Quantification of rainbow trout (Oncorhynchus mykiss) estrogen receptor-α messenger RNA and its expression in the ovary during the reproductive cycle

J J Nagler, M Krisfalusi and D G Cyr

Center for Reproductive Biology, Department of Biological Sciences, University of Idaho, Moscow, Idaho 83844–3051, USA
1Human Health Research Center, INRS-Institut Armand Frappier, University of Québec, Pointe-Claire, Québec, Canada G5H 3Z4

(Requests for offprints should be addressed to J J Nagler, Center for Reproductive Biology, Department of Biological Sciences, University of Idaho, Moscow, Idaho 83844–3051, USA)

ABSTRACT

This study developed a quantitative reverse transcription-polymerase chain reaction (RT-PCR) method to measure estrogen receptor-α (ERα) mRNA in the rainbow trout (Oncorhynchus mykiss). Using RT-PCR, and primers based on the known ERα DNA sequence in this species, cDNA sequences representing most of the protein coding region were obtained from ovary poly A(+) RNA. Using these DNA sequences as probes in Northern blot hybridizations confirmed that a single transcript of 4·2 kilobases in poly A(+) RNA could be detected in liver and ovary RNA. For the quantitative RT-PCR assay an internal standard RNA molecule was produced to control for inherent inter-tube differences in amplification efficiency and permit accurate quantification of ERα mRNAs. The quantitative RT-PCR assay proved to be highly specific for ERα mRNA with a detection limit of 6·9 fg, which corresponds to 273 fg ERα mRNA/µg total RNA. The quantitative RT-PCR assay was used to measure the levels of ERα mRNA in ovaries of rainbow trout at different stages of reproductive development. Ovarian ERα mRNA expression was found during two distinct periods of reproductive development, in pre-vitellogenic ovaries of fish with ovarian follicle diameters (OFDs) ≤100 µm and in mid-vitellogenic ovaries with OFDs >1000 µm. ERα mRNA could not be detected in the ovaries of fish with OFDs >100 µm but ≤1000 µm. The highest levels of ERα mRNA were found in late vitellogenic ovaries of fish with OFDs >2000 µm.

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INTRODUCTION

In vertebrates studied to date, the genomic action of the sex steroid estradiol (E₂) is facilitated through specific proteins, called estrogen receptors (ERs) (Auchus & Fuqua 1994). Estrogen receptors are nuclear proteins which, when complexed with E₂, act as transcriptional regulators of estrogen responsive genes (Katzenellenbogen 1996). They are members of a large superfamily of steroid hormone receptors that share a number of structural similarities. Steroid receptors have regions or ‘domains’, denoted A to F, that have been shown to have identical functions (Tsai & O’Malley 1994). The A-B domain is responsible for transcriptional activation, the C-domain DNA binding, and the E-domain ligand binding. The C- and E-domains of ERs are highly conserved across animal species (Tan et al. 1996).

Estrogen receptors are found in many vertebrate tissues including the ovary, which is the principal tissue responsible for the biosynthesis of E₂. In fishes, the specific binding of radiolabeled E₂ to ovarian nuclear extracts in the spotted seatrout (Cynoscion nebulosus) provided the first conclusive evidence that fish ovaries contain ER proteins (Smith & Thomas 1990). Subsequently, similar to mammals (Saunders 1998), the cDNAs for two different ER forms, denoted ERα and ERβ have been found in fish ovaries (Chang et al. 1999, Tchoudakova et al. 1999, Xia et al. 1999, 2000). The rainbow trout (Oncorhynchus mykiss) is unusual in that only ERα forms are apparent in the ovary (Pakdel et al. 1999, Nagler & Krisfalusi 2000).
In female fishes, E2 regulates a number of well-documented physiological processes outside the ovary that relate to reproduction. Indeed, one of the long established actions of E2 on the liver of the ovary that relates to reproduction. Indeed, one of the well-documented physiological processes outside E2 stimulates the release of growth factors and undergoes theca and granulosa cell proliferation and also possible. The previtellogenic ovarian follicle development before the period of yolk deposition is also possible. The previtellogenic ovarian follicle undergoes theca and granulosa cell proliferation and oocyte enlargement. Studies in mammals show that E2 stimulates the release of growth factors and upregulates follicle-stimulating hormone receptors within the ovary that promote development (Richards 1994). The synthesis and release of growth factors within the ovary is necessary for the growth and development of the granulosa and thecal cells that surround the oocyte (Dorrington et al. 1993). It is possible that E2 could interact with ERs in the ovaries of fish to stimulate the release of growth factors necessary for proper ovarian development. The activity of E2 in regard to these processes has not been explored in fish because of their small size and low yield of total RNA. Total RNA was washed twice with 70% ethanol, dried, solubilized in diethylpyrocarbonate (DEPC)-treated water, and stored at −20 °C. Total RNA was quantified by absorbance at OD 260.

Poly A(+) RNA was isolated with the Oligotex kit (Qiagen, Santa Clarita, CA, USA) according to the suppliers instructions. The yield of poly A(+) RNA from ovaries with OFDs >1000 µm was consistently low with the Oligotex kit, presumably due to contaminating yolk proteins in the total RNA preparations. It was found that pretreating the total RNA samples with protease K (GibcoBRL, Grand Island, NY, USA) before poly A(+) RNA isolation dramatically improved the yield. Therefore, total RNA (500 ìg) from ovaries with OFDs >1000 µm was precipitated with 3 M sodium acetate to a final concentration of 0·3 M and 2 volumes of 100% ethanol, followed by storage at −20 °C overnight. The following day the samples were centrifuged at 15 000 × g (4 °C) for 15 min, the pellet was washed twice with 70% ethanol, and dried. The RNA pellet was resuspended in 200–400 ìl proteinase K digestion buffer (50 mg of proteinase K/ml) (Sambrook et al. 1989). This solution was incubated for 1·5 h at 37 °C, and the RNA was extracted with an equal volume of 1:1 phenol:chloroform (pH 7·5). The supernatant containing the RNA was then used directly with the Oligotex kit procedure. The amount of poly A(+) RNA was quantified by absorbance at OD 260.

**Materials and Methods**

**Tissue collection**

Female rainbow trout of the Mount Lassen strain, at different stages of reproductive development (0·5–3 years old), were obtained from local aquaculture operations. Ovaries and livers from freshly killed fish were placed in sterile tubes, immediately frozen in liquid nitrogen, and stored at −80 °C. The stage of reproductive development of individual females was based on the mean maximum ovarian follicle diameter (OFD; µm) calculated from 10 of the largest follicles randomly selected from a sample of each ovary. The OFDs were determined by microscopy using eyepiece graticules. Rainbow trout ovaries were placed in the following size classes based on their OFDs: ≤100, ≤250, ≤500 µm (previtellogenic), ≤1000, ≤1500, ≤2000 µm (vitellogenic), and >2000 µm (late vitellogenic).

**Total and poly A(+) RNA isolation**

Total RNA was isolated from 0·2–2 g pieces of individual ovary or liver samples using an acidic phenol-chloroform extraction procedure (Chomczynski & Sacchi 1987) and precipitation with 100% isopropanol. The ovaries of females with OFDs ≤100 µm were pooled from 5 fish because of their small size and low yield of total RNA. Total RNA was washed twice with 70% ethanol, dried, solubilized in diethylpyrocarbonate (DEPC)-treated water, and stored at −20 °C. Total RNA was quantified by absorbance at OD 260.

Poly A(+) RNA was isolated with the oligotex kit (Qiagen, Santa Clarita, CA, USA) according to the suppliers instructions. The yield of poly A(+) RNA from ovaries with OFDs >1000 µm was consistently low with the oligotex kit, presumably due to contaminating yolk proteins in the total RNA preparations. It was found that pretreating the total RNA samples with protease K (GibcoBRL, Grand Island, NY, USA) before poly A(+) RNA isolation dramatically improved the yield. Therefore, total RNA (500 ìg) from ovaries with OFDs >1000 µm was precipitated with 3 M sodium acetate to a final concentration of 0·3 M and 2 volumes of 100% ethanol, followed by storage at −20 °C overnight. The following day the samples were centrifuged at 15 000 × g (4 °C) for 15 min, the pellet was washed twice with 70% ethanol, and dried. The RNA pellet was resuspended in 200–400 ìl proteinase K digestion buffer (50 mg of proteinase K/ml) (Sambrook et al. 1989). This solution was incubated for 1·5 h at 37 °C, and the RNA was extracted with an equal volume of 1:1 phenol:chloroform (pH 7·5). The supernatant containing the RNA was then used directly with the oligotex kit procedure. The amount of poly A(+) RNA was quantified by absorbance at OD 260.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Reverse transcription reactions were performed using the GibcoBRL Superscript II kit. Poly A(+) RNA (1 µg) or total RNA (2·5 µg) was combined with 50 pmol ER4 primer (5’-TCTGAGCTGGGGAGCCATA-3’) or ER3a primer (5’-CTCGCCACGTGTACTAGCG-3’) and DEPC-treated
water to a final volume of 12 µl. The sequences of the ER4 and ER3a primers were derived from bases 1908–1927 and 1173–1192 respectively of the rainbow trout liver ER sequence (Pakdel et al. 1990). The tubes were heated at 70 °C for 10 min and cooled to room temperature for 15 min before the addition of the appropriate volumes of the remaining reagents necessary for RT according to the Superscript II kit protocol (final volume= 20 µl). The tubes were placed in a thermal cycler (Perkin Elmer 2400, Perkin Elmer, Foster City, CA, USA) and heated at 42 °C for 2 min followed by the addition of 200 units (1 µl) reverse transcriptase, then heated at 42 °C for 50 min, 95 °C for 5 min, and 4 °C for 5 min. A portion (2 µl) of each RT reaction was added to the PCR reagents according to the GibcoBRL protocol, along with 50 pmol ER3 primer (5′-GAGCTGTCGACATGATCGC-3′) in the case of RT reactions with ER4, or 50 pmol ER7 primer (5′-ATGTACCCTGAGGAGACACG-3′) in the case of RT reactions with ER3a, to a final volume of 50 µl. The sequences of the ER3 and ER7 primers were derived from bases 1173–1192 and bases 222–241 respectively of the rainbow trout liver ER sequence (Pakdel et al. 1990). A manual hotstart was performed by heating the tubes to 95 °C for 1 min, then cooling the tubes to 85 °C before adding 5 U (1 µl) of Taq DNA polymerase (GibcoBRL). This was followed by 30 cycles of 94 °C for 15 s, 68 °C for 30 s, 72 °C for 40 s, and upon completion final cooling to 5 °C. Typically, 5 µl of the reaction mixture was loaded on a 1% agarose gel containing ethidium bromide and electrophoresed in 1 × Tris EDTA buffer (Ausubel et al. 1998). DNA size markers (1 kb; GibcoBRL) were run in a well adjacent to experimental samples.

Cloning and sequencing

RT-PCR products were ligated into the pCR II plasmid vector and used to transform INV αF′ cells supplied in the Original TA Cloning kit (Invitrogen, Carlsbad, CA, USA). Plasmid DNA minipreps were made from single bacterial colonies grown overnight on LB-agar plates containing kanamycin with the QIAprep kit (QIAGEN). Plasmid DNA from clones containing the appropriate sized insert, as determined by EcoRI (GibcoBRL) restriction digest, were selected for sequencing. DNA sequencing, in both directions, was carried out using an automated system (ABI PRISM, Perkin-Elmer or IR2 System, LI-COR, Lincoln, NB, USA). Clones from two separate RT-PCRs were analyzed to construct the final cDNA sequence from which the amino acid sequence was deduced.

Northern hybridization

Northern blot analyses were conducted as described in Nagler and Cyr (1997), using poly A(+) RNA isolated from rainbow trout liver and ovary as described previously. The RT-PCR product generated with ER3 and ER4 primers was used as a cDNA probe for Northern blot analyses. The ER cDNA probe was random prime labeled (Oligolabeling kit, Amersham Pharmacia Biotech, Piscataway, NJ, USA) with [α-32P]dCTP and hybridized with the membranes containing the poly A(+) RNA. Following the hybridization, the membrane was washed in SSC buffer (Nagler & Cyr 1997) and exposed to a Molecular Imaging Screen-BI (Bio-Rad, Hercules, CA, USA) for 24 h. Results of the exposure were collected with a GS-525 Molecular Imaging System (Bio-Rad) and analyzed with Molecular Analyst software (Bio-Rad).

Quantitative RT-PCR

Specific primers were selected from the rainbow trout liver ER cDNA sequence (Pakdel et al. 1990) using OLIKO software (version 5-0, NBI, Plymouth, MN, USA) and synthesized by Gibco-BRL. The forward primer (5′-AGCTAGCCCTTCCCTCACC-3′) was selected from the A-B domain (bases 442–461) of the rainbow trout liver ER sequence (Pakdel et al. 1990), while the reverse primer (5′-GGGCGCAATACCGGTTATTC-3′) was selected from the D-domain (bases 904-925). These primers generate a PCR product of 484 base pairs (bp) in length. The primers span a large intron in the ER gene (Valotaire et al. 1993) ensuring amplification from RNA rather than any contaminating genomic DNA that might be present. To confirm the primers described above were amplifying the expected fragment of the ovary ERα cDNA, the 484 bp RT-PCR product was cloned into the pCR II plasmid vector and sequenced as described above. The 484 bp sequence contains a single SacII restriction site located near the 3′-end of the fragment. A double-stranded exogenous DNA fragment was synthesized to insert into the SacII site of the 484 bp native ERα sequence to create a larger DNA template with the same priming sites. Two 54-mer primers were designed complementary to each other (5′-TTCCCCGCGCCACCACAAAACTGACTGTGAGAGAICCTGGCAAGAACCGAGATCTCCAG-3′ and 5′-TTCCCCGGCGCGCGGCGGCTGCTAAGTCTCCAGGTGTGAGAGACTGGCA-3′) such that they produced a 75 bp product following PCR amplification; this DNA fragment had a G:C content similar to the native ERα cDNA. Both
primers contained SacII restriction enzymes sites (underlined). The pCR II plasmid, containing the 484 bp ERα DNA, was digested with SacII (New England BioLabs, Beverly, MA, USA) and treated with calf intestinal alkaline phosphatase (Gibco-BRL) to prevent re-ligation. Following linearization, the digested vector was gel-isolated to remove enzymes and the 75 bp DNA insert was ligated into the SacII site with T4 ligase (Invitrogen). This plasmid was re-cloned and sequenced to confirm that a 560 bp DNA fragment was present between the specific priming sites. This larger DNA, containing an exogenous fragment, was used as a template to synthesize RNA by in vitro transcription to produce an internal standard for quantitative RT-PCR, similar to the method of Kagami et al. (1996) for mammalian ERs. Briefly, plasmid DNA was purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1, pH 7-5) and linearized with SpeI (GibcoBRL). The plasmid DNA was purified with the QIAquick PCR Purification kit (QIAGEN) and transcribed in vitro with T7 polymerase according to the instructions supplied with the Ribomax kit (Promega, Madison, WI, USA). Following transcription, the DNA template was digested with RQ1 RNase-free DNase (Promega) and removed with a phenol:chloroform:isoamyl alcohol (25:24:1, pH 7-5) extraction. Three separate aliquots of the internal standard-RNA (IS-RNA) produced were quantified by absorbance at OD260. The IS-RNA stock solution was aliquoted in 50 µl volumes and stored at −80 °C.

Prior to RT, tubes containing ovarian total RNA (2.5 µg), IS-RNA (1–10 pg) and 2 pmol 3' -primer were heated to 70 °C for 10 min and cooled to room temperature. The remaining RT reagents, 0.5 mM of each dNTP, 10 mM dithiothreitol, 200 U Superscript II reverse transcriptase (GibcoBRL), and RT buffer were added to a final volume of 20 µl. RT was conducted for 52 min at 42 °C, followed by heating to 95 °C for 5 min. Samples were diluted 1:4 or 1:8 in sterile water and held at 4 °C until PCR. Polymerase chain reaction was carried out in 25 µl reaction volumes by adding the following components: PCR buffer (GibcoBRL), 2 mM MgSO4, 50 mM of each dNTP, 2 pmol 5’-primer, 1.25 U Platinum Taq DNA Polymerase High Fidelity (GibcoBRL). In addition, a minimum of 5 × 10^5 c.p.m. radiolabeled 5'-primer end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (GibcoBRL) using the forward labeling reaction (Ausubel et al. 1998) were added to each PCR for detection purposes. Serially diluted cDNA template, corresponding to 62.5, 125, 187.5, 250, 312.5, 375 ng total RNA was added to each PCR, and the following thermal profile used: 2 cycles of 94 °C for 25 s, 68 °C for 30 s, and 72 °C for 40 s, followed by 21–24 cycles of 94 °C for 15 s, 66 °C for 15 s, and 72 °C for 40 s. To ensure that amplification fell within the exponential phase of PCR, 5 µl aliquots were withdrawn from test RT-PCRs after the completion of every cycle from 18–30. Negative control RT-PCRs containing no template were included in each assay. In addition, the structural integrity of the mRNA in all ovarian RNA samples was tested by amplifying the gene for a constitutively expressed enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), by RT-PCR. The GAPDH forward (5’-GGGTGAGGTGAGCATGGAGGACG-3’) and reverse (5’-GCCCAGCAGGCCGAGGT-3’) primers used in the RT-PCR were derived from bases 212–234 and bases 771–798 respectively of the rainbow trout GAPDH DNA sequence (GenBank Accession #AF027130; Abnet et al. 1999). The RT-PCR conditions were as described earlier, with the exception that the annealing temperature used in the PCR was 64 °C.

For quantification, 5 µl aliquots of the PCRs were resolved on 2% agarose gels, the gels dried, and exposed to a Molecular Imaging Screen-BI (Bio-Rad) for 12–18 h. Results of the exposure were collected with a GS-525 Molecular Imaging System (Bio-Rad) and analyzed with Molecular Analyst software (Bio-Rad). The signal intensity of the two bands (ER: 485 bp and IS-RNA: 560 bp) in arbitrary pixel density units (directly proportional to d.p.m.) were plotted against the logarithms of the starting amounts of total RNA and IS-RNA. Regression equations were calculated for the ERα mRNA and the IS-RNA and, provided the slopes of the lines were parallel (i.e. not significantly different at P<0.05), the amount of ERα mRNA was determined by interpolation (Wang & Mark 1990).

**Statistical analysis**

Regression analysis was performed on Cricket Graph III software (Macintosh). Data are presented as the mean ± the standard error of the mean (s.e.m.). An ANOVA (P<0.05) was used to determine if significant differences existed. Tukey’s test was used to identify significant differences (P<0.05) between groups. All statistical analyses used SigmaStat software (Jandel Scientific Software, San Rafael, CA, USA).

**RESULTS**

Single DNA products were obtained in two separate RT-PCRs using ovarian poly A(+) RNA with primer combinations ER3-ER4 and ER7-ER3a. The sizes of the DNA fragments obtained were
754 bp using ER3-ER4, and 970 bp using ER7-ER3a. Subsequent DNA sequencing of the RT-PCR products showed them to be identical. When placed end-to-end from ER7 to ER4, the nucleotide sequence covers 1705 out of 1724 bases from the protein coding region beginning at the ATG start codon (data not shown). A comparison of the deduced amino acid sequences from the ovary ERα (this study) and rainbow trout ERα long and short isoforms (Genbank Accession #s AJ242740 and AJ242741; Pakdel et al. 1999) show 100% similarity.

Northern hybridization analyses showed the presence of a single mRNA in the liver and ovary poly A(+) RNA that hybridized to the radiolabeled ovarian cDNA probe (Fig. 1). It appears that the amount of ERα mRNA in the liver is greater than in the ovary. The mRNAs in each case were about 4.2 kb in size as estimated from the adjacent RNA standards run on the gel.

A quantitative RT-PCR assay specific for the rainbow trout ERα mRNA was developed. Only two DNA bands, derived from ovarian ERα mRNA (484 bp) and the IS-RNA (560 bp), were evident on the agarose gels following RT-PCR (Fig. 2A). Serial dilutions of the RT reactions in the PCR step consistently gave lines with parallel slopes from which the unknown amount of ERα mRNA in each sample of ovarian total RNA was determined (Fig. 2B). Interassay variability, as assessed by the amount of ERα mRNA measured in ovarian total RNA samples from 3 independent RT-PCRs was always <20%. The lower limit of detection was 6.9 fg ERα mRNA, which corresponds to 273 fg ERα mRNA/µg total RNA.

Ovarian ERα mRNA expression, as measured by quantitative RT-PCR, was found during two distinct periods of reproductive development, in pre-vitellogenic ovaries of fish with OFDs ≤100 µm and in vitellogenic ovaries with OFDs >1000 µm (Fig. 3). The highest levels of ERα mRNA were found in late vitellogenic ovaries of fish with OFDs >2000 µm; the mean level of 1.76 ± 0.03 pg ERα mRNA/µg ovarian total RNA was significantly higher (P<0.05) than all earlier sampling times. Estrogen receptor-α mRNA was below the limit of detection in ovaries of fish with OFDs >100 µm but ≤1000 µm. In an effort to detect ERα mRNA in ovaries with OFDs >100 µm but ≤1000 µm, poly A(+) RNA was isolated and used in the quantitative RT-PCR assay, and the number of cycles in the

![Figure 1](image1.png)

**Figure 1.** Northern blot analysis of 2 µg poly A(+) RNA from rainbow trout ovary (o) and liver (l) hybridized with a radiolabeled ovarian DNA probe. The fish used had ovaries with a mean ovarian follicle diameter >2000 µm. The mobility and size in kilobases (kb) of the RNA standards are indicated on the left.

![Figure 2](image2.png)

**Figure 2.** Analysis of a typical quantitative RT-PCR assay, showing (A) the radioactive image after electrophoresis on a 2% agarose gel of serially diluted aliquots of the IS-RNA and ERα mRNA from the PCR, and (B) the linear regression of the ERα mRNA (▲) and IS-RNA (●) data.
and 4) were used.

This study confirms that ERα mRNA transcripts are present in the rainbow trout ovary. Using primers based on the rainbow trout liver ER cDNA sequence (Pakdel et al. 1990), a large portion of a similar cDNA was amplified using RT-PCR from rainbow trout ovarian poly A(+) RNA. The ovarian nucleotide sequence obtained covers 99% of the protein coding region (Valotaire et al. 1993), and a comparison of the deduced amino acid sequences from the rainbow trout ovary (Pakdel et al. 1999) and liver ERs (Pakdel et al. 1990) show them to be identical. In support of the RT-PCR analysis, Northern blot hybridization confirmed that a similar sized mRNA (i.e. 4-2 kb) exists in both liver and ovary that binds to the ovary ERα-derived DNA probe. The size of the liver mRNA (4-2 kb) is larger than that reported by Pakdel et al. (1990); this may be accounted for by differences in RNA standards used in each study.

A highly reproducible quantitative RT-PCR method was developed to measure the level of ERα mRNA in total RNA samples from rainbow trout ovaries. The assay utilizes an IS-RNA that is included in the same tube with the ovarian total RNA beginning with the RT step. This strategy is similar to those employed in quantitative RT-PCR assays with steroid hormone receptors in mammalian species (Young et al. 1994, Kagami et al. 1996), and is an improvement on the semi-quantitative RT-PCR assay for rainbow trout ER mRNA reported by Guigen et al. (1999). The IS-RNA controls for differences in enzymatic efficiencies during the RT and PCR steps of the assay, and is crucial for quantification of unknown ERα mRNA. Any fluctuations in amplification efficiency that occur will be reflected in DNA products produced from both the IS-RNA and the target ERα mRNA.

The rainbow trout ovary contains multiple ERα forms; no ERβ has yet been discovered, unlike the catfish (Xia et al. 1999, 2000), goldfish (Carassius auratus) (Tchoundakova et al. 1999) or tilapia (Oreochromis niloticus) (Chang et al. 1999). Pakdel et al. (1999) described two different ERα isoforms, long and short, that differ by the addition of 45 amino acids at the N-terminal end of the long isoform. The remainder of the DNA sequence is identical in both long and short isoforms. The quantitative RT-PCR assay in this study will detect both of these ERα isoforms because the primers used in this assay are designed based on a region that is complementary to both isoforms. In addition, Nagler and Krisfalusi (2000) have noted that an ER mRNA, that is distinct from those reported by Pakdel et al. (1999), is also present in the rainbow trout ovary. This novel ER has a DNA sequence which is markedly different in the regions (A-B and D domains) where the primers anneal in this quantitative RT-PCR assay and is therefore not detected. DNA sequencing of products amplified by the quantitative RT-PCR assay show this method to

**FIGURE 3.** The amount of ERα mRNA in the ovaries of rainbow trout measured by quantitative RT-PCR at different points during the reproductive cycle. Data are presented as means ± s.e.m.; n=3–5 fish/group. nd= non-detectable. *P<0.05 compared with other groups.

**FIGURE 4.** Presence or absence of rainbow trout ERα (754 base pairs; lanes 1 and 3) and GAPDH (586 base pairs; lanes 2 and 4) DNA products amplified using RT-PCR. In the RT-PCRs 2-5 µg rainbow trout ovary total RNA from a representative fish with ovarian follicles >2000 µm (lanes 1 and 2) or <500 µm (lanes 3 and 4) were used.

PCR was increased to 35. These modifications failed to produce any detectable ERα mRNA. To establish that the mRNA was intact in the ovarian RNA samples used in these analyses, a 586 bp segment of GAPDH mRNA was amplified by RT-PCR (Fig. 4). In all RNA samples GADPH was amplified by RT-PCR, confirming the structural integrity of the mRNA in all RNA samples.

**DISCUSSION**

This study confirms that ERα mRNA transcripts are present in the rainbow trout ovary. Using primers based on the rainbow trout liver ER cDNA sequence (Pakdel et al. 1990), a large portion of a similar cDNA was amplified using RT-PCR from rainbow trout ovarian poly A(+) RNA. The ovarian nucleotide sequence obtained covers 99% of the protein coding region (Valotaire et al. 1993), and a comparison of the deduced amino acid sequences from the rainbow trout ovary (Pakdel et al. 1999) and liver ERs (Pakdel et al. 1990) show them to be identical. In support of the RT-PCR analysis, Northern blot hybridization confirmed that a similar sized mRNA (i.e. 4-2 kb) exists in both liver and ovary that binds to the ovary ERα-derived DNA probe. The size of the liver mRNA (4-2 kb) is larger than that reported by Pakdel et al. (1990); this may be accounted for by differences in RNA standards used in each study.

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be specific for the ERα isoforms reported by Pakdel et al. (1999).

The need for a quantitative RT-PCR assay arose because of the low abundance of ERα mRNA in the rainbow trout ovary. This became apparent in our initial studies using Northern blot hybridizations which showed much higher levels of ERα mRNA in liver, for example, compared with ovary. ERα mRNA signals in rainbow trout ovary could only be reproducibly obtained by Northern analysis using a minimum of 2 µg poly A(+) RNA. The necessity of isolating poly A(+) RNA from large numbers of ovarian samples would be prohibitive, and in the case of juvenile fish with small ovaries, impossible because of the small amount of total RNA typically obtained. The quantitative RT-PCR approach with the inherent amplification of DNA in the PCR step overcomes the limitations of Northern blotting and permits measurement of ERα mRNA in the femtogram range. As indicated above, the amount of ERα mRNA in the ovary is less than in the other tissue, the liver, for which we have quantitative data using RT-PCR in rainbow trout. It is not known how ERα mRNA levels in a given fish tissue relate to the amount of ERα protein present. The levels of ERα mRNA and ERα protein in these and other tissues of rainbow trout are needed in future studies before conclusions can be made.

An important finding of this study was that two distinct periods of ERα expression are apparent in the rainbow trout ovary during reproductive development. Estrogen receptor-α mRNA was first detected in the ovaries of fish with OFDs ≤ 100 µm. Once fish had developed to the point that OFDs were ≤ 250 µm, ERα mRNA could not be detected. This indicates that ERα mRNA expression is present in very immature ovaries, but that transcription of ERα mRNA is rapidly downregulated as ovarian development progresses. The mechanism by which ERα mRNA transcription in the immature rainbow trout ovary is downregulated is unknown. It appears that ERα mRNA levels decrease to exceedingly low levels once OFDs attain 250 µm through to 1000 µm. Our efforts to detect ERα transcripts in these ovary samples by using poly A(+) RNA in the quantitative RT-PCR (i.e. instead of total RNA) and including more cycles during PCR amplification failed to show any signal. The mRNAs are not degraded in these RNA samples because GAPDH mRNA could be successfully amplified in all samples. However, at some point during the cycle, in vitellogenic ovaries with OFDs between 1000 and 1500 µm, the ER mRNA is again detectable and the amount of transcript increases to peak levels in late vitellogenic ovaries. The increase in ERα mRNA at this point of the reproductive cycle may be controlled by the ER ligand E2. The plasma concentration of E2 steadily increases during the reproductive cycle of rainbow trout to peak shortly before spawning (Scott et al. 1980), similar to the increase in ovarian ERα mRNA levels. Estrogen receptor transcription in the liver of rainbow trout is upregulated in a dose–responsive fashion by E2 (Pakdel et al. 1989, MacKay et al. 1996), and E2 has post-transcriptional effects that result in increased stability of the liver ER mRNA (Flouriot et al. 1996). Therefore, E2 is a likely candidate for the upregulation of ERα mRNA in vitellogenic ovaries.

The presence of ovarian ERα mRNA in rainbow trout implies that a functional ERα protein will also be present. Although this has yet to be demonstrated, it raises the possibility that E2 could have important regulatory role(s) within the fish ovary. During fish early embryonic development, ERs are implicated in sex determination (Guigen et al. 1999) and treatment of male fish with E2 results in intersex (Krisfalusi & Nagler 2000) or phenotypic sex reversal (Johnstone et al. 1978, Yamazaki 1983). The presence of ERα mRNA in immature rainbow trout ovaries containing OFDs ≤ 100 µm in this study suggests that ERs may be important for normal sexual differentiation of the ovaries in genetic females. The function of ERs in the ovary, later during active vitellogenin uptake, could be involved with regulating VTG uptake. Interestingly, the pattern of ovarian ERα mRNA expression in vitellogenic rainbow trout is opposite to that of the vitellogenin receptor (VTGR) mRNA in the ovary (Perazzolo et al. 1999). It has been shown by in situ hybridization that VTGR mRNA was not present in ovaries containing OFDs from 35–200 µm, but that when the OFDs had increased to 250 µm all oocytes were expressing VTGR mRNA. The expression of VTGR mRNA is apparent until the OFDs are >1000 µm when it disappears. This period of ovarian development, based on OFDs, is precisely when ER mRNA is non-detectable in the rainbow trout ovary. When VTG mRNA is no longer detectable ER mRNA appears and steadily increases to peak levels during late vitellogenesis. Although estrogens may not directly influence VTG mRNA transcription in rainbow trout, they could regulate the cycling of VTGR in vitellogenic oocytes and synchronize ovarian development in the mid to late phase of the reproductive cycle.

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