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# Molecular Cloning of Estrogen Receptor α in the Masu Salmon, *Oncorhynchus masou*

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A cDNA encoding the masu salmon, *Oncorhynchus masou*, estrogen receptor α (msERα) was cloned from the pituitary gland by polymerase chain reaction (PCR). This cDNA contains an open reading frame encoding 513 amino acid residues, and the calculated molecular weight of this protein is about 56,430 Dalton. The amino acid sequences of the DNA binding and ligand binding domains of msERα showed high homology to those of other fish species (84-100%). Reverse transcription PCR analysis showed that the mRNA level of msERα in the pituitary was slightly higher in estradiol-17β (E2) injected masu salmon than that of control fish. To test the biological activity of msERα, the cDNA was ligated to a mammalian expression vector and transfected into a gonadotrope-derived cell line, LβT2, with a reporter plasmid including estrogen responsive element. Expression of the reporter protein, luciferase, was E2 and msERα- dependent. The masu salmon ERα is structurally conserved among teleost species and functions as a transcriptional activator in the pituitary cells.

**Keywords:** Masu salmon, Estrogen receptor α, cDNA cloning, Pituitary, Transcription

## Introduction

Estrogens play a role not only in growth, differentiation, and homeostasis of male and female reproductive organs, but also in liver, and cardiovascular system (Korach, 1994). It is generally accepted that the action of estrogen is mediated by a specific estrogen receptor (ER) present in the nucleus of target cells. The effects of estrogens are mediated by at least two receptors, i.e. ERα and ERβ, which are members of the nuclear receptor family (Katzenellenbogen and Korach, 1997). The nuclear receptor superfamily is a group of ligand-dependent transcriptional regulatory proteins that function by binding to specific DNA sequences named hormone response elements in the promoters of target genes (Mangelsdorf et al., 1995).

Estrogen-activated ERs regulate gene expression directly by binding to a specific *cis*-element called the estrogen responsive element (ERE) or indirectly by interacting with other transcription factors in the nucleus. ER is divided into six distinct regions (A-F domain), which have been shown to represent functionally independent domains. The C terminal region of the ligand binding domain of this protein (E/F domain) harbors an essential ligand-dependent transactivation function, activation function 2 (AF2), whereas the N terminus of some nuclear receptors includes activation function 1 (Mangelsdorf et al., 1995). C domain contains 8 cysteine residues, which form the zinc finger. This domain is the most conserved region in the nuclear receptor family including ERs.

In teleost species, ERs including γ-type have been identified by cloning the ER cDNAs from African catfish, *Clarias gariepinus* (Teves et al., 2003); Atlantic croaker, *Micropogonias undulates* (Hawkins et al., 2000); Atlantic salmon, *Salmo salar* (Rogers et al., 2000); channel catfish, *Ictalurus punctatus* (Xia et al., 1999, 2000); eelpout, *Zoarces viviparus* (Andreassen et al., 2003); gilthead sea bream, *Sparus aurata* (Munoz-Cueto et al., 1999; Socorro et al., 2000); goldfish, *Carassius auratus* (Tchoudakova et al., 1999; Ma et al., 2000; Choi and Habibi, 2003); Japanese eel, *Anguilla japonica* (Todo et al, 1996); mummichog, *Fundulus heteroclitus* (Urushitani et al., 2003); rainbow trout, *Oncorhynchus mykiss* (Pakdel et al., 1991, 2000); sheepshead minnow, *Cyprinodon variegates* (Karels and Brouwer, 2003); wrasse, *Halichoeres trimaculatus* (Kim et al., 2002) and zebrafish, *Danio rerio* (Bardet et al., 2002; Menuet et al., 2002). The estrogenic signaling pathway via ER was well studied in the liver including vitellogenin (VTG) gene regulation. In the liver ER has been shown to be autoregulated by estrogen both at the transcriptional and protein level (MacKay et al., 1996; Pakdel et al., 2000). Apart from the liver, ER mRNA has been identified in other tissues of

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fish, particularly in the brain (Salbert et al., 1993; Anglade et al., 1994), gonad (Tchoudakova et al., 1999; Socorro et al., 2000; Xia et al., 2000; Andreassen et al., 2003; He et al., 2003), and pituitary gland (Choi and Habibi, 2003; Teves et al., 2003; Menuet et al., 2003).

To understand better the function of estrogen receptor in the pituitary of fish species, an ERα cDNA containing the partial N terminus region and complete C-F domains from the pituitary gland of masu salmon (*Oncorhynchus masou*) was cloned. In addition, transcriptional activity of the msERα was examined in a mouse gonadotrope-derived cell line, LβT2.

## Material and Methods

### RNA preparation and cDNA synthesis

Maturing female masu salmons (~100 g) were kindly provided by the Yangyang Inland Fisheries Research Institute, National Fisheries Research and Development Institute, Korea. mRNA was extracted from their pituitary glands and liver using a mRNA purification kit (Amersham Phamacia, Sweden), following manufacturer's instructions. cDNA was synthesized from the extracted mRNA using first-strand cDNA synthesis kit and *Not*I-d$(T)_{18}$ primer (Amersham Phamacia) according to manufacturers protocol. The synthesized first-strand cDNA was used in the following cDNA amplification.

### cDNA amplification and cloning of masu salmon ERα (msERα)

Table 1 shows the nucleotide sequence of oligonucleotide primers used for cDNA synthesis and amplification of msERα. Degenerated ERα primers were based on a highly conserved region common to salmon species ERα (GenBank Accession No. AJ242740). An aliquot of the first-strand reaction was amplified with primers #1 and #3 for msERα (Table 1). Polymerase chain reaction (PCR) was performed in 50 μl final volume containing 5 μl of 10x reaction buffer, 2 mM $MgCl_2$, 200 μM dNTP, 2 μM of each primer, and 2.5 U LA *Taq* DNA polymerase (Takara Biomedicals, Japan). After an initial 5 min denaturing step at 94°C, 30 cycles of amplification were performed using a cycle profile of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1.5 min. After the last cycle, elongation was extended to 10 min at 72°C. PCR product was cut with *Eco* RI and *Xho* I, purified by gel-extraction method and cloned into *Eco* RI *Xho* I sites of pcDNA3 vector (Invitrogen, USA). An expected insert size was determined by *Eco* RI - *Xho* I restriction digestion and gel electrophoresis analysis. Plasmid DNA was sequenced in both strands by chain-termination method using a Big Dye Terminator Ready Reaction Mix (Perkin-Elmer/Applied Biosystems, USA) and an Applied Biosystems Prism 377 DNA Sequencer. The sequence identity was determined using the NCBI Blast search program.

### *In vivo* hormone treatment and reverse transcription-PCR (RT-PCR)

Cultured precocious masu salmon of mixed sexes (~50 g) were obtained from a commercial dealer. Each group was transferred to a glass aquarium containing 50 L of freshwater and kept in the tank under 14 h light-10 h dark photoperiod at 15-18°C (N=10).

Estradiol-17β (E2; Sigma, USA) was initially dissolved in ethanol and diluted with saline buffer to a final concentration (2.5 mg/ml). Fish received intraperitoneal injections of the E2 solution (5 mg/kg/0.1 ml) or vehicle as a control. After treatment of 3 days, pituitary glands were taken from the fish of each group. After decapitation, the pituitary gland was removed, immediately frozen in liquid nitrogen and stored at -80°C until mRNA purification.

For RT-PCR experiment, total RNA was extracted from female pituitaries (N=3) using an RNA extraction kit and reverse transcribed to first strand cDNA using MMLV reverse transcriptase and oligo-dT primer (Bioneer, Korea) according to manufactures instructions. PCR was performed in 50 μl final volume containing 5 μl of 10x reaction buffer including 1.0 μl of synthesized cDNA, 15 mM $MgCl_2$, 2.5 mM dNTP, 1.6 μM of each primer (#2 and #3 for ERα; #4 and #5 for β-actin) (Table 1), and 2.5 U *Taq* DNA polymerase (Bioneer, Korea). After an

**Table 1.** Primers used in the polymerase chain reactions

| Primer | Direction | Sequence |
|---|---|---|
| #1 (ER) | F | 5-CGGAATTCATGGTGTTTGTGTCCTCC-3' |
| #2 (ER) | F | 5-ATGCAGTCATACTACCTGGAG-3' |
| #3 (ER) | R | 5-CCGCTCGAGTCACGGAATGGGCATCT-3' |
| #4 (Actin) | F | 5-AGACATCAAGGAGAAGCTGTG-3' |
| #5 (Actin) | R | 5-TCCAGACGGAGTATTTAC-3' |

F=Forward; R=Reverse

initial 5 min denaturing step at 94°C, 30 cycles of amplification were performed using a cycle profile of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1.5 min. After the last cycle, elongation was extended to 7 min at 72°C. PCR products were separated by 1.0% agarose gel electrophoresis containing 200 ng/ml ethidium bromide.

### Transient transfections and luciferase assay

LβT2 cells, a mouse gonadotrope-derived cell line, were generously provided by Dr. Pamela L. Mellon (University of California, San Diego, USA). The cells were cultured at 37°C with 5% $CO_2$ in DMEM medium (GIBCO BRL, USA) containing 10% fetal bovine serum and 1% antibiotic-antimycotic (10,000 U/ml of penicillin, 10 mg/ml of streptomycin sulfate and 25 ug/ml amphotericin B; GIBCO BRL). The cells were grown in 24-well plates with medium supplemented with 10% charcoal-stripped serum. Cells were transfected 24 hr later with 100 ng of a luciferase reporter plasmid (pGL3; Promega, USA) containing an estrogen responsive element (ERE) of vitellogenin promoter in *Xenopus* (Jung et al., 2001) and 50-100 ng of msERα in pcDNA3 plasmid vector (Invitrogen, USA) by liposome-mediated method (Lipofectamine, Invitrogen) according to the protocol of the manufacturer. All of the plasmid DNAs were purified by Qiagen Midi column (Germany). After 24 hr, cells were treated for 20 hr with 1 μM E2 or equal volume of ethanol as a control. Subsequently, the cells were washed with ice-cold phosphate-buffered saline and lysed with 100 μl of 1x cell lysis buffer (Promega, USA). Luciferase assay was performed using a microplate-luminometer (BERTHOLD, Germany) and assay buffer (1 mM luciferin, 2.16 mM ATP, 10.8 mM $MgCl_2$, 90 mM $KH_2PO_4$) as previously reported (Jung et al., 2001).

## Results and Discussion

### Cloning and sequence analysis of masu salmon estrogen receptor α

The nucleotide and deduced amino acid sequences of the cloned msERa are shown in Fig. 1, and deposited in DDBJ/EMBL/GenBank-linked GENENURI under Accession No. KS109190 (GenBank Accession No. AY520443). The cDNA contains 1542 bp and an open reading frame encoding 513 amino acid residues, and the molecular weight of this protein is about 56,430 Dalton. When the overall nucleotide sequence of the msERa was compared with those of fish species, there were sequence similarities ranging from 85 to 98%. Although

```
   1 atggtgtttgtgtcctccagcccccagctcagcccccagctcagc
     M  V  F  V  S  S  S  P  Q  L  S  P  Q  L  S
  46 cccttcctccacccccaagccaccatggtctccccagccagtca
     P  F  L  H  P  P  S  H  H  G  L  P  S  Q  S
  91 tactacctggagacctcgtccacacccttatacaggtcgagtgtg
     Y  Y  L  E  T  S  S  T  P  L  Y  R  S  S  V
 136 gtaaccaatcagctgtcagcgtcagaggagaagctctgcatcgcc
     V  T  N  Q  L  S  A  S  E  E  K  L  C  I  A
 181 tccgataggcagcagtcatacagtgcagcagggtcaggggtcagg
     S  D  R  Q  Q  S  Y  S  A  A  G  S  G  V  R
 226 gtgtttgagatggccaacgagacgaggtactgtgcggtctgcagc
     V  F  E  M  A  N  E  T  R  Y  C  A  V  C  S
 271 gactttgcctctgggtaccactacggagtttggtcctgcgagggc
     D  F  A  S  G  Y  H  Y  G  V  W  S  C  E  G
 316 tgcaaagccttcttcaaaaggagcatccaaggtcacaatgactac
     C  K  A  F  F  K  R  S  I  Q  G  H  N  D  Y
 361 atgtgccctgcgactaaccagtgtacaatggacaggaatcgtagg
     M  C  P  A  T  N  Q  C  T  M  D  R  N  R  R
 406 aagagctgccaggcctgccgcctcagaaagtgttatgaagtgggg
     K  S  C  Q  A  C  R  L  R  K  C  Y  E  V  G
 451 atggtgaaaggaggcttgcgtaaggaccggggtgggcgggttctc
     M  V  K  G  G  L  R  K  D  R  G  G  R  V  L
 496 aggaaggataagcggtattgtggccctgctggtgacagagagaaa
     R  K  D  K  R  Y  C  G  P  A  G  D  R  E  K
 541 ccctacggtgacctagagcacaggacagcgcccccctcaggacggg
     P  Y  G  D  L  E  H  R  T  A  P  P  Q  D  G
 586 gttaggaacagcagcagcagtctcaatggtggtggaggatggcgt
     V  R  N  S  S  S  S  L  N  G  G  G  G  W  R
 631 gggcctagaatcaccatgcctcctgaacaggtgctgttcctgctg
     G  P  R  I  T  M  P  P  E  Q  V  L  F  L  L
 676 caggggcagagcctccggccctgtgttctcgtcagaaggtggcc
     Q  G  A  E  P  P  A  L  C  S  R  Q  K  V  A
 721 cgcccctacacagaggtcaccatgatgaccctgctcaccagcatg
     R  P  Y  T  E  V  T  M  M  T  L  L  T  S  M
 766 gctgacaaggagctggtgcacatgatcgcttgggctaagaaagta
     A  D  K  E  L  V  H  M  I  A  W  A  K  K  V
 811 ccaggtttccaggagctgtctctccatgaccaggtgcagctgctg
     P  G  F  Q  E  L  S  L  H  D  Q  V  Q  L  L
 856 gagagttcctggctggaggtgctgatgatcggactcatatggcgg
     E  S  S  W  L  E  V  L  M  I  G  L  I  W  R
 901 tccatccactgccctgggaaactcatcttcgcccaggacctcata
     S  I  H  C  P  G  K  L  I  F  A  Q  D  L  I
 946 ctggacaggagtgaaggggactgtgtggagggcatggctgagatc
     L  D  R  S  E  G  D  C  V  E  G  M  A  E  I
 991 ttcgacatgctcctggccactgtgtctcgcttccgcatgctcaag
     F  D  M  L  L  A  T  V  S  R  F  R  M  L  K
1036 ctgaagcctgaggagtttttgtgcctcaaagccatcatcttgctc
     L  K  P  E  E  F  L  C  L  K  A  I  I  L  L
1081 aactctggtgccttctccttctgttctaactctgtggagtccctc
     N  S  G  A  F  S  F  C  S  N  S  V  E  S  L
1126 cacaacagctcggcagtggaaagcatgctggacaacatcaccgac
     H  N  S  S  A  V  E  S  M  L  D  N  I  T  D
1171 gccctcatccaccacatcagccattcaggagcctctgtgcagcag
     A  L  I  H  H  I  S  H  S  G  A  S  V  Q  Q
1216 caacccagacggcaggcccagctcctgctcctgctctcacacatc
     Q  P  R  R  Q  A  Q  L  L  L  L  L  S  H  I
1261 agacatatgagcaacaaaggcatggagcacctttacagcataaaa
     R  H  M  S  N  K  G  M  E  H  L  Y  S  I  K
1306 tgtaagaacaaagtgcctctgtatgacctgctcctggagatgctg
     C  K  N  K  V  P  L  Y  D  L  L  L  E  M  L
1351 gatggtcaccggctccaatccccaggcaaagtggcccaagctggg
     D  G  H  R  L  Q  S  P  G  K  V  A  Q  A  G
1396 gaacagaccgagggcccctctaccaccactaccacctccacaggc
     E  Q  T  E  G  P  S  T  T  T  T  T  S  T  G
1441 tccagcatagggccgatgcgaggcagccaggatacccacatcaga
     S  S  I  G  P  M  R  G  S  Q  D  T  H  I  R
1486 agccctggggtactccagtatggctcccccagctcagaccagatg
     S  P  G  V  L  Q  Y  G  S  P  S  S  D  Q  M
1531 cccattccgtga 1542
     P  I  P  *
```

**Fig. 1.** The nucleotide and deduced amino acid sequences of masu salmon estrogen receptor α. The numbers in the left margin refer to the first nucleotide on the corresponding line. A stop codon is indicated by an asterisk and the primers used in cloning are underlines. The nucleotide sequence data reported in this paper was submitted to the DDBJ/EMBL/GenBank-linked GENENURI under Accession No. KS109190 (GenBank Accession No. AY520443).

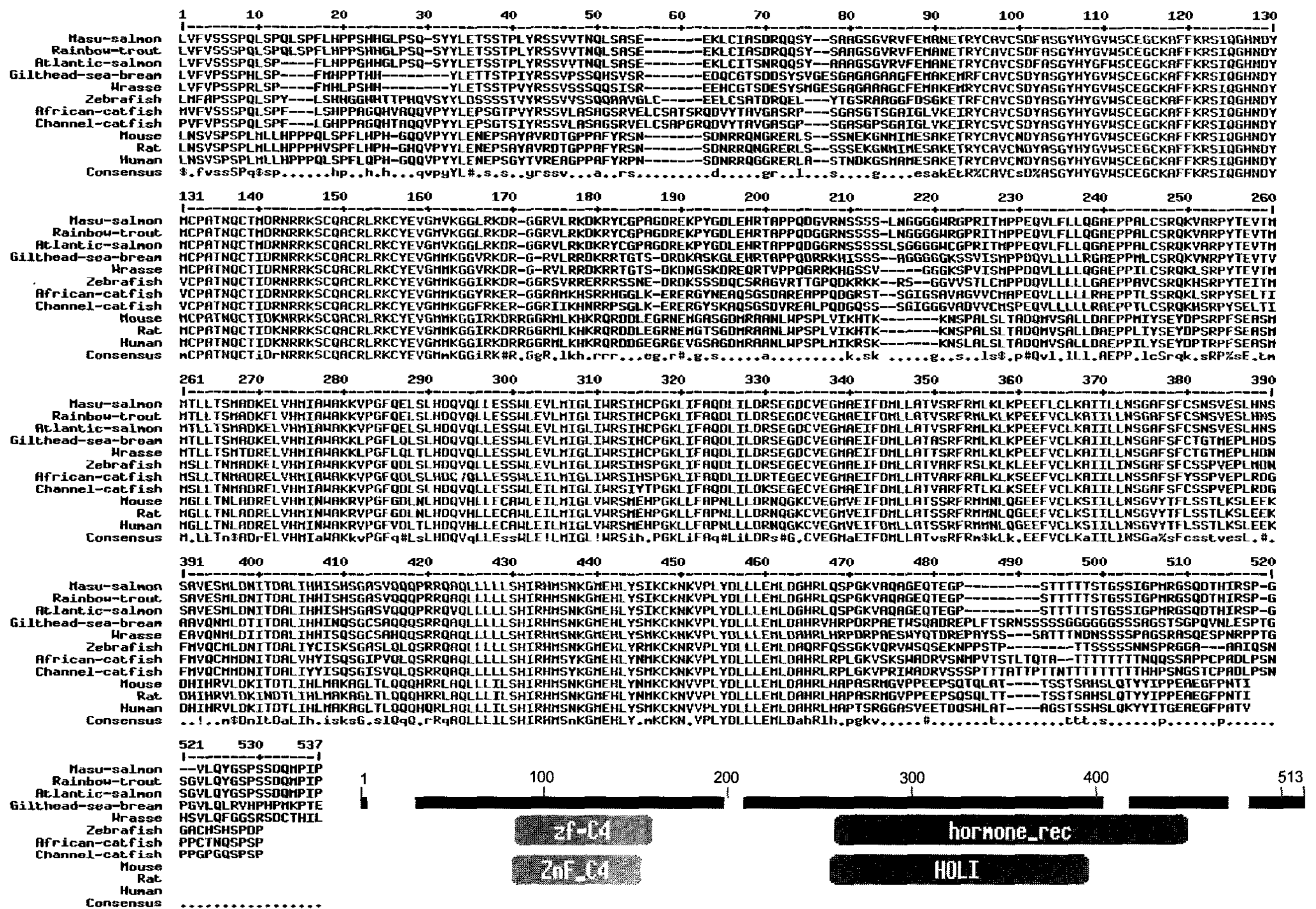

**Fig. 2.** Multiple sequence alignment of estrogen receptor a family in vertebrates and conserved core domains (zf-C4, zinc finger domain; hormone_rec, hormone binding domain) of masu salmon estrogen receptor α. The sequences used in the alignment include rainbow trout (AJ242740), Atlantic salmon (X89959), gilthead sea bream (AJ006039), wrasse (AY305026), zebrafish (AB037185), African catfish (X84743), channel catfish (AF061275), mouse (P19785), rat (X61098) and human (M12674). The alignment was performed using the MultAlin program (www.toulouse.inra.fr/multalin.html). The amino acid numbers of each protein are indicated.

the complete N terminus of the msERa could not be obtained, the most conserved regions of fish estrogen receptors, i.e. zinc finger domain (DNA binding domain, DBD) and ligand binding domain (LBD), were identified in the present study (Fig. 2). Moreover, the DBD and LBD regions of ERα proteins showed high identities between mammals and masu salmon (68-92%), but overall amino acid sequence had low identities (49-50%). The sequence homologies and phylogenetic relationships of various ERs suggest that masu salmon ER should be considered as an ERα-like receptor, and is also true for most other teleost ERs described to date (Munoz-Cueto et al., 1999; see Fig. 3).

### Effect of estradiol-17β on estrogen receptor α mRNA level in the pituitary

To understand the pituitary ER in teleost, feedback effect of estradiol-17β (E2) on msERα transcript was investigated by RT-PCR products in the pituitary of masu salmon treated by E2. RT-PCR analysis showed that the PCR product of msERα was slightly higher in E2-injected masu salmon pituitary than that of control fish (Fig. 4). In mammals, it was found that the estrogen up-regulates mRNA expression levels of ERα and ERβ in the pituitary (Tena-Sempere et al., 2001) and subsequently regulates transcription of downstream target genes. Although little is known about the effect of estrogen on the pituitary ERs in teleost species, potential activity of E2 on gonadotropin mRNA levels in the pituitary was found in many species (Sohn et al., 1998; for review see Yaron et al., 2003). To investigate detailed pituitary-specific mRNA expression of msERα, further studies will be necessary.

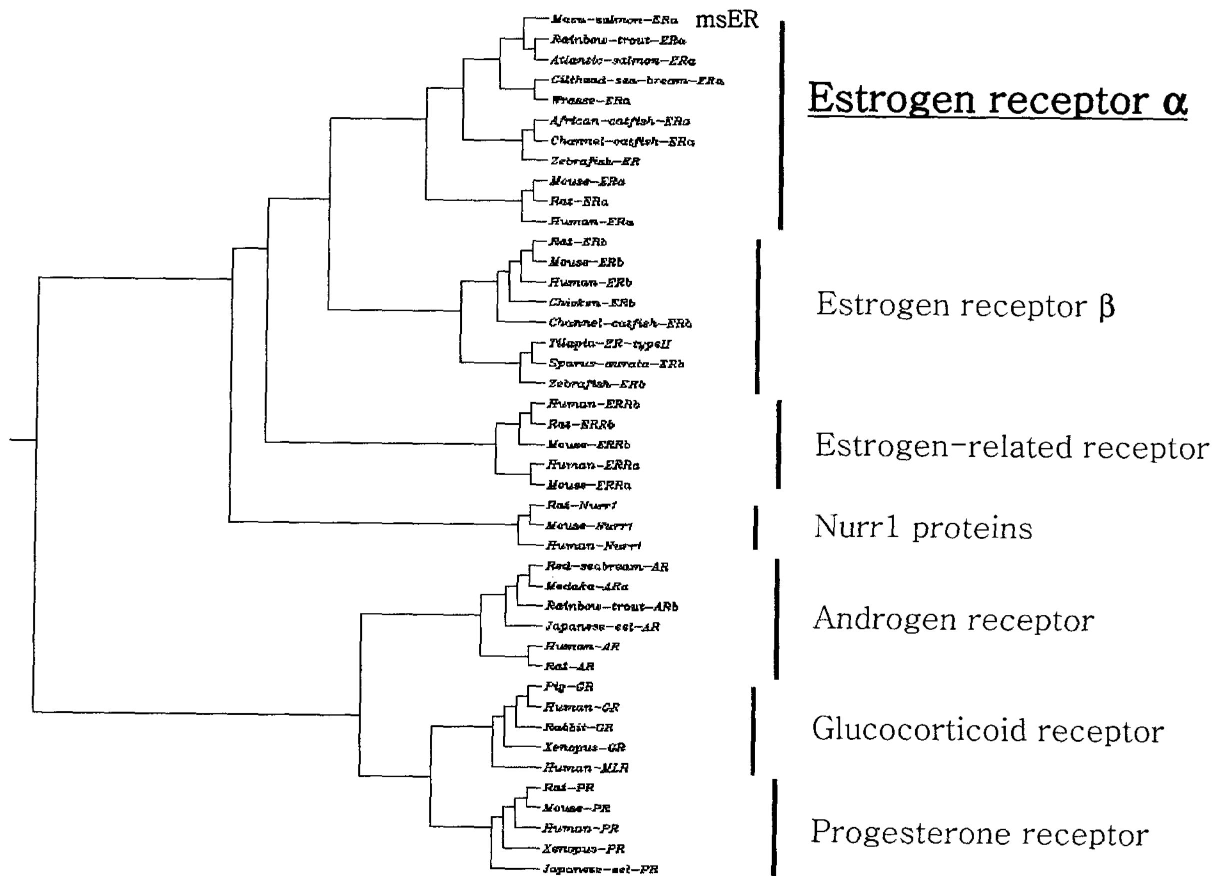


**Fig. 3.** Neighbor-joining phylogenetic tree analysis of nuclear receptor family in different fish and mammalian genes based on amino acid sequences. The sequences were aligned using ClustalW multiple alignment software (www.ddbj.nig.ac.jp/E-mail/clustalw-e.html).

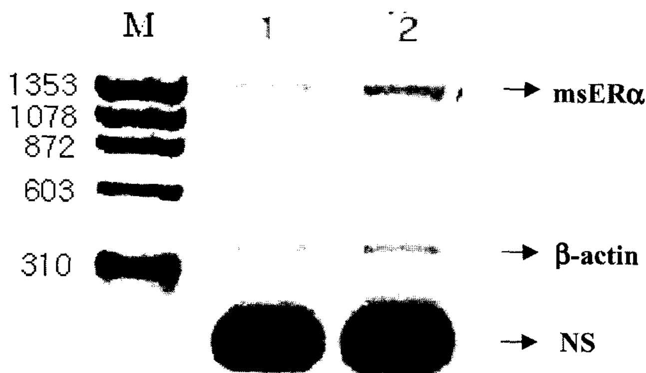


**Fig. 4.** Effect of estradiol-17β (E2) on estrogen receptor α mRNA in maturing female masu salmon. The level of ERα expression was analyzed by reverse transcription polymerase chain reaction method (see Materials and Methods) (lane 1: control fish; lane 2: E2-injected fish, 5 mg/kg body weight). NS, nonspecific bands (oligo dimers).

**Transcriptional activity of masu salmon estrogen receptor α**

To verify the implication of msERα in the pituitary cells, transient transfection experiments were conducted. A mouse gonadotrope-derived cell line, LβT2, was chosen for the reason of the presence of ER and a representative cell model for gonadotropin gene expression (Vasilyev et al., 2002). The transcriptional activity of the msERα was assessed by cotransfection of a luciferase reporter driven by a promoter under the control of one estrogen responsive element (ERE-Luc). The luciferase reporter was activated about 2-fold in the presence of msERα and E2 (Fig. 5). Although the reporter activity was not so strong, estrogen-dependent activation was shown in this cell line. Pakdel and colleagues reported that transcriptional activity of N-terminal deletion mutant construct (Δ1-142) of rainbow trout ER was similar to the full-length receptor (Metivier et al., 2000), confirming that ligand-dependent AF-2 domain in C terminus of ER has a dominant transactivation function. Testing the natural promoter of masu salmon pituitary with msERα should provide information about the importance of physiological roles of estrogen signals in the pituitary of teleost species.

In the present study, a partial cDNA encoding the estrogen receptor (msERα) was cloned from the pituitary gland in the masu salmon. The deduced amino acid sequence of the msERα showed high homology of the DNA binding and ligand bind-

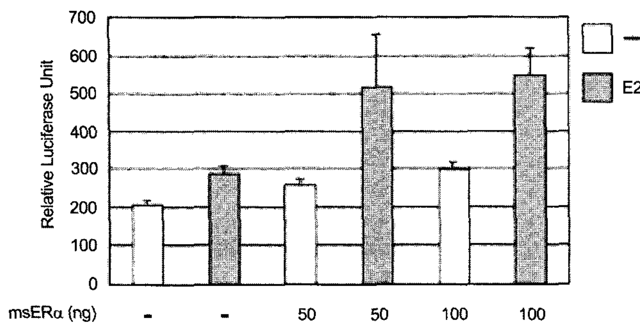


**Fig. 5.** Transcriptional activity of masu salmon estrogen receptor α. LβT2 cells were transfected with expression plasmid (50 or 100 ng) containing masu salmon estrogen receptor α (msERα) and a reporter plasmid including luciferase driven by estrogen responsive element (100 ng). - and E2 indicate the absence and presence of 1 μM estradiol-17β. Luciferase expression levels from duplicate samples were shown with standard deviations.

ing domains with those of other fish species. Studies on estrogen responsibility of msERα using RT-PCR analysis and promoter assay suggested that msERα is dependent on E2 and stimulates transcription of genes including estrogen responsive element in the pituitary cells.

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