresed on a 5% polyacrylamide gel. After autoradiography, the gel was dried and analyzed using a BAS 2000 Bio-Image Analyzer. After confirming that the efficiency of the amplification of ER and actin PCR products was the same, the intensity of the ER signal was normalized over that of the internal control (related ER value).

Accuracy test

Total RNA from liver of SPH-injected Japanese eel was extracted and used for the accuracy test. Ten tubes of RT-products were used for the within-assay coefficient of variation (CVw), while the between-assay coefficient of variation (CVb) was calculated from 5 assays (assays in triplicate). CVw and CVb for the ER RT-PCR assay were 6.3% and 5.1% respectively.

Southern blot analysis

The PCR product amplified by ER primers was electrophoresed on a 2% agarose gel and then blotted onto a nylon membrane (Hybond-N$^+$, Amersham International) using alkaline transfer buffer (0.1 M NaOH and 1.5 M NaCl). The membrane was pre-hybridized at 65°C for 3 hr in 6 × SSC. Hybridization was carried out under the same conditions for 16 hr with ^{32}P-labeled probe. For this purpose, the entire Japanese eel ER coding region (Todo *et al.*, 1996) was labeled with the Random Primer Labeling Kit (NEN) in the presence of [α-^{32}P]dCTP. Following hybridization, the membrane was washed at 65°C for 3 × 10 min with 6 × SSC containing 0.1% SDS, for 2 × 10 min with 3 × SSC containing 0.1% SDS and for 10 min with 1 × SSC containing 0.1% SDS, and then analyzed using a BAS 2000 Bio-Image Analyzer (Fujifilm).

Northern blot analysis

Poly(A)$^+$ RNA was isolated by oligo(dT)-latex beads (Oligotex-dT30<super>, Takara). The poly(A)$^+$ RNA was subjected to 1% agarose gel electrophoresis under denaturing conditions, then vacuum blotted onto a nylon membrane (Hybond-N$^+$, Amersham International). Northern hybridization was done in a manner analogous to that described for Southern blot (above). However, after probing for ER, membranes were stripped and re-probed with ^{32}P-labeled actin (β and γ).

Statistical analysis

Data are presented as the mean ± SE. The relative levels of ER mRNA from initial and experimental groups were compared statistically for significant differences using an unpaired t-test (2-tailed).

RESULTS

Japanese eel actin cDNA sequence determinations

The nucleotide sequences amplified by the actin primers were determined. It was revealed that the actin primers amplified two species of actins (Fig. 1). The homology of the nucleotide sequence of both actin fragments was 89.2%, while the deduced amino acid sequence of these fragments displayed 100% matching. Furthermore, cDNAs of both actins were cloned from the liver of the same individual (details will be published by Kazeto *et al.*). Based on the amino-terminal end of the primary structure deduced from these cloned cDNAs (data not shown), actins were identified as β-actin (upper line of Fig. 1) and γ-actin (lower line). The nucleotide sequence of the β-actin fragment showed high homology with that of β-actin in human (Ponte *et al.*, 1984; 91%), rat (Nudel *et al.*,

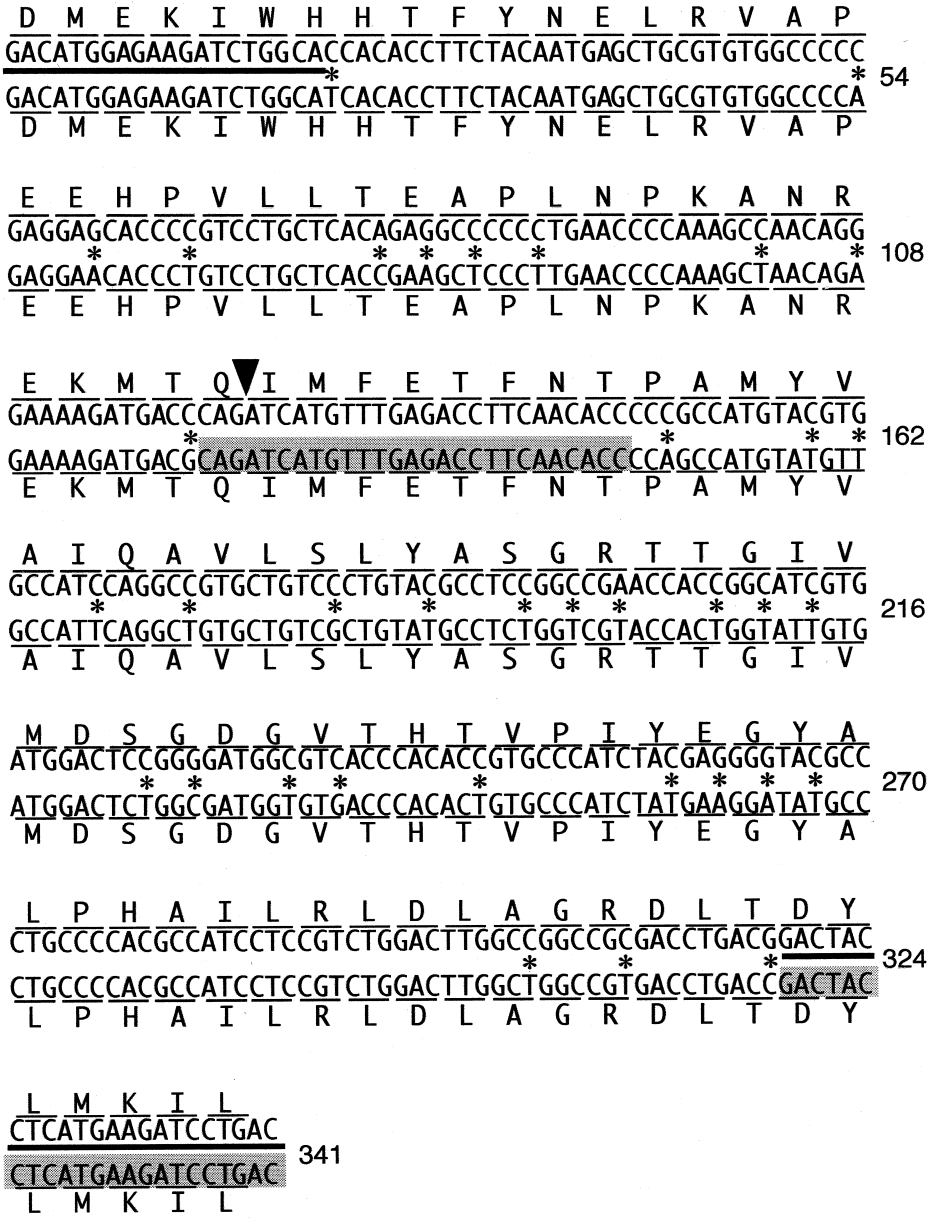

Fig. 1. The nucleotide and deduced amino acid sequences of the PCR products amplified by β-actin primers in the Japanese eel. Mismatches are indicated by asterisks. The β-actin primers used in RT-PCR assay are underlined. β-actin primers used previously by Nagae *et al.* (1997) are represented by the shaded boxes, while the arrow head indicates the anticipated beginning of exon 4.

1983; 88.9%), chicken (Kost *et al.*, 1983; 89.2%), sea bream (Santos *et al.*, 1997; 89.8%) and grass carp (Liu *et al.*, 1989; 93.3%). The γ-actin cDNA fragment was 100% matched with the partial nucleotide sequence of Japanese eel β-actin previously determined (Nagae *et al.*, 1997) and also showed high homology with that of cytoskeletal γ-actin in human (Erba *et al.*, 1986; 87.4%), rat (Brown *et al.*, 1990; 88.0%), and mouse (Tokunaga *et al.*, 1988; 88.0%).

PCR products from cDNA and genomic DNA

The method established in a previous study (Nagae *et al.*, 1997) required treatment of total RNA with DNase I, because previous primers annealed to sequences on the same exon and hence, the amplified fragments could either be from genomic DNA or from total RNA. In this study, to omit the DNase I treatment, the primers were selected to anneal to different exons enabling identification of the source of the PCR products. To investigate whether the primers annealed to the same exon or not, total RNA and genomic DNA from liver samples from SPH-injected females were analyzed by PCR using ER or actin primers. ER and actin RT-PCR products from hepatic total RNA samples showed single bands (Fig. 2) 289 bp and 341 bp in size, respectively, as expected. The PCR products from ER and actins from liver genomic DNA were different and longer in size than those from total RNA. From genomic DNA, one band was detected in the ER PCR

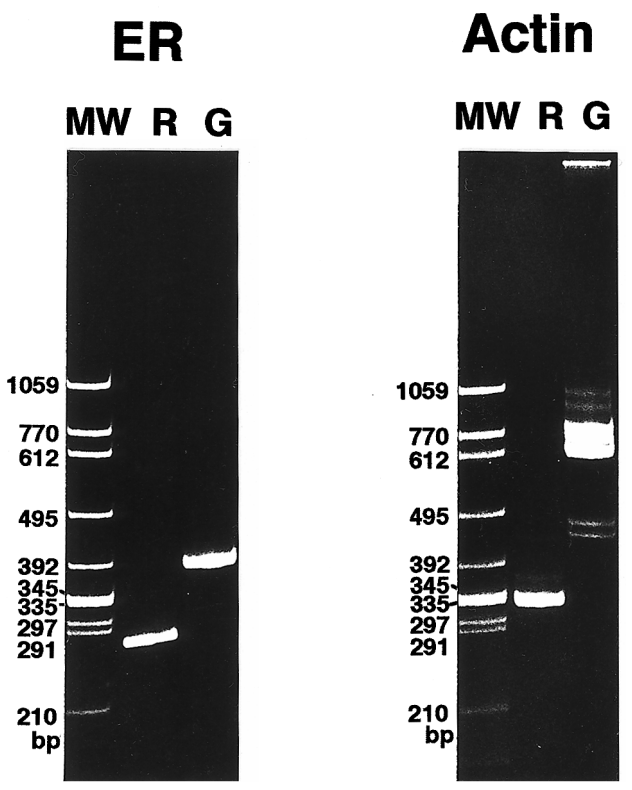

Fig. 2. Amplification of ER and actin fragments in liver from chum salmon pituitary homogenate-injected Japanese eel, using total RNA (R) and genomic DNA (G). PCR products for ER and actins were separated on a 5% polyacrylamide gel. The gel was stained by ethidium bromide. ER: ER PCR product. Actin: actin PCR product. MW: molecular weight marker.

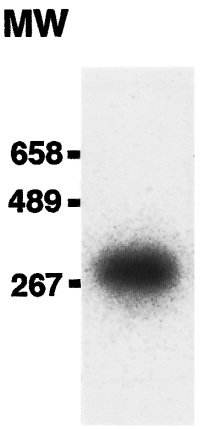

Fig. 3. Southern blot analysis of the PCR product amplified by ER primers. *In vitro* synthesized cDNA from liver of SPH-injected Japanese eel was amplified by PCR using ER primers. The PCR product was probed with a α-^{32}P random-labeled cDNA fragment encoding the ER. Following exposure, signals were analyzed by a BAS 2000 Bio-Image Analyzer.

product, whereas the actin PCR product exhibited multiple bands.

Southern blot analysis

The ER coding region probe specifically hybridized to a single band, approximately 289 bp in size, of the ER PCR products (Fig. 3).

Validation of the ER RT-PCR assay

Using the reaction conditions described above, aliquots were taken from the PCR tubes at the end of 16, 18, 20, 22 cycles and loaded on a 5% polyacrylamide gel. Radioactivity of the PCR products from each cycle was evaluated and plotted on a semi-logarithmic graph (Fig. 4). Between the 16th and 18th cycle, the ER and actin PCR products displayed parallel amplification curves in all tested tissues.

Transcription of ER mRNA in the liver

In order to quantify liver ER mRNA levels, RT-PCR was performed for five SPH injected and five initial fish, while Northern blot was carried out on livers from two SPH injected and 2 initial females. The relative value of ER mRNA in the liver of initial fish was about 0.15 by RT-PCR analysis (Fig. 5A). After SPH injection, the contents of liver ER mRNA increased about 2.6 fold ($p < 0.01$) in comparison with initials. Northern blot analysis for livers from initial and SPH-injected fish, using the coding region probe, is shown in Fig. 5B. In livers from initial and SPH-injected eels, two mRNA bands, corresponding to approximately 5.6 and 3.8 kb, were detected. The intensity of these bands increased after SPH injection, whereas actin levels ($\beta + \gamma$) did not change. The relative values of ER signals were 0.08 and 0.09 in initial, and 0.18 and 0.15 in SPH-injected fish (duplicated measurements).

Japanese eel ER mRNA transcription in various tissues

The relative levels of ER mRNA in various tissues were measured by RT-PCR in initial fish and SPH-treated fish (Fig. 6). ER mRNA levels were detectable in all tissues that we measured by this assay. In control fish, the levels of ER mRNA in the pituitary and coelomic epithelium were relatively high at 0.2 and 0.1 respectively (similar to liver levels), but did not change in SPH-treated fish. Values of ER mRNA detected in the brain and ovary were very low (around 0.01) and did not appear to be affected by SPH injection.

DISCUSSION

In this study, we employed RT-PCR to quantify mRNA for ER in various tissues. Using this assay we were able to detect the ER mRNA in all tissues that we tested from initial and SPH-injected fish. The efficiency of both RT and PCR steps allows the amplification and detection of low mRNA transcript levels. The exponential amplification of target cDNA was necessary to enhance sensitivity in RT-PCR, although it increased the difficulty in precisely quantifying the initial levels of mRNA. Thus, for accurate quantitation of target mRNA, it is essential to amplify the transcripts of the internal control gene together with those of the target gene and check the efficiency of the amplification in each sample (Rappolee *et al.*, 1988;

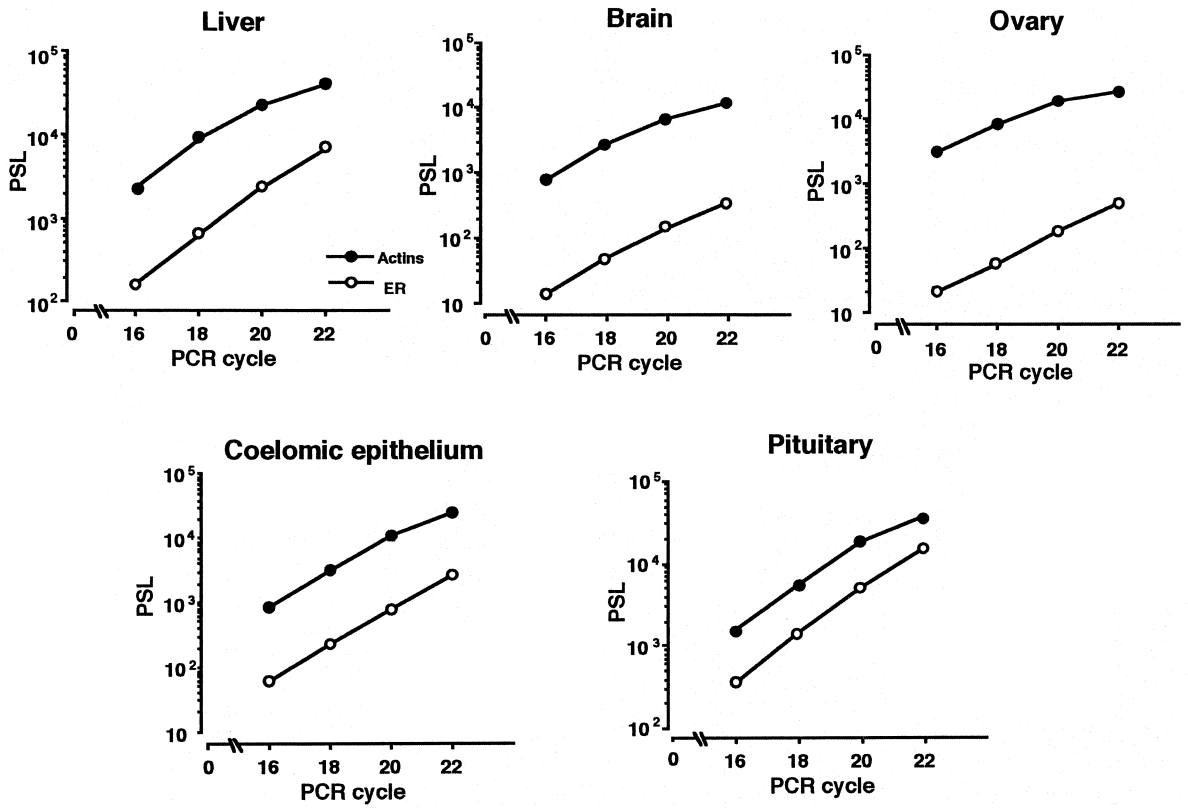

Fig. 4. The radioactivity associated with ER and actin (β + γ) PCR products in various tissues. PCR products were taken at the end of 16, 18, 20, 22 cycles and separated on 5% PAGE. The radioactivity of ER and actin bands is plotted on a semi-logarithmic graph. PSL is an arbitrary unit representing radioactivity used by the BAS 2000 Bio-Image Analyzer.

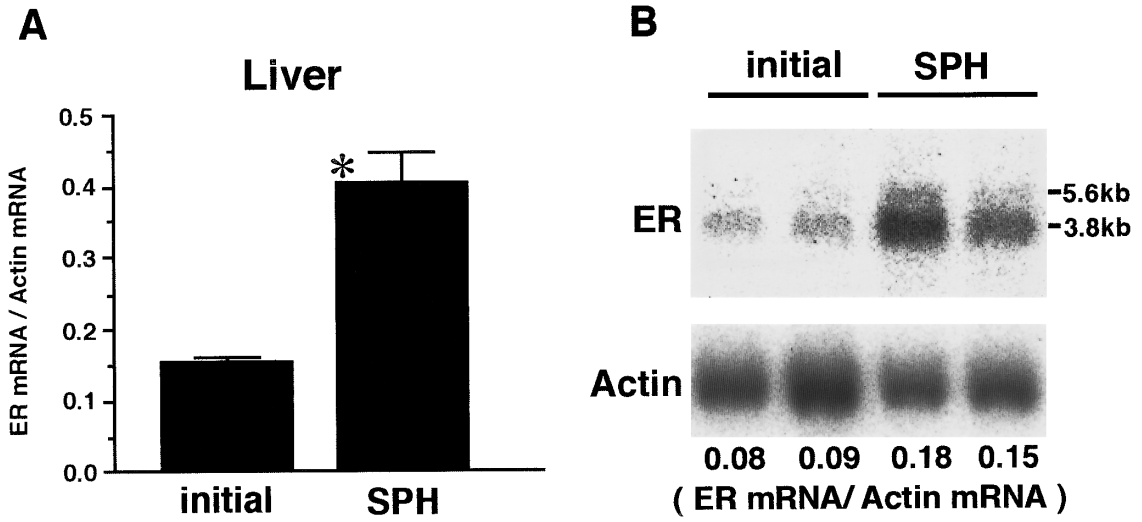

Fig. 5. RT-PCR and Northern blot analysis of ER mRNA levels in the liver from initial and SPH-injected fish. (**A**) Total RNA (250 ng) from the liver was used for RT-PCR. Radioactivity of the two bands representing ER and actin (β + γ) fragments was evaluated by a BAS 2000 Bio-Image Analyzer. Relative expression was calculated as the ratio of radioactivity associated with ER over that of β + γ actins. Vertical bars represent the SEM. initial: initial fish (N = 5). SPH: SPH-injected fish (N = 5). ∗ : Significantly different from initial values ($p < 0.01$). (**B**) Northern blot analysis of poly(A)$^+$ RNA (5 μg) from the liver was performed using the ER coding and actins cDNA probes. Hybridization was first carried out with the ER probe and, after 2 days BAS exposure, the same membrane was re-hybridized with the actin cDNA probe and again exposed overnight.

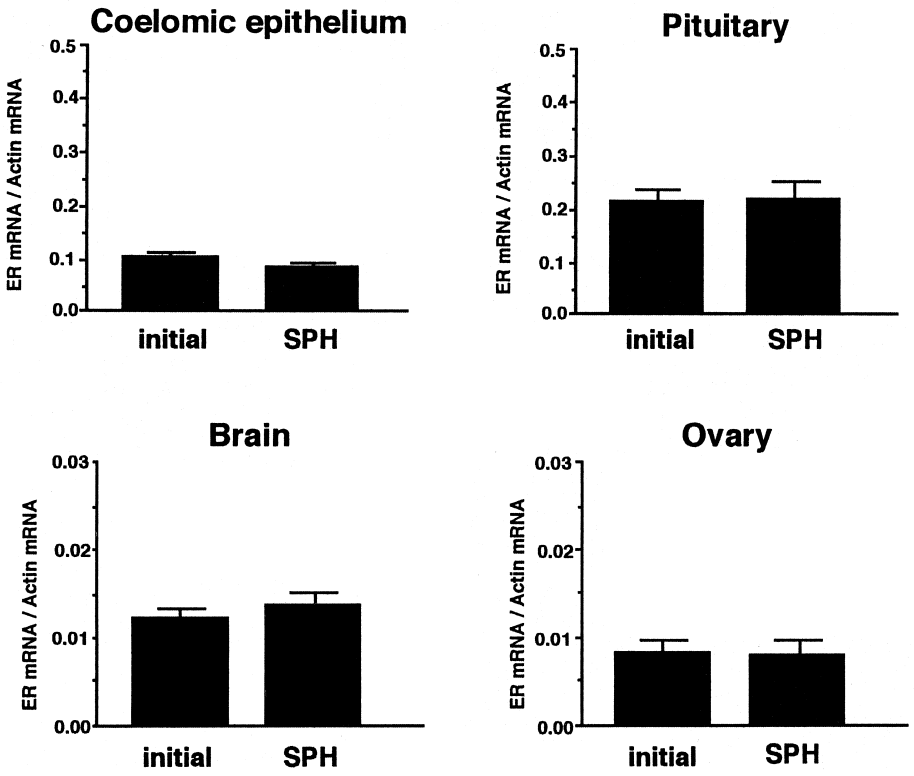

Fig. 6. ER mRNA levels in various tissues (pituitary, coelomic epithelium, brain and ovary) from initial and SPH-injected fish, using quantitative RT-PCR. Vertical bars represent the SEM. initial: initial fish (N = 5). SPH: SPH-injected fish (N = 5).

Chelly *et al.*, 1988). We selected actin as the internal control to normalize the data and determined the sequence of partial cDNAs generated by PCR.

The nucleotide sequence of the β-actin fragment mostly corresponded to grass carp β-actin (93.3%; Liu *et al.*, 1989) and shared high homology (approximately 90%) with that of other species, e.g., human (Ponte *et al.*, 1984), rat (Nudel *et al.*, 1983), chicken (Kost *et al.*, 1983) and sea bream (Santos *et al.*, 1997). The high homology was also displayed in the nucleotide sequence of the γ-actin fragment when compared with cytoskeletal γ-actin in other species, e.g., human (Erba *et al.*, 1986), rat (Brown *et al.*, 1990) and mouse (Tokunaga *et al.*, 1988). The cytoskeletal β- and γ-isoforms are very similar and differ at only four amino acids in human (Tokunaga *et al.*, 1988). Tokunaga *et al.* (1988) reported these changes of amino acids to be located in the amino-terminal regions. The amino-terminal regions of Japanese eel β-actin (Kazeto *et al.*, unpublished results) completely matched those of sea bream β-actin (Santos *et al.*, 1997), while the same region of Japanese eel γ-actin differed by only one amino acid from γ-actin in documented mammalian species (Kazeto *et al.*, unpublished results). The cloning of cytoskeletal actins revealed that the PCR assay amplified both β- and γ-actin as internal controls. Furthermore, the β-actin primers previously used by Nagae *et al.* (1997) were also found to match actin sequences for 100% indicating that β- and γ-actin were also amplified as internal control in the previously reported assay.

The actin PCR products from genomic DNA were longer in size than those from total RNA and displayed several bands. This result indicates that the pair of actin primers anneal on different exons, while the actin primers used previously (Nagae *et al.*, 1997) anneal on the same exon. Changing the actin primers enables us to distinguish the mRNA PCR products from the genomic DNA PCR products (attributed to contamination) by their length and thus, to skip the process of DNase I treatment because it is not necessary to completely remove genomic DNA from samples. In mammals, a large numbers of actin-related sequences, mainly pseudogenes, were found scattered throughout the genome (Soriano *et al.*, 1982; Ponte *et al.*, 1983; Ng *et al.*, 1985). Among these, many β and γ actin pseudogenes have been identified and sequenced (Moos and Gallwitz, 1983; Tokunaga *et al.*, 1985; Leube and Gallwitz, 1986; Begg *et al.*, 1988; Peter *et al.*, 1988). Multiple PCR products, amplified from genomic DNA, therefore probably derived from actin pseudogenes. In contrast, the PCR product for ER using total RNA yielded only one band, which was shorter in size than that from genomic DNA. Thus the pair of ER primers behaved like the pair of actin primers and annealed on different exons. Furthermore, Southern blot analysis using the ER coding region probe, showed that the ER primer specifically amplified ER.

The actin and ER PCR products displayed parallel amplification curves between the 16th and 18th cycle in tissues we tested. This implies that the two products were amplified with the same efficiency between the 16th and the 18th cycle, and therefore, the counts at the end of the 16th or 18th cycles

were used for calculations after checking of the amplification curves in each sample. Moreover, both within- and between-assay coefficients of variation were low for the RT-PCR assay, indicating accurate estimation of ER values. Nevertheless, this quantitative assay using RT-PCR suffers from overestimation of target mRNA if PCR cycles are excessively repeated. Hence, quantitation of target mRNA must be performed before PCR products reach a plateau.

Northern blot analysis, using the entire coding region probe, showed that two ER mRNAs with length of approximately 5.6 and 3.8 kb were expressed in the liver. Todo *et al*. (1996) reported that multiple ER mRNAs (5.6, 3.8 and 1.2 kb) were expressed in the Japanese eel liver using a partial ER fragment as probe. The 1.2 kb mRNA was not detected when using the entire coding region probe in this study. The reason for the difference in the expression of the 1.2 kb mRNA is unclear, but it may have occurred on account of the different probes. Moreover, it is necessary to determine if the 5.6 and 3.8 kb mRNAs originated by alternative splicing from a single copy gene or by transcription from different genes. Further analysis of multiple ER mRNA is needed to solve these problems. Todo *et al*. (1996) suggested that both the 5.6 and 3.8 kb mRNAs encode the functional ER because their sizes are longer than the coding region of Japanese eel. Northern blot analysis, using ^{32}P-labeled ER primer as probe showed that both the 5.6 and 3.8 kb mRNAs are specifically amplified by ER primers in this RT-PCR assay (data not shown). These results demonstrate that our quantitation system can be used to measure the two ER mRNAs expected to encode the functional ER corresponding to approximately 5.6 and 3.8 kb mRNA.

RT-PCR analysis established in this way demonstrated that ER levels of the liver in early vitellogenic stages were increased compared with those in pre-vitellogenic stages. The increase of ER mRNA in the early vitellogenic stage correlated to changes in the serum E_2 and vitellogenin levels (Okumura *et al*., data unpublished). The result of RT-PCR increased similarly to that of Northern blot analysis, again implying that the target ER mRNA was accurately quantified by this RT-PCR system. However, differences in ER expression between developmental changes were relatively smaller when using Northern blot compared with RT-PCR. This may be attributed to the use of different individuals between experiments. In tissues other than the liver, no significant changes were observed in levels of ER mRNA between pre-vitellogenic and early vitellogenic fish. In other species, tissue-specific regulation of ER mRNA in many different target tissues has been reported (Maxwell *et al*., 1987; Shupnik *et al*., 1989; Ninomiya *et al*., 1992; Salbert *et al*., 1993). Therefore, the differential expression between the liver and other tissues in the Japanese eel may indicate tissue-specific regulation of ER during the maturational period. Furthermore, evaluation of ER mRNA levels during ovarian development using quantitative RT-PCR for various tissues will be necessary to establish the tissue-specific regulatory effects of ER during sexual maturation in the eel.

Recently, an ER subtype termed ERβ has been cloned in human (Mosselman *et al*., 1996), rat (Kuiper *et al*., 1996), and mouse (Tremblay *et al*., 1997). In the rat, the tissue distribution and relative expression of ERβ was shown to be quite different to that of ERα (Kuiper *et al*., 1997). In fish, no information is available about the ERα and ERβ subtypes. In this study, two types (5.6 and 3.8 kb) of ER mRNAs were detected by Northern blot. If ERα and ERβ exist in the Japanese eel, it will be necessary to clone them and separately determine the quantity of ERα and ERβ mRNA. The RT-PCR quantitative system will be particularly suitable to respectively measure ERα and ERβ mRNA, owing to selection of specific primer combinations.

In conclusion, we have developed a sensitive quantitation system using RT-PCR to measure Japanese eel ER mRNA levels. ER mRNA was detectable in all tissues that we measured by this assay. Furthermore, the results of RT-PCR analysis for various tissues suggest the tissue-specific regulation of ER. The advantage of this quantitative system is that only 250 ng total RNA are required for quantitative analysis. Thus, the use of this method for the quantitation of ER mRNA from hepatic cells cultured *in vitro* will yield new and highly needed information on ER regulation.

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REFERENCES

Begg C, Delius H, Leader D (1988) Duplicated region of the mouse genome containing a cytoplasmic γ-actin processed pseudogene associated with long interspersed repetitive elements. J Mol Biol 203: 677–687

Brown CW, McHugh KM, Lessard JL (1990) A cDNA sequence encoding cytoskeletal gamma-actin from rat. Nucleic Acids Res 18: 5312

Chelly J, Kaplan JC, Maire P, Gautron S, Kahn A (1988) Transcription of the dystrophin gene in human muscle and non-muscle tissues. Nature 333: 858–860

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159

Erba HP, Gunning P, Kedes L (1986) Nucleotide sequence of the human gamma cytoskeletal actin mRNA: anomalous evolution of vertebrate non-muscle actin genes. Nucleic Acids Res 14: 5275–5294

Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, Chambon P (1986) Human oestrogen receptor cDNA: Sequence and expression and homology to v-erb-A. Nature 320: 134–139

Koike S, Sakai M, Muramatsu M (1987) Molecular cloning and characterization of rat estrogen receptor cDNA. Nucleic Acids Res 15: 2499–2513

Kost TA, Theodorakis N, Hughes SH (1983) The nucleotide sequence of the chick cytoplasmic β-actin gene. Nucleic Acids Res 11: 8287–8301

Krust A, Green S, Argos P, Kumar V, Walter P, Bornert JM, Chambon

P (1986) The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. EMBO J 5: 891–897

Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JÅ (1996) Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93: 5925–5930

Kuiper GGJM, Carlsson BO, Grandien KAJ, Enmark E, Häggblad J, Nilsson S, Gustafsson JÅ (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor α and β. Endocrinology 138: 863–870

Leube R, Gallwitz D (1986) Structure of a processed gene of human cytoplasmic γ-actin. Nucleic Acids Res 14: 6339

Liu Z, Zhu Z, Roberg K, Faras AJ, Guise KS, Kapuscinski AR, Hackett PB (1989) The β-actin gene of carp (*Ctenopharyngodon idella*). Nucleic Acids Res 17: 5850

Liu Z, Zhu Z, Roberg K, Faras A, Guise K, Kapuscinski AR, Hackett PB (1990) Isolation and characterization of β-actin gene of carp (*Cyprinus carpio*). DNA Sequence 1: 125–136

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evance RM (1995) The nuclear receptor superfamily: the second decade. Cell 83: 835–839

Maxwell BL, McDonnell DP, Conneely OM, Schulz TZ, Greene GL, O'Malley BW (1987) Structural organization and regulation of the chicken estrogen receptor. Mol Endocrinol 1: 25–35

Moos M, Gallwitz D (1983) Structure of two human β-actin-related processed genes, one of which is located next to a simple repetitive sequence. EMBO J 2: 757–761

Mosselman S, Polman J, Dijkema R (1996) Identification and characterization of a novel human estrogen receptor. FEBS Lett 392: 49–53

Nagae M, Adachi S, Yamauchi K (1997) Changes in transcription of pituitary glycoprotein hormone α and gonadotropin IIβ subunits during ovarian development induced by repeated injections of salmon pituitary homogenate in the Japanese eel, *Anguilla japonica*. Fish Physiol Biochem 17: 179–186

Ng SY, Gunning P, Eddy R, Ponte P, Leavitt J, Shows T, Kedes L (1985) Evolution of the functional human β-actin gene and its multi-pseudogene family: Conservation of noncoding regions and chromosomal dispersion of pseudogenes. Mol Cell Biol 5: 2720–2732

Ninomiya Y, Mochii M, Eguchi G, Hasegawa T, Masushige S, Kato S (1992) Tissue-specific response of estrogen receptor gene expression to estrogen in chick. Biochem Biophys Res Commun 187: 1374–1380

Nudel UR, Zakut R, Shani M, Neuman S, Levy Z, Yaffe D (1983) The nucleotide sequence of the rat cytoplasmic β-actin gene. Nucl Acids Res 11: 1759–1771

Pakdel F, Le Gac F, Le Goff P, Valotaire Y (1990) Full-length sequence and in vitro expression of rainbow trout estrogen receptor cDNA. Mol Cell Endocrinol 71: 195–204

Peter B, Man Y, Begg C, Gall I, Leader D (1988) Mouse cytoskeletal γ-actin: Analysis and implications of the structure of cloned cDNA and processed pseudogenes. J Mol Biol 203: 665–676

Ponte P, Gunning P, Blau H, Kedes L (1983) Human actin genes are single copy for α-skeletal and α-cardiac actin but multicopy for β- and γ-cytoskeletal genes: 3' untranslated regions are isotype specific but are conserved in evolution. Mol Cell Biol 3: 1783–1791

Ponte P, Ng SY, Engel J, Gunning P, Kedes L (1984) Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human β-actin cDNA. Nucleic Acids Res 12: 1687–1696

Rappolee D, Mark D, Banda MJ, Werb Z (1988) Wound macrophages express TGF-α and other growth factors *in vivo*: analysis by mRNA phenotyping. Science 241: 708–712

Salbert G, Atteke C, Bonnec G, Jego P (1993) Differential regulation of the estrogen receptor mRNA by estradiol in the trout hypothalamus and pituitary. Mol Cell Endocrinol 96: 177–182

Santos CRA, Power DM, Kille P, Llewellyn L, Ramsurn V, Wigham T, Sweeney GE (1997) Cloning and sequencing of a full-length sea bream (*Sparus aurata*) β-actin cDNA. Comp Biochem Physiol 117B: 185–189

Soriano P, Szabo P, Bernadi G (1982) The scattered distribution of actin genes in the mouse and human genomes. EMBO J 1: 579–583

Shupnik MA, Gordon MS, Chin WW, (1989) Tissue-specific regulation of rat estrogen receptor mRNAs. Mol Endocrinol 3: 660–665

Tachiki H, Nakagawa T (1993) Induction of spawning in female cultured eel *Anguilla japonica*. Bull Aichi Fish Res Int 1: 79–80

Todo T, Adachi S, Yamauchi K (1996) Molecular cloning and characterization of Japanese eel estrogen receptor cDNA. Mol Cell Endocrinol 119: 37–45

Tokunaga K, Yoda K, Sakiyama S (1985) Structure of the processed gene of mouse cytoplasmic γ-actin transposed into a BAMS sequence: Inscrtion has created 13 base-pair direcr repeats. Nucleic Acids Res 13: 3031–3042

Tokunaga K, Takeda K, Kamiyama K, Kageyama H, Takenaga K, Sakiyama S (1988) Isolation of cDNA clones for mouse cytoskeletal gamma-actin and differential expression of cytoskeletal actin mRNAs in mouse cells. Mol Cell Biol 8: 3929–3933

Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguère V (1997) Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 11: 353–365

Wallace RA (1985) A Comprehensive Synthesis. In "Developmental Biology Vol 1" Ed by LW Browder, Plenum Press, New York, pp 127–177

Weiler IJ, Lew D, Shapiro DJ (1987) The *Xenopus laevis* estrogen receptor: sequence homology with human and avian receptors and identification of multiple estrogen receptor messenger ribonucleic acids. Mol Endocrinol 1: 355–362

White R, Lees JA, Needham M, Ham J, Parker M (1987) Structural organization and expression of mouse estrogen receptor. Mol Endocrinol 1: 735–744

Yamamoto K, Yamauchi K (1974) Sexual maturation of Japanese eel and production of eel larvae in the aquarium. Nature 251: 220–222

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