Cloning and Expression of Genomic and Complementary DNAs Encoding an Estrogen Receptor in the Medaka Fish, Oryzias latipes

Authors: Kawahara, Tetsuya, Okada, Hisashi, and Yamashita, Ichiro

Cloning and Expression of Genomic and Complementary DNAs Encoding an Estrogen Receptor in the Medaka Fish, *Oryzias latipes*

Tetsuya Kawahara, Hisashi Okada, and Ichiro Yamashita*

Center for Gene Science, Hiroshima University, Kagamiyama 1-4-2, Higashi-hiroshima 739-8527, Japan

ABSTRACT—Genomic and complementary DNA fragments encoding an estrogen receptor (ER) homolog were cloned from the medaka fish, *Oryzias latipes*. The genomic DNA contains nine introns. The cDNA encodes a protein of 620 amino acids that is composed of the complete set of the conserved ER domains. Sequence comparison among fish and human ER genes suggests that the human gene may have lost two introns during the evolution. ER-specific mRNA was expressed in large amounts in adult tissues such as brain, ovary, testis, and female liver. As expected by the fact that embryos and fry are highly sensitive to and sex-reversed by estrogen, ER mRNA was detected in them but at a basal level. Oral administration of estrogen elevated the hepatic expression of ER mRNA, suggesting an autoregulatory loop for transcriptional activation. However, estrogen did not affect the expression in embryos and fry, suggesting that the positive loop is silent at the juvenile stage and that the basal expression is not due to the absence of estrogen. We also found that estrogen inhibits the embryonic development of blood vessels, providing a new, simple method of bioassay for estrogenic activity.

INTRODUCTION

Estrogen is a female sex steroid but regulates a wide variety of physiological processes such as the reproductive cycle (follicular development and spermatogenic process) (Lubahn *et al.*, 1993) and bone homeostasis (Turner *et al.*, 1994). The effects of this hormone are mediated classically by the estrogen receptor (ER) that acts as a ligand-dependent transcription factor (Beato *et al.*, 1995). Members of this family display a conserved domain organization that is composed of six domains (A to F) (Krust *et al.*, 1986).

Estrogen can also affect testicular developments and ultimately reverse it to ovary formation, if applied early enough during development of lower vertebrates such as bird (Scheib, 1983), reptile (Dorizzi *et al.*, 1991), amphibia (Hayes, 1998), and fish (Yamamoto, 1969). In chickens (Scheib, 1983) and turtles (Dorizzi *et al.*, 1991), estrogens are synthesized by morphologically undifferentiated female gonads, but at reduced levels in male gonads. However, it is controversial that estrogen is a natural inducer of ovary formation, because pharmacological studies using anti-estrogens (that bind to ER and compete with estrogens) do not provide convincing results. Treatment with anti-estrogens does not disrupt ovarian development but disturb it only slightly showing partial musculinization (some testicular appearance of female

gonads), whereas the treatment completely inhibits the male to female gonadal sex reversal caused by estrogens (Scheib, 1983; Dorizzi *et al.*, 1991). Furthermore, it remains to be seen whether exogenous estrogens can cause the sex reversal at the equivalent concentrations found in undifferentiated female embryos. Treatment with aromatase inhibitors (that block the synthesis of estrogen from androgen, a male sex steroid) causes females to develop testes (Elbrecht and Smith, 1992; Richard-Mercier *et al.*, 1995). This sex reversal should be interpreted as that the accumulated androgen elicited the male gonadal differentiation but not as widely believed that the absence of estrogen caused the alternative gonadal development, because of the well-identified positive role of androgen in the testicular development (Yamamoto, 1969).

In contrast, histological and ultrastructural studies in some fishes including medaka reveal that steroid hormone biosynthesis and steroid-producing cells appear after the completion of gonadal sex differentiation (Iwasaki, 1973; Takahashi and Iwasaki, 1973; Kagawa and Takano, 1979; Schreibman *et al.*, 1982; van den Hurk *et al.*, 1982; Kanamori *et al.*, 1985). Furthermore, pharmacological studies in medaka support the absence of sex steroids during the sex differentiation and provide sufficient evidence for an estrogen-independent mechanism for ovarian development (Kawahara and Yamashita, 1999). Numerous studies have been done using a lot of animals from fish to birds to clarify the possible role of estrogen and ER in the female sex determination (Clinton, 1998; Hayes, 1998; Jeyasuria and Place, 1998; Nakamura *et al.*, 1998;

* Corresponding author: Tel. +81-824-24-6271;
                                              FAX. +81-824-22-7184.
                                              E-mail: iyama@hiroshima-u.ac.jp

Patino, 1997; Pieau *et al.*, 1998). However, at present, there is no convincing evidence supporting it: the results obtained from these studies can not be explained solely by the action of estrogen-activated ER. Our working model is as follows: (1) in medaka fish, ER is expressed in both sexes but inactive in male because of the lack of estrogen, activated only in female by the estrogen-independent mechanism as proposed in other biological systems (Power *et al.*, 1991; Smith *et al.*, 1993; Kato *et al.*, 1995; Bunone *et al.*, 1996; Das *et al.*, 1997; Zwijsen *et al.*, 1998); however, we can not exclude the possibility that female sex determination is governed by an unknown factor that is not related to estrogenic actions but shares with ER the ability to activate downstream genes for female sex development; and (2) in chickens and turtles, there are at least two pathways for ovarian development; one involves the estrogen-activated ER and another is independent of estrogen. To elucidate the possible role of ER in the sex determination of medaka fish, we first cloned an ER homolog and, by using it as a probe, examined its expression in embryos, fry, and adult fish.

MATERIALS AND METHODS

Isolation of genomic and complementary DNAs encoding an ER homolog

Genomic and female liver cDNA libraries of medaka, constructed in λEMBLIII and λZAPII, respectively, were kindly provided by Yasumasu and Yamagami (Sophia University, Japan). We first screened for genomic DNAs from a total of 5×10^5 phages by hybridization with human ERα cDNA (Green *et al.*, 1986). The same number of phages were screened for cDNAs using as probes human ERα cDNA and the medaka genomic DNAs obtained (encoding the C or D domain). After extraction of phage DNAs from positive clones, genomic and complementary DNAs were subcloned into the plasmids pUC118 and pBluescript SK (–), respectively.

RNA analysis

We used the d-rR strain of medaka, in which the genotype of sex can be judged by body color after hatching with more than 99 % reliability (Yamamoto, 1969). Breeding and pharmacological studies were performed as described (Kawahara and Yamashita, 1999). The stages of embryonic development were identified according to the methods of Iwamatsu (1994). Total RNA was extracted using RNeasy Mini Kit (QIAGEN) from embryos, fry, and tissues of adult fish after chilling in liquid nitrogen and homogenization with pellet mixers. RNA blot analysis was done using as probes the 350-bp *Eco*RI-*Sal*I fragment (encoding parts of E and F domains) of plasmid pMER and the 650-bp *Xho*I-*Bam*HI fragment of plasmid pMACT1 for detection of ER and β- actin mRNAs, respectively. RT-PCR analysis was done using RNA PCR Kit (Takara) with the primers as follows for generation of the 320-bp cDNA encoding a part of E domain: 5'-GTAGGAGGTC-ATAAAGAGGG (used for reverse transcription) and 5'-CTTCCG-TGTGCTCAAACTCA. One μg of total RNA was used with 20 amplification cycles to keep the linearity between the intensity of an amplified band and a content of ER mRNA. The amplified band was visualized after electrophoresis by DNA blot analysis using the same probe as described. Whole mount *in situ* hybridization was performed as described (Inohaya *et al.*, 1997) with digoxigenin-labeled anti-sense and sense RNA probes generated from the ER cDNA lacking the 468-bp *Nar*I fragment that encompasses the C and D domains and from the 650-bp fragment of β-actin cDNA as described.
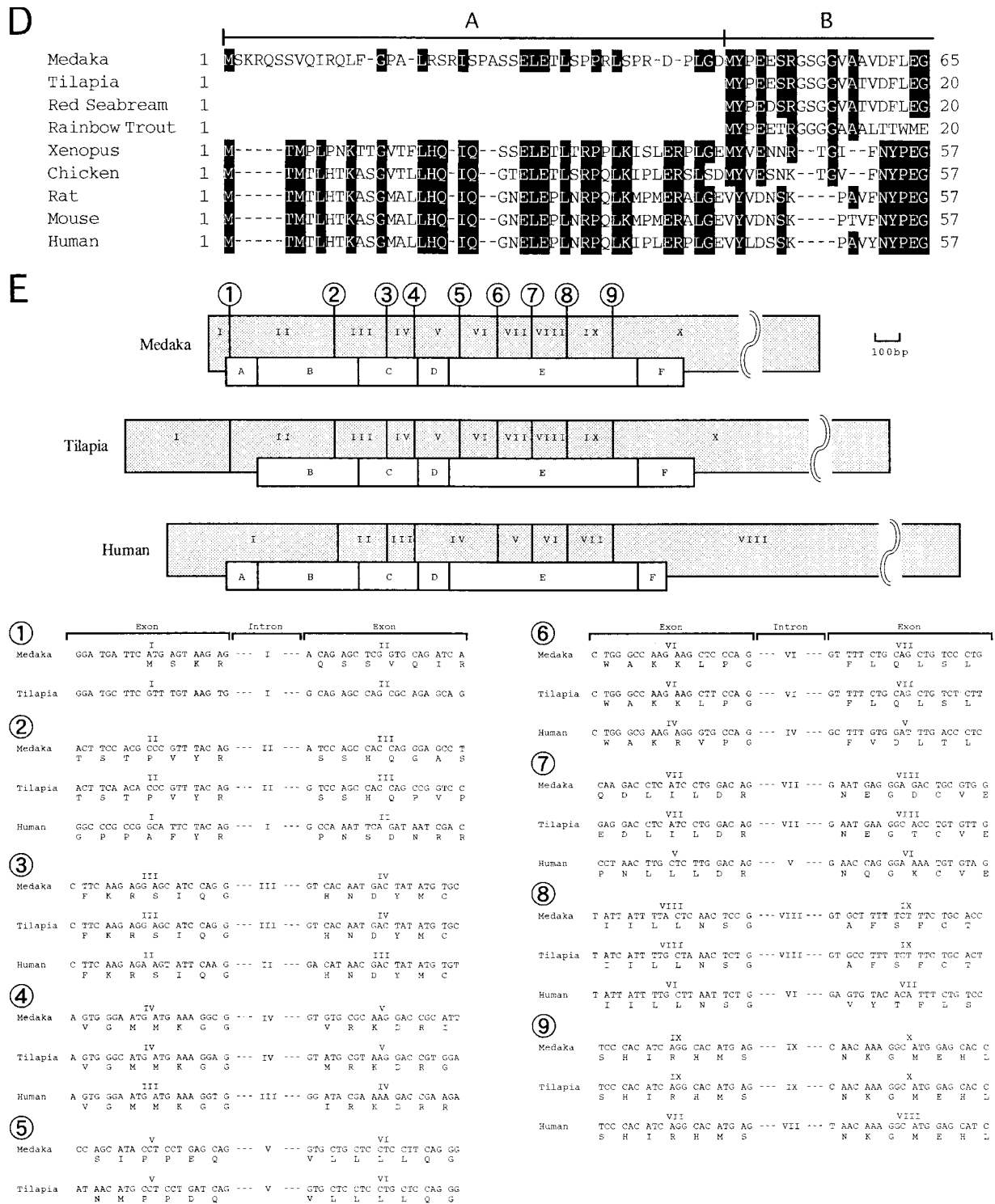
RESULTS AND DISCUSSION

Isolation and characterization of genomic and complementary DNAs encoding an ER homolog of medaka fish

We obtained 5 positive phage clones from the genomic library, which hybridized with the human ERα cDNA, and recloned into the plasmid pUC118 after analysis with restriction enzymes. Nine inserts of the plasmids obtained encompassed the entire sequence of the ER gene (Fig. 1A). DNA blot analysis of genomic DNA fragments generated by a number of restriction enzymes revealed only the predicted bands, suggesting a single gene in the genome (data not shown). We next obtained 3 positive cDNAs, in which two were estimated to be identical by the restriction mapping (Fig. 1B). Nucleotide sequences were determined for the genomic DNA (accession number AB033491) and the longest cDNA (accession number D28954) (only the latter sequence is shown in Fig. 1C). The ER gene contains nine introns ranging from 168 to 1702 bp in length. The cDNA is composed of three regions: 75 bp of a 5' untranslated region with three stop codons, 1860 bp of a coding region, and 829 bp of a 3' untranslated region with putative polyadenylation signal sequences. The coding region encodes a protein of 620 amino acids with a calculated molecular weight of 67.7 KDa. The deduced protein may be classified into an α-type ER because it is most homologous to known α-type ERs with overall amino acid identity of 72 and 36% to the tilapia α- and β-type ERs (Chang *et al.*, 1999), respectively. The medaka ER is also composed of the six conserved domains (A to F) (Fig. 1B). This is the first fish ER with "A" domain (Fig. 1D), the significance of which is presently unknown. We also found that positions of most introns are strictly conserved among the ER genes from fish (medaka, tilapia, and rainbow trout) and human except that the human gene lacks two introns corresponding to the introns 1 and 5 of medaka ER gene (Fig. 1E). The human gene may have arisen from an ancestral ER gene by reverse transcription of prematurely spliced mRNA and chromosomal integration.

Tissue-specific expression and regulation of the medaka ER mRNA

Tissue-specific expression of the ER mRNA was analyzed by RNA blotting. Total RNA was prepared from liver, ovary, and testis of adult fish. ER mRNA was expressed in large amounts in ovary, testis, and female liver, but at a greatly reduced level in male liver (Fig. 2A). Expressions of ER mRNA in embryos and fry were also analyzed by RT-PCR (Fig. 3). In preliminary experiments, our conditions for RT-PCR were semi-quantitative because dilutions of samples resulted in corresponding linear decreases in signals for both ER and β-actin mRNAs (data not shown). Furthermore, the amplified cDNA had the same electrophoretic mobility as that expected to be derived from ER mRNA but not genomic sequence. As expected, ER mRNA was expressed during the development of embryos (Fig. 3A) and fry (Fig. 3B) of both sexes, at nearly a constant level except relatively low expression at the

A

Exon number   I   II   III   IV   V   VI   VII   VIII   IX   X

Exon size (bp) 86 420 215 117 191 154 139 134 184 1124
Intron size (bp)   630   628   433 332 174   1702   962 602 168   1 Kb

pgMER11
pgMER21
pgMER51
pgMER31,41
pgMER13,23
pgMER12,22

B

F→   ←R

I   II   III   IV   V   VI   VII   VIII   IX   X

A   B   C   D   E   F

100bp

E9
E3,8

C

```
        ex1                                                          ex1  ex2
   1 GTCTCGCTGCTAGATGCCTGTCAGGCAGGCAGAGAGGAAGCAGCCCGTGTTGCGCAGCACATCTGAGGATGATTCATGAGTAAGACAGAGCTCGGTGCAGATCAGGCAGCTGTTCGGA
                                                                            M  S  K  R  Q  S  S  V  Q  I  R  Q  L  F  G    15

 121 CCAGCACTCAGATCCAGGATCAGCCCAGCCTCCTCAGAGCTGGAGACCCTCTCCCCACCTCGCCTCTCGCCCCGTGACCCCCTCGGTGACATGTACCCTGAAGAGAGCCGGGGTTCTGGA
      P  A  L  R  S  R  I  S  P  A  S  S  E  L  E  T  L  S  P  P  R  L  S  P  R  D  P  L  G  D  M  Y  P  E  E  S  R  G  S  G    55

 241 GGGGTGGCTGCTGTGGACTTTTTGGAAGGGACGTACGACTATGCCGCCCCCAACCCTGCCACGACTCCCCTTTACAGCCAGTCCAGCACCGGCTACTACTCTGCTCCCCTGGAAACAAAC
      G  V  A  A  V  D  F  L  E  G  T  Y  D  Y  A  A  P  N  P  A  T  T  P  L  Y  S  Q  S  S  T  G  Y  Y  S  A  P  L  E  T  N    95

 361 GGACCCCCCTCAGAAGGCAGTCTGCAGTCCCTGGGCAGTGGGCCGACGAGCCCTCTGGTGTTTGTGCCCTCCAGCCCCAGACTCAGTCCCTTTATGCATCCACCCAGCCACCACTATCTG
      G  P  P  S  E  G  S  L  Q  S  L  G  S  G  P  T  S  P  L  V  F  V  P  S  S  P  R  L  S  P  F  M  H  P  P  S  H  H  Y  L    135
                            ex2  ex3
 481 GAAACCACTTCCACGCCCGTTTACAGATCCAGCCACCAGGGAGCCTCCAGGGAGGACCAGTGCGGCTCCCGGGAGGACACGTGCAGCCTGGGGGAGTTAGGCGCCGGAGCCGGGGCTGGG
      E  T  T  S  T  P  V  Y  R  S  S  H  Q  G  A  S  R  E  D  Q  C  G  S  R  E  D  T  C  S  L  G  E  L  G  A  G  A  G  A  G    175
                                                                                                                           ex3
 601 GGGTTTGAGATGGCCAAAGACACGCGTTTCTGCGCCGTGTGCAGCGACTACGCCTCTGGGTACCACTATGGGGTGTGGTCTTGTGAGGGCTGCAAGGCCTTCTTCAAGAGGAGCATCCAG
      G  F  E  M  A  K  D  T  R  F  C  A  V  C  S  D  Y  A  S  G  Y  H  Y  G  V  W  S  C  E  G  C  K  A  F  F  K  R  S  I  Q    215
        ex4                                                                                                            ex4  e
 721 GGTCACAATGACTATATGTGCCCAGCGACCAATCAGTGCACTATTGACAGAAATCGGAGGAAGAGCTGCCAGGCTTGTCGTCTTAGGAAGTGTTACGAAGTGGGAATGATGAAAGGCCGT
      G  H  N  D  Y  M  C  P  A  T  N  Q  C  T  I  D  R  N  R  R  K  S  C  Q  A  C  R  L  R  K  C  Y  E  V  G  M  M  K  G  R    255
      x5
 841 GTGCGCAAGGACCGCATTCGCATTTTACGGCGTGACAAACGGCGGACAGGCGTTGGTGATGGAGACAAGGTTGTAAAGGGTCAGGAGCATAAAACGGTGCATTATGATGGAAGGAAACGC
      V  R  K  D  R  I  R  I  L  R  R  D  K  R  R  T  G  V  G  D  G  D  K  V  V  K  G  Q  E  H  K  T  V  H  Y  D  G  R  K  R    295
                                                                                       ex5  ex6
 961 AGCAGCACAGGAGGAGGAGGAGGAGGAGGAGGAGGAAGACTGTCTGTGACCAGCATACCTCCTGAGCAGGTGCTGCTCCTCCTTCAGGGCGCCGAGCCCCGATACTCTGCTCGCGTCAG
      S  S  T  G  G  G  G  G  G  G  G  R  L  S  V  T  S  I  P  P  E  Q  V  L  L  L  L  Q  G  A  E  P  P  I  L  C  S  R  Q    335
                                                                                          ex6  ex7
1081 AAGTTGAGCCGACCGTACACCGAGGTCACCATGATGACCCTGCTCACCAGCATGGCAGACAAGGAGCTGGTCCACATGATCGCCTGGGCCAAGAAGCTCCCAGGTTTTCTGCAGCTGTCC
      K  L  S  R  P  Y  T  E  V  T  M  M  T  L  L  T  S  M  A  D  K  E  L  V  H  M  I  A  W  A  K  K  L  P  G  F  L  Q  L  S    375
                                                                                                                           ex
1201 CTGCACGATCAGGTGCTGCTGCTGGAGAGCTCGTGGCTGGAGGTGCTCATGATCGGCCTCATTTGGAGGTCCATCCACTGTCCCGGGAAGCTCATCTTTGCACAAGACCTCATCCTGGAC
      L  H  D  Q  V  L  L  L  E  S  S  W  L  E  V  L  M  I  G  L  I  W  R  S  I  H  C  P  G  K  L  I  F  A  Q  D  L  I  L  D    415
      7  ex8
1321 AGGAATGAGGGAGACTGCGTGGAAGGCATGACGGAGATCTTCCACATGCTGCTGGCCACTGCTTCCCGCTTCCGTGTGCTCAAACTCAAACCTGAGGAATTCGTCTGCCTCAAAGCTATT
      R  N  E  G  D  C  V  E  G  M  T  E  I  F  D  M  L  L  A  T  A  S  R  F  R  V  L  K  L  K  P  E  E  F  V  C  L  K  A  I    455
              ex8  ex9
1441 ATTTTACTCAACTCCGGTGCTTTTTCTTTCTGCACCGGCACCATGGAGCCCACTTCACAACAGCGCGGCGGTTCAGAGCATGCTGGACACCATCACAGACGCACTCATTCATTACATCAGT
      I  L  L  N  S  G  A  F  S  F  C  T  G  T  M  E  P  L  H  N  S  A  A  V  Q  S  M  L  D  T  I  T  D  A  L  I  H  Y  I  S    495
                                                                               ex9  ex10
1561 CAGTCGGGTTACTTGGCCCAGGAGCAGGCGAGACGGCAGGCCCAGCTGCTCCTGCTGCTCTCCCACATCAGGCACATGAGCAACAAAGGCATGGAGCACCTCTACAGCATGAAGTGCAAG
      Q  S  G  Y  L  A  Q  E  Q  A  R  R  Q  A  Q  L  L  L  L  L  S  H  I  R  H  M  S  N  K  G  M  E  H  L  Y  S  M  K  C  K    535

1681 AACAAAGTCCCTCTTTATGACCTCCTACTGGAGATGCTCGATGCCCACCGCCTGCACCACCCCGTCAGAGCACCCCAGTCCTTGTCCCAAGTCGACAGAGACCCTCCCTCCACCAGCAGC
      N  K  V  P  L  Y  D  L  L  L  E  M  L  D  A  H  R  L  H  H  P  V  R  A  P  Q  S  L  S  Q  V  D  R  D  P  P  S  T  S  S    575

1801 GGCGGGGGTGGAATCGCTCCCGGTTCTATATCAGCATCTCGAGGCAGAATCGAGAGTCCGAGCAGAGGCCCCTTTGCTCCCAGTGTCCTTCAGTATGGAGGGTCGCGTCCTGACTGCACC
      G  G  G  G  I  A  P  G  S  I  S  A  S  R  G  R  I  E  S  P  S  R  G  P  F  A  P  S  V  L  Q  Y  G  G  S  R  P  D  C  T    605

1921 CCGGCCCTTCAAGACTGAGCACACAGTCCAAGGCCCTTTTTTTGTGGCTCAAGGGTTCAGGTTGGGACAAGGTGATGCTTGATTTAATTTTAAGAATTATTTATAAATAAGAGTGGCGCT
      P  A  L  Q  D  *                                                                                                       620

2041 GAGAGGAGAAGCTCCCACAATGAACTGCCTCTGCTTGGTCCAGCTTTTGTGCAGTCACTTTAATCTGCTTATATTCATCTCCTTTGTAAACCTGAGCGTCTCTTTAGCAGCTTTTTTTTT
2161 CTCTCCAAACAGCATGTGGTAGATTGTAAGGTTGCGTCCCATGAGGTCTGGTGATTTCAAGAAAATGAGCAGCTAATGTTTTCTGTAACCGTCTTGACCCAAGTGCACTTCCTCTTGGAT
2281 TAAAGGGGCTAATGGGCATTATTTTGTCTCTTGTACATATGGGATGGCTAAGAATAATGAGAGTAATTGTCAGATTTTGTGTAGAACTTACCCACAAATGCAATTTTAAAATAAGATTTA
2401 AAAACAAAAGAGGCAAGATCAAACCTGAGAGCACTGAAGACACGCTGTAGAAAGCTGGGTAAATTTGTTATCCACGTCTATCTCTGGAAAGGACTTTGTTCTCTGTGCGTGCAGCTCATT
2521 TACTCTGAACTTGCTACTTGTTGAACATTTGTGCACTTGTCCGTGTTTTTCTAGCACTGTAGCTTATGAACGCTGAGAAAGAATCTAATGCTTTGATGCACAGATTTGCCTTGTATTGTA
                                                                                                                            e
2641 CATCTCAGCCACAAACGTACTTTTCGTCCACAAGTTGACTGACTGCACCTTGATTAAATTGTCTAAAAGTTCATTTAAATGTTGAATTCTGTGAAAATTAAAAAGGCAATTCCTGTTTCT
      x10
2761 ATTT
```

Fig. 1. Genomic and cDNA sequences for medaka ER. (**A**) Schematic map of the ER gene, showing the exons (I to X) and introns. Inserts in the plasmids recovered are also shown. (**B**) Schematic alignment of the cDNA and the deduced ER protein, composed of the six conserved domains (A to F). The cDNAs recovered (E3, E8, and E9), the fragments used as probes in RNA blot analysis (thick line) and *in situ* hybridization (thin line), and the oligonucleotide primers (F and R) used in RT-PCR are also shown. (**C**) Nucleotide sequence of the cDNA and the deduced protein sequence. The positions of exons are marked. Polyadenylation signals are underlined. (**D**) Alignment of "A" domains from medaka and other vertebrates indicated. The sequences were extracted from the EMBL/GenBank/DDBJ database with accession numbers as follows: medaka (D28954), tilapia *Oreochromis aureus* (X93557), red seabream (AB007453), rainbow trout (M31559), *Xenopus* (L20735), chicken (X03805), rat (Y00102), mouse (M38651), and human (M11457). Conserved (more than 50%) amino acids are boxed. Gapped sequences are indicated by bars. (**E**) Schematic map of ER cDNAs and ERs from fish (medaka and tilapia) and human. Exon-intron junctions of ER genomic sequences from medaka (AB033491), tilapia (X93555-93561, Z46665–46669), and human (AF123494–123500) are also indicated. Intron numbers are circled. Organization of the rainbow trout ER gene is described previously (Roux *et al.*, 1993).
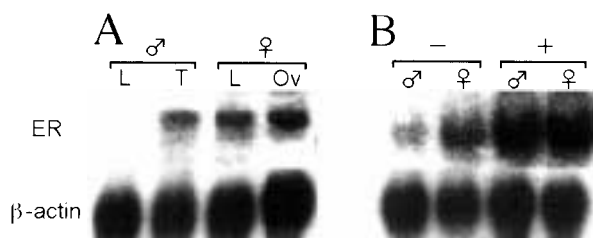
Fig. 2. RNA blot analysis of ER and β-actin mRNAs. (**A**) Total RNA was extracted from liver (L), testis (T), and ovary (Ov) of adult fish. (**B**) Total RNA was extracted from liver of adult fish that had been treated with oral administration of estrogen (2 mg of 17β-estradiol per g of diet) for 7 days (+) or mock treated (–).
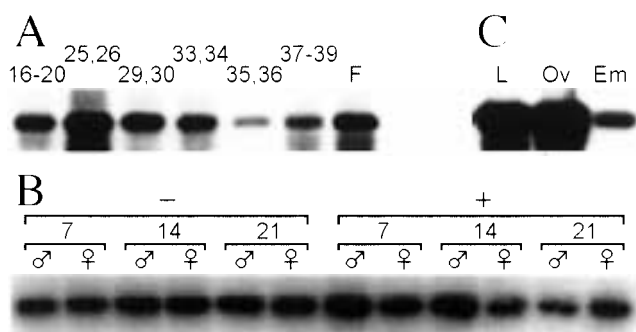
Fig. 3. RT-PCR analysis of ER mRNA. (**A**) Total RNA was extracted from embryos at the indicated stages and newly hatched fry (F). (**B**) Newly hatched fry were reared by estrogen-containing diet (20 μg of 17β-estradiol per g of diet; +) or normal diet (–) for the indicated days, and total RNA was extracted. (**C**) Total RNA was extracted from liver (L) and ovary (Ov) of adult female fish and from embryos at stages 37 to 39 (Em).

embryonic stages 35 and 36. The presence of ER mRNA in both sexes during the sex determination period (at or after hatching) is consistent with our model. Treatment of adult fish with oral administration of estrogen elevated the hepatic expression of ER mRNA regardless of the sex (Fig. 2B). This autoregulatory positive loop may be conserved throughout the evolution, because the treatment with estrogen of rainbow trout (Drean *et al.*, 1995) and a frog, *Xenopus laevis* (Barton and Shapiro, 1988), also elevates the hepatic expression of ER mRNA. The positive transcriptional loop in rainbow trout is governed by ER- binding sequences (a palindromic estrogen-response element and 7 half-sites) located near the mRNA start site (Drean *et al.*, 1995). The medaka ER gene also contains 14 half-sites (not shown). However, the treatment with sufficient amounts of estrogen for the male-to-female sex reversal did not affect the ER mRNA expression in embryos (data not shown) and fry within 3 weeks after hatching (Fig. 3B). This suggests that the expression at these stages is not under the control of estrogen and that the low level expression in embryos and fry (see below) is not due to the absence of estrogen.

Spatial pattern of ER mRNA expression was analyzed by *in situ* hybridization to whole mounts of embryos, fry, and adult tissues. Specific signals were detected in adult tissues such as the ventral preoptic area and the pituitary gland of

brain, ovary, testis, and female liver (Fig. 4), consistent with the biological functions of ER in these tissues (Salbert *et al.*, 1991; Lubahn *et al.*, 1993; Jobling *et al.*, 1996). However, we could not detect any significant signals for ER mRNA (but could easily for β-actin mRNA) in embryos and fry (data not shown), indicating the basal level expression at the juvenile stage. This was confirmed by RT-PCR analysis: the expression was ~ 20 folds higher in liver and ovary of adult female fish than in embryos (Fig. 3C). There are certain tissues such as the bone that are highly responsive to estrogens but only express a low level of ER (50- to 100-fold fewer estrogen-binding sites present in osteoblast than in sexual tissues) (Eriksen *et al.*, 1988; Komm *et al.*, 1988).

Embryonic expression of ER is not limited to medaka fish. ER is detected in all cell types of the mouse embryo at the blastocyst stage (Hou *et al.*, 1996). Mouse embryos implant at this stage and estrogen is an absolute requirement for this process. ER may therefore have a general role during early development. To examine whether medaka embryos display the sensitivity to estrogen in addition to the sex reversal, embryos at the stages of 9 to 11 (approximately 8 hr after fertilization) were immersed in the presence of estrogen. After 3-day incubation at the concentration of 4 mg of 17β-estradiol per liter, a 20-time increased concentration of estrogen that is sufficient for the complete sex reversal, the development of blood vessels was inhibited in most embryos whereas the embryos developed normally to stage 24 and had the blood island (Fig. 5). Specificity of estrogen could not
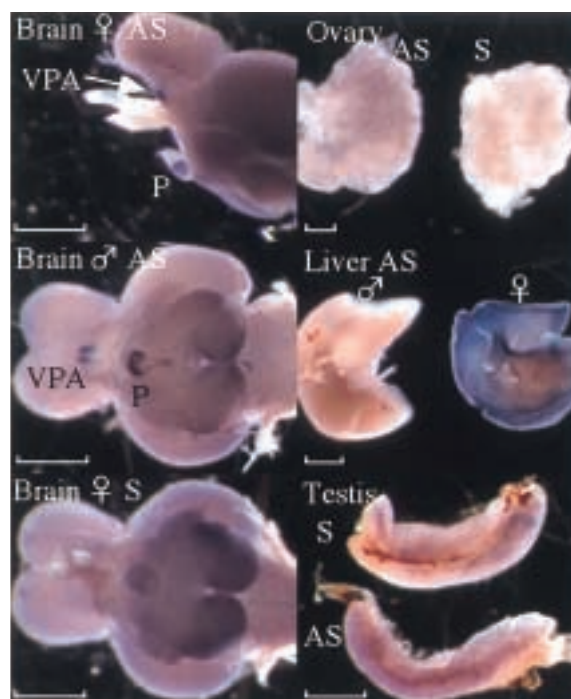
Fig. 4. Tissue-specific expression of ER mRNA. *in situ* Hybridization with anti-sense (AS) and sense (S) probes to whole mounts of brain, liver, ovary, and testis of adult fish. Ventral preoptic area (VPA) and pituitary gland (P) are marked in lateral (top) and ventral (middle) views of brain. Bar, 0.5 mm.
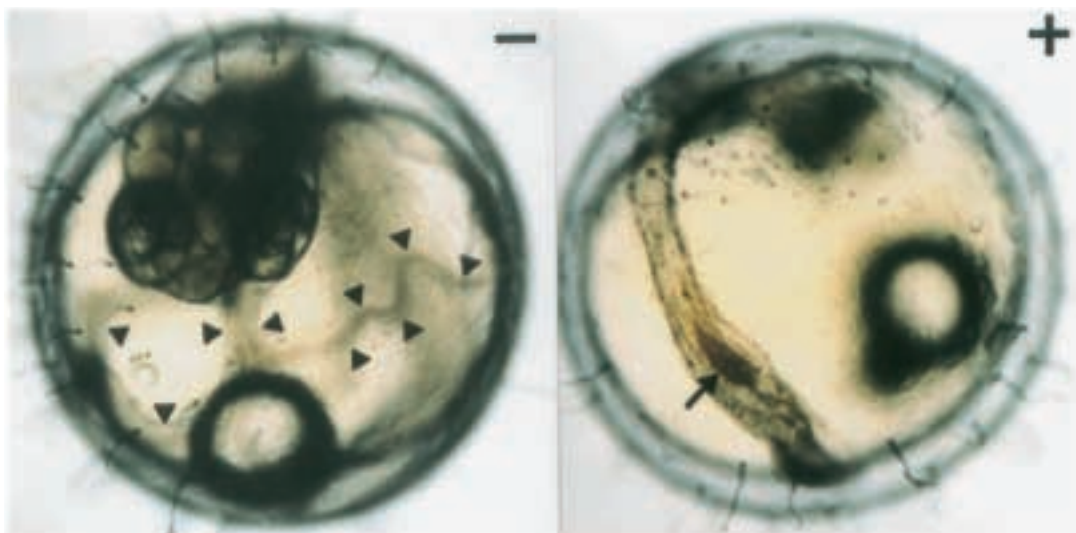
Fig. 5. Inhibition of the embryonic blood vessel development by estrogen. The embryos 8 hr after fertilization were incubated in Yamamoto's salt solution in the presence (+) or the absence (–) of 17β-estradiol (4 mg/l) for 3 days. Normally developed blood vessels are marked by arrowheads. The blood island in the estrogen-treated embryo is marked by an arrow.

be studied because of an insoluble nature of anti- estrogens at the concentrations more than 100-time that of estrogen, which is usually required for the anti-estrogenic action. However, transgenic embryos overexpressing medaka ER (from one of the transgenic lines recently established in our lab) displayed hypersensitivity specifically to estrogen: the development of blood vessels was inhibited by 17β-estradiol at the concentration of 2 μg/l but not by 17α-estradiol, a biologically inactive isomer, at the concentration of 2 mg/l (unpublished data). These results indicate that the embryonic development of blood vessels is specifically inhibited by estrogen and suggest that regulation of ER activity is essential to embryonic development. This biological assay is simple, rapid, and highly sensitive, thus, may be used for monitoring of environmental xeno-estrogens (Gimeno *et al.*, 1996; Gray and Metcalfe, 1997) such as alkylphenolic compounds that have been detected in sewage effluents at μg/l concentrations (Stephanou and Giger, 1982; Kubeck and Naylor, 1990; Lee and Peart, 1995).

In this study, we obtained the genomic and complementary DNAs for medaka α- type ER. It is presently unknown whether there exist other ER homologs in medaka such as a β-type one that has been cloned from mammals (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996; Tremblay *et al.*, 1997) and fish (Chang *et al.*, 1999). Recently, we obtained preliminary data that suggest the existence of variants of the ERα expressed in embryos and fry. Cloning and expression studies of these ER homologs may facilitate to elucidate the possible roles of ER in gonadal sex determination of lower vertebrates.

REFERENCES

Barton MC, Shapiro DJ (1988) Transient administration of estradiol-17β establishes an autoregulatory loop permanently inducing estrogen receptor mRNA. Proc Natl Acad Sci USA 85: 7119–7123

Beato M, Herrlich P, Schutz G (1995) Steroid hormone receptors: many actors in search of a plot. Cell 83: 851–857

Bunone G, Briand PA, Miksicek RJ, Picard D (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 15: 2174–2183

Chang X, Kobayashi T, Todo T, Ikeuchi T, Yoshiura M, Kajiura-Kobayashi H, Morrey C, Nagahama Y (1999) Molecular cloning of estrogen receptors α and β in the ovary of a teleost fish, the tilapia (*Oreochromis niloticus*). Zool Sci 16: 653–658

Clinton M (1998) Sex determination and gonadal development: a bird's eye view. J Exp Zool 281: 457–465

Das SK, Taylor JA, Korach KS, Paria BC, Dey SK, Lubahn DB (1997) Estrogenic responses in estrogen receptor-α deficient mice reveal a distinct estrogen signaling pathway. Proc Natl Acad Sci USA 94: 12786–12791

Dorizzi M, Mignot T-M, Guichard A, Desvages G, Pieau C (1991) Involvement of oestrogens in sexual differentiation of gonads as a function of temperature in turtles. Differentiation 47: 9–17

Drean YL, Lazennec G, Kern L, Saligaut D, Pakdel F, Valotaire Y (1995) Characterization of an estrogen-responsive element implicated in regulation of the rainbow trout estrogen receptor gene. J Mol Endocrinol 15: 37–47

Elbrecht A, Smith RG (1992) Aromatase enzyme activity and sex determination in chickens. Science 255: 467–470

Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC, Riggs BL (1988) Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241: 84–86

Gimeno S, Gerritsen A, Bowmer T, Komen H (1996) Feminization of male carp. Nature 384: 221–222

Gray MA, Metcalfe CD (1997) Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to p-nonylphenol. Environ

Toxicol Chem 16: 1082–1086

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

Hayes TB (1998) Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. J Exp Zool 281: 373–399

Hou Q, Paria BC, Mui C, Dey SK, Gorski J (1996) Immunolocalization of estrogen receptor protein in the mouse blastocyst during normal and delayed implantation. Proc Natl Acad Sci USA 93: 2376–2381

Inohaya K, Yasumasu S, Araki K, Naruse K, Yamazaki K, Yasumasu I, Iuchi I, Yamagami K (1997) Species-dependent migration of fish hatching gland cells that commonly express astacin-like proteases in common. Develop Growth Differ 39: 191–197

Iwamatsu T (1994) Stages of normal development in the medaka *Oryzias latipes*. Zool Sci 11: 825–839

Iwasaki Y (1973) Histochemical detection of Δ^5-3β-hydroxysteroid dehydrogenase in the ovary of medaka, *Oryzias latipes*, during annual reproductive cycle. Bull Fac Fish Hokkaido Univ 23: 177–184

Jeyasuria P, Place AR (1998) Embryonic brain-gonadal axis in temperature-dependent sex determination of reptiles: a role for P450 aromatase (CYP19). J Exp Zool 281: 428–449

Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP (1996) Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environ Toxicol Chem 15: 194–202

Kagawa H, Takano K (1979) Ultrastructure and histochemistry of granulosa cells of pre- and post-ovulatory follicles in the ovary of the medaka, *Oryzias latipes*. Bull Fac Fish Hokkaido Univ 30: 191–204

Kanamori A, Nagahama Y, Egami N (1985) Development of the tissue architecture in the gonads of the medaka *Oryzias latipes*. Zool Sci 2: 695–706

Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270: 1491–1494

Kawahara T, Yamashita I (2000) Estrogen-independent ovary formation in the medaka fish, *Oryzias latipes*. Zool Sci 17: 65–68

Komm BS, Terpening CM, Benz DJ, Graeme KA, Gallegos A, Korc M, Greene GL, O'Malley BW, Haussler MR (1988) Estrogen binding, receptor mRNA and biological response in osteoblast-like osteosarcoma cells. Science 241: 81–84

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

Kubeck E, Naylor CG (1990) Trace analysis of alkylphenol ethoxylates. JAOCS 67: 400–405

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

Lee B, Peart TE (1995) Determination of 4-NP in effluent and sludge from sewage treatment plants. Anal Chem 67: 1967–1980

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O (1993) Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90: 11162–11166

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

Nakamura M, Kobayashi T, Chang XT, Nagahama Y (1998) Gonadal sex differentiation in teleost fish. J Exp Zool 281: 362–372

Patino R (1997) Modifications of the reproductive system of fishes by means of exogenous chemicals. Prog Fish-Cult 59: 118–128

Pieau C, Dorrizzi M, Richard-Mercier N, Desvages G (1998) Sexual differentiation of gonads as a function of temperature in the turtle *Emys orbicularis*: endocrine function, intersexuality and growth. J Exp Zool 281: 400–408

Power RF, Mani SK, Codina J, Conneely OM, O'Malley BW (1991) Dopaminergic and ligand-independent activation of steroid hormone receptors. Science 254: 1636–1639

Richard-Mercier N, Dorizzi M, Desvages G, Girondot M, Pieau C (1995) Endocrine sex reversal of gonads by the aromatase inhibitor Letrozole (CGS 20267) in *Emys orbicularis*, a turtle with temperature-dependent sex determination. Gen Comp Endocrinol 100: 314–326

Roux MGL, Theze N, Wolff J, Pennec JPL (1993) Organization of a rainbow trout estrogen receptor gene. Biochim Biophys Acta 1172: 226–230

Salbert G, Bonnec G, Goff PL, Boujard D, Valotaire Y, Jego P (1991) Localization of the estradiol receptor mRNA in the forebrain of the rainbow trout. Mol Cell Endocrinol 76: 173–180

Scheib D (1983) Effects and role of estrogens in avian gonadal differentiation. Differentiation 23 (Suppl): S87–S92

Schreibman MP, Berkowitz EJ, van den Hurk R (1982) Histology and histochemistry of the testis and ovary of the platyfish, *Xiphophorus maculatus*, from birth to sexual maturity. Cell Tissue Res 224: 81–87

Smith CL, Conneely OM, O'Malley BW (1993) Modulation of the ligand-independent activation of the human estrogen receptor by hormone and antihormone. Proc Natl Acad Sci USA 90: 6120–6124

Stephanou E, Giger W (1982) Persistent organic chemicals in sewage effluents. 2. Quantitative determinations of nonylphenols and nonylphenol ethoxylates by glass capillary gas chromatography. Environ Sci Technol 16: 800–805

Takahashi H, Iwasaki Y (1973) The occurrence of histochemical activity of 3β-hydroxysteroid dehydrogenase in the developing testes of *Poecilia reticulata*. Dev Growth Differ 15: 241–253

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

Turner RT, Riggs BL, Spelsberg TC (1994) Skeletal effects of estrogen. Endocrinol Rev 15: 275–300

van den Hurk R, Lambert JGD, Peute J (1982) Steroidgenesis in the gonads of rainbow trout fry (*Salmo gairdneri*) before and after the onset of gonadal sex differentiation. Reprod Nutr Dev 22: 413–425

Yamamoto T (1969) Sex differentiation In "Fish Physiology 3" Ed by WS Hoar, DJ Randall Academic Press, New York, pp 117–175

Zwijsen RML, Buckle RS, Hijmans EM, Loomans CJM, Bernards R (1998) Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1. Genes Dev 12: 3488–3498