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

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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;

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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

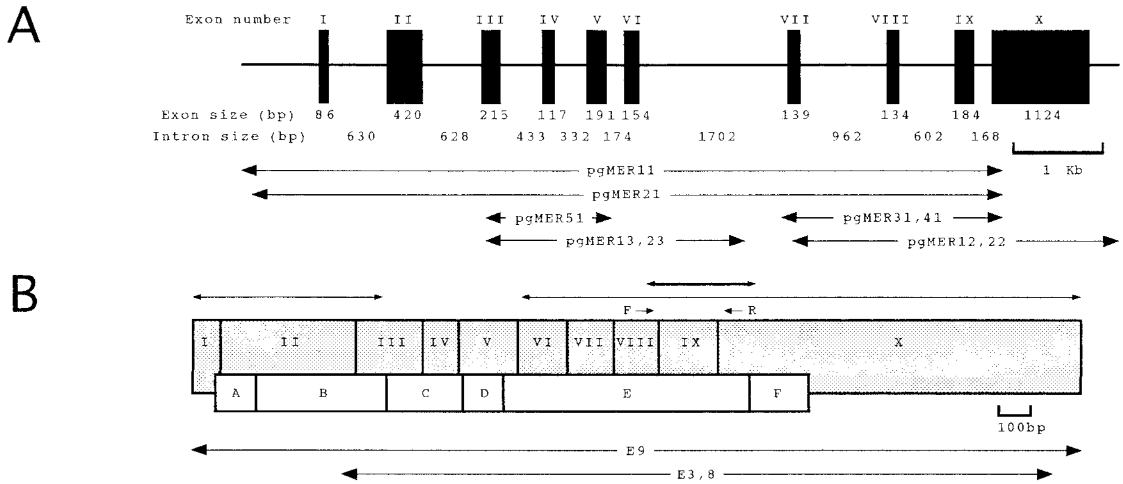
We obtained 5 positive phage clones from the genomic library, which hybridized with the human ERα cDNA, and recloned into the plasmid pUC118 after analysis with restriction enzymes. Nine inserts of the plasmids obtained encompassed the entire sequence of the ER gene (Fig. 1A). DNA blot analysis of genomic 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 of both sexes (Fig. 3B), at nearly a constant level except relatively low expression at the

A

B



C

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      ex1                                                                                ex1  ex2
   1 GTCTCGCTGCTAGATGCCTGTCAGGCAGGCAGAGAGGAAGCAGCCCGTGTTGCGCAGCACATCTGAGGATGATTCATGAGTAAGAGACAGAGCTCGGTGCAGATCAGGCAGCTGTTCGGA
                                                                                      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 GGTCACAATGACTATATGTGCCCAGCGACCAATCAGTGCACTATTGACAGAAATCGGAGGAAGAGCTGCCAGGCTTGTCGTCTTAGGAAGTGTTACGAAGTGGGAATGATGAAAGGCGGT
     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  G    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 AGCAGCACAGGAGGAGGAGGAGGAGGAGGAGGAGGAAGACTGTCTGTGACCAGCATACCTCCTGAGCAGGTGCTGCTCCTCCTTCAGGGCGCCGAGCCCCCGATACTCTGCTCGCGTCAG
     S  S  T  G  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 ATTTTACTCAACTCCGGTGCTTTTTCTTTCTGCACCGGCACCATGGAGCCACTTCACAACAGCGCGGCGGTTCAGAGCATGCTGGACACCATCACAGACGCACTCATTCATTACATCAGT
     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 GAGAGGAGAAGCTCCCACAATGAACTGCCTCTGCTTGGTCCAGCTTTTGTGCAGTCACTTTAATCTGCTTATATTCATCTCCTTTGTAAACCTGAGCGTCTCTTTAGCACCTTTTTTTTG
2161 CTCTCCAAACAGCATGTGGTAGATTGTAAGGTTGCGTCCCATGAGGTCTGGTGATTTCAAGAAAATGAGCAGCTAATGTTTTCTGTAACCGTCTTGACCCAAGTGCACTTCCTCTTGGAT
2281 TAAAGGGGCTAATGGGCATTATTTTGTCTCTTGTACATATGGGATGGCTAAGAATAATGAGAGTAATTGTCAGATTTTGTGTAGAACTTACCCACAAATGCAATTTTAAAATAAGATTTA
2401 AAAACAAAAGAGGCAAGATCAAACCTGAGAGCACTGAAGACACGCTGTAGAAAGCTGGGTAAATTTGTTATCCACGTCTATCTCTGGAAAGGACTTTGTTCTCTGTGCCTGCAGCTCATT
2521 TACTCTGAACTTGCTACTTGTTGAACATTTGTGCACTTGTCCGTGTTTTTCTAGCACTGTAGCTTATGAACGCTGAGAAAGAATCTAATGCTTTGATGCACAGATTTGCCTTGTATTGTA
                                                                                                                           e
2641 CATCTCAGCCACAAACGTACTTTTCGTCCACAAGTTGACTGACTGCACCTTGATTAAATTGTCTAAAAGTTCATTTAAATGTTGAATTCTGTGAAAATTAAAAAGGCAATTCCTGTTTCT
     x10
2761 ATTT
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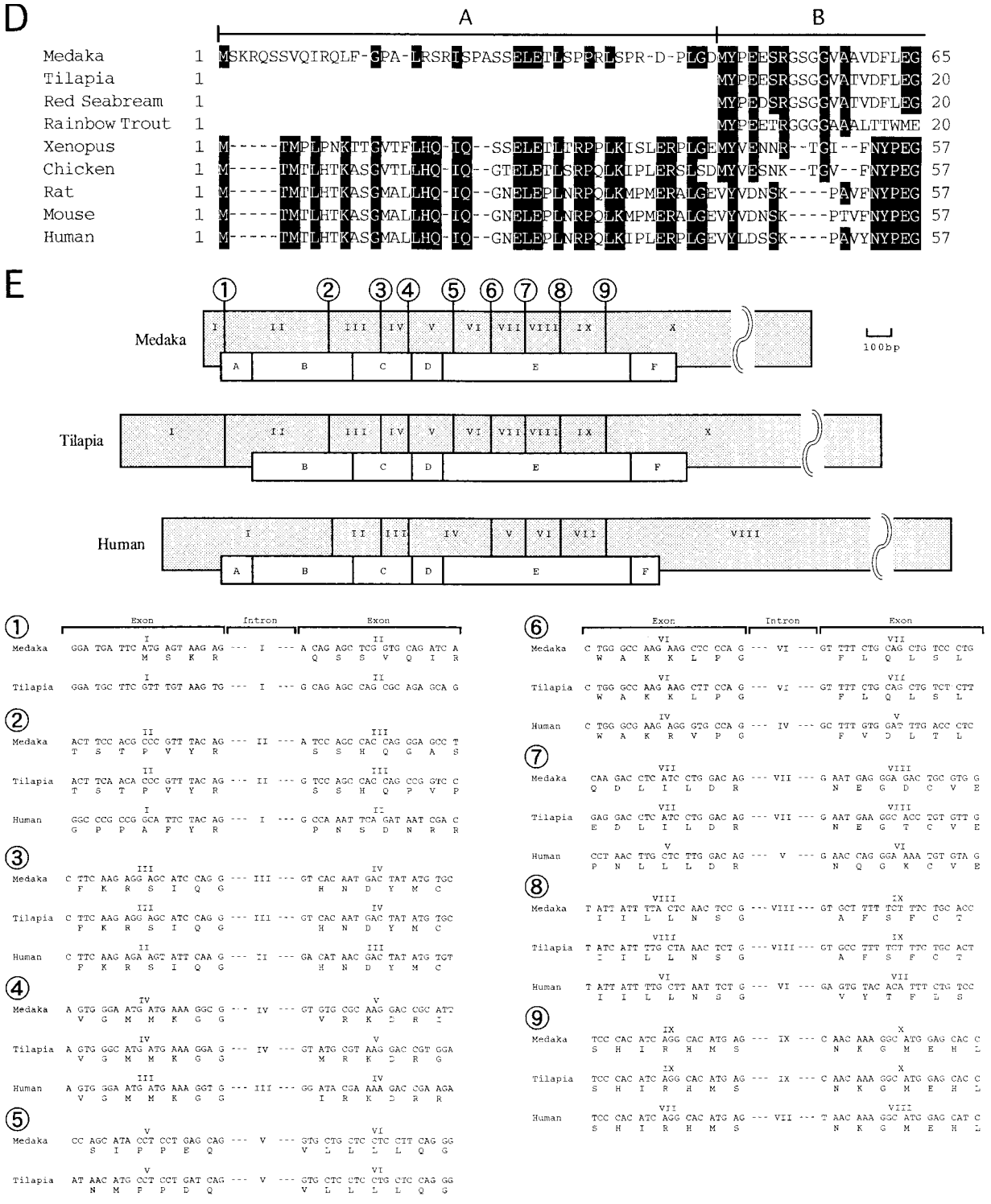

Fig. 1. Genomic and cDNA sequences for medaka ER. (**A**) Schematic map of the ER gene, showing the exons (I to X) and introns. Inserts in the plasmids recovered are also shown. (**B**) Schematic alignment of the cDNA and the deduced ER protein, composed of the six conserved domains (A to F). The cDNAs 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).

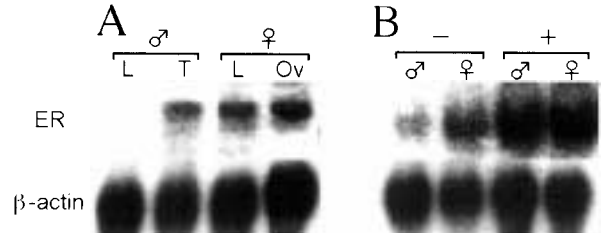

Fig. 2. RNA blot analysis of ER and β-actin mRNAs. (**A**) Total RNA was extracted from liver (L), testis (T), and ovary (Ov) of adult fish. (**B**) Total RNA was extracted from liver of adult fish that had been treated with oral administration of estrogen (2 mg of 17β-estradiol per g of diet) for 7 days (+) or mock treated (–).

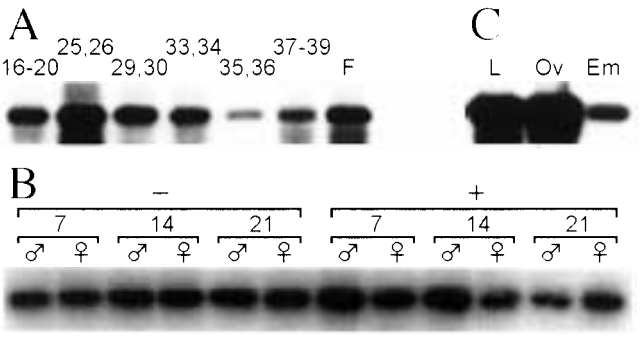

Fig. 3. RT-PCR analysis of ER mRNA. (**A**) Total RNA was extracted from embryos at the indicated stages and newly hatched fry (F). (**B**) Newly hatched fry were reared by estrogen-containing diet (20 μg of 17β-estradiol per g of diet; +) or normal diet (–) for the indicated days, and total RNA was 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

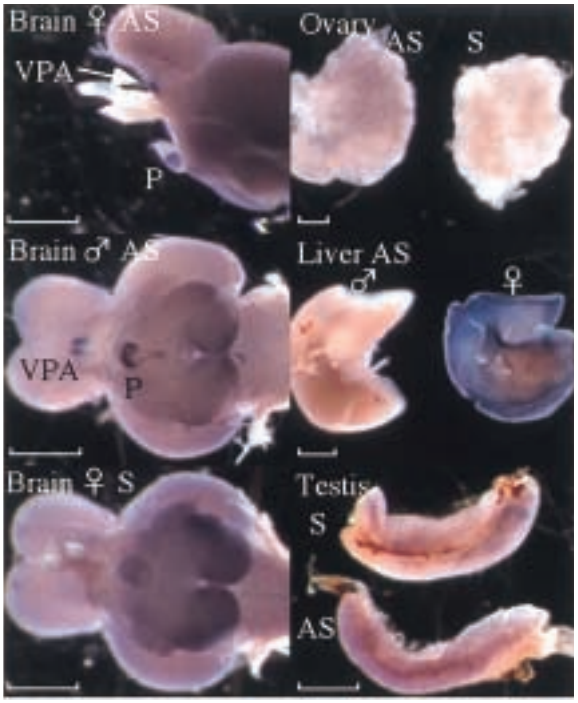

Fig. 4. Tissue-specific expression of ER mRNA. *in situ* Hybridization with anti-sense (AS) and sense (S) probes to whole mounts of brain, liver, ovary, and testis of adult fish. Ventral preoptic area (VPA) and pituitary gland (P) are marked in lateral (top) and ventral (middle) views of brain. Bar, 0.5 mm.

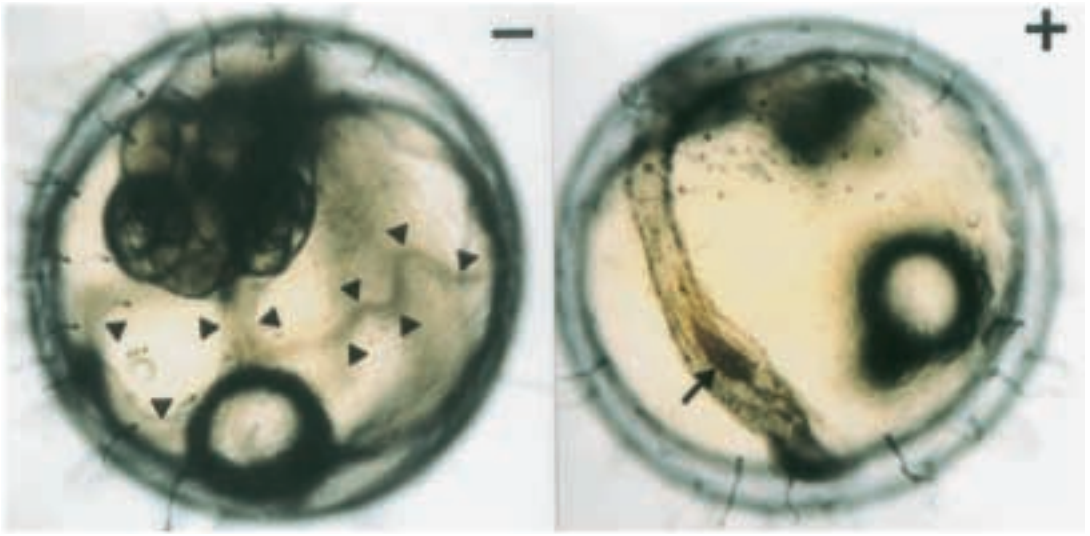

Fig. 5. Inhibition of the embryonic blood vessel development by estrogen. The embryos 8 hr after fertilization were incubated in Yamamoto's salt solution in the presence (+) or the absence (–) of 17β-estradiol (4 mg/l) for 3 days. Normally developed blood vessels are marked by arrowheads. The blood island 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.

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