Title	Hepatic estrogen-responsive genes relating to oogenesis in cutthroat trout (Oncorhynchus clarki) : The transcriptional induction in primary cultured hepatocytes and the in vitro promoter transactivation in responses to estradiol-17 beta
Author(s)	Nagata, Jun; Mushirobira, Yuji; Nishimiya, Osamu; Yamaguchi, You; Fujita, Toshiaki; Hiramatsu, Naoshi; Hara, Akihiko; Todo, Takashi
Citation	General and Comparative Endocrinology, 310, 113812 https://doi.org/10.1016/j.ygcen.2021.113812
Issue Date	2021-09-01
Doc URL	http://hdl.handle.net/2115/86759
Rights	© 2021. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
Rights(URL)	http://creativecommons.org/licenses/by-nc-nd/4.0/
Type	article (author version)
File Information	text_21_04_28.pdf

Hepatic estrogen-responsive genes relating to oogenesis in cutthroat trout (*Oncorhynchus clarki*): the transcriptional induction in primary cultured hepatocytes and the *in vitro* promoter transactivation in responses to estradiol-17β

Jun Nagata [a, 1, *], **Yuji Mushirobira** [b], **Osamu Nishimiya** [c], **You Yamaguchi** [d] **Toshiaki Fujita** [e], **Naoshi Hiramatsu** [a], **Akihiko Hara** [a], **Takashi Todo** [a]

[a] *Division of Marine Life Science, Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido, 041-8611, Japan*

[b] *Institute for East China Sea Research, Organization for Marine Science and Technology, Nagasaki University, 1551-7 Taira, Nagasaki, 851-2213, Japan*

[c] *South Ehime Fisheries Research Center, Ehime University, 25-1 Uchidomari, Ainan, Ehime 798-4206, Japan*

[d] *Division of Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido, 041-8611, Japan*

[e] *Faculty of Engineering, Hachinohe Institute of Technology, 88-1 Obiraki, Myo, Hachinohe, Aomori 031-8501, Japan*

[1] *Present address: Fisheries Research Department, Abashiri Fisheries Research Institute, Hokkaido Research Organization, 1-1, Masuura, Abashiri, Hokkaido 099-3119, Japan*

[*] *Corresponding author: E-mail address, nagata-jun@hro.or.jp*

Abstract (365 words)

Estradiol-17β (E2) regulates transcription of estrogen-responsive genes *via* estrogen receptors (Esr). In many teleost species, choriogenin (*chg*), vitellogenin (*vtg*) and *esr* genes are transactivated by E2 in the liver. This study aimed i) to compare expression properties of all subtypes of these genes (*chg*: *chgHα*, *chgHβ*, *chgL*; *vtg*: *vtgAs*, *vtgC*; *esr*: *esr1a*, *esr1b*, *esr2a*, *esr2b*) in response to estrogen stimulation, and ii) to confirm how each of four Esr subtypes is involved in the transcriptional regulation of these estrogen-responsive genes in cutthroat trout hepatocytes. In hepatocytes in primary culture, all *chg* and *vtg* subtype mRNA levels, and those of *esr1a*, were increased by E2 treatment (10^{-6} M) at 24 and 72 h post initiation (hpi), but *esr1b*, *esr2a* and *esr2b* mRNA levels were not. Treatment of hepatocytes with various concentrations of E2 (10^{-11} ~ 10^{-6} M) induced dose-dependent increases in the levels of all *chg* and *vtg* subtype mRNAs at 24 and 72 hpi. At both time points, the lowest dose that induced a significant increase in the expression levels of mRNAs (LOEC) for E2 differed among the genes; LOECs were estimated as 10^{-11} M for *chgHα* at 24 hpi, as 10^{-9} M for *vtgC* at 72 hpi, and as 10^{-10} M for other mRNAs at both 24 and 72 hpi. Meanwhile, the levels of *esr1a* mRNA exhibited a dose-dependent increase at 24 and 72 hpi, but the LOEC shifted from 10^{-9} M at 24 hpi to 10^{-7} M at 72 hpi because of a decrease in mRNA levels at treatment groups exposed to high concentrations of E2. All Esr subtypes transactivated *chg*, *vtg* and *esr1a* promoters in the presence of E2 *in vitro*. The activation levels indicated that promoter activity of *chgHα* ≥ *vtgAs* > *chgHβ* > *chgL* ≥ *vtgC* ≥ *esr1a* when mediated by Esr1a, *chgHβ* > *chgHα* > *chgHL* > *vtgAs* ≥ *vtgC* ≥ *esr1a* by Esr1b, *chgHβ* ≥ *chgL* > *chgHα* ≥ *vtgAs* > *vtgC* > *esr1a* by Esr2a, and *chgHβ* ≥ *chgHα* ≥ *vtgAs* > *chgL* ≥ *vtgC* >*esr1a* by Esr2b. Collectively, different Esr subtypes were distinctly different in their ability to transactivate estrogen-responsive target genes, resulting in differential expression of *chg*, *vtg* and *esr1a* genes in the estrogen-exposed hepatocytes.

Keywords: estrogen, estrogen receptor, choriogenin, vitellogenin, transactivation

1. Introduction

Estrogens are steroid hormones that regulate vertebrate reproduction, development, growth and sexual homeostasis (Heldring et al., 2007). Estrogens, estradiol-17β (E2) in most cases, generally act on target cells through a nuclear estrogen receptor (Esr/ESR). The Esr/ESR is a member of the nuclear receptor superfamily of ligand-activated transcription factors, which includes receptors for steroid hormones, for thyroid hormones, for vitamins and for ligands that have yet to be identified (Sladek, 2011). The molecular mechanism underlying E2-Esr/ESR dependent transactivation of its target gene is generally accepted to be as follows: after E2 binds to Esr/ESR in the cell, the complex of E2 and Esr/ESR forms a homodimer that binds to estrogen-responsive elements (ERE) that are present in the promoter regions of targeted genes to induce expression of the target gene. The most typical ERE is composed of two head-to-head GGTCA half sites separated by three nucleotides (5'-GGTCAnnnTGACC-3', Walker et al., 1984).

Circulating E2 regulates expression of hepatic genes that are important for oocyte development in teleosts. As is well known, synthesis of vitellogenin (Vtg), the precursor of yolk protein, is induced by E2 in the liver of oviparous vertebrates. In addition to Vtg, choriogenin (Chg), the precursor of chorion protein, is produced in the livers of many teleosts, including salmonids (Hara et al., 2016). Chg is a glycoprotein belonging to the zona pellucida (ZP) superfamily (Goudet et al., 2008). Both Vtg and Chg are secreted into the blood stream, transported to oocytes, and incorporated in oocytes as yolk proteins and deposited onto oocytes as chorions, respectively. Yolk proteins serve as a source of nutrients for embryonic development and larval growth. Chorions protect the eggs and the embryos from physical and environmental stressors (Grierson and Neville, 1981; Songe et al., 2016).

So far, cDNAs encoding ESR orthologs have been cloned and characterized in various vertebrates, including teleosts. The presence of two forms of ESR, designated as ESR1 and ESR2, has been confirmed in most vertebrates, while most teleosts exhibit at least three distinct subtypes of Esrs, i.e., Esr1 (also designated as Erα), Esr2a (also known as Erγ or Erβ1) and Esr2b (also known as Erβ or Erβ2) (Choi and Habibi, 2003; Halm et al., 2004; Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2004; Nagler et al., 2007). Of the *esr* subtypes, hepatic *esr1* (*esr1a* in salmonids) expression is high in the liver of vitellogenic females (Nagler et al., 2012; Sabo-Attwood et al., 2004) and induced by E2 treatment (Boyce-Derricott et al., 2009; Filby and Tyler, 2005; Sabo-Attwood et al., 2004). In rainbow trout (*Oncorhynchus mykiss*), an additional *esr1* subtype (*esr1b*) has been identified (Nagler et al., 2007), considered to be a minor subtype in terms of its mRNA levels in the liver of vitellogenic females and E2-treated fish (Boyce-Derricott et al., 2009; Nagler et al., 2012). So far, the functionality of Esr1b protein (ligand-binding and estrogen-dependent transactivation qualities) has not been confirmed yet. As for Esr2 subtypes, recent studies using zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*) have demonstrated their possible involvement in the expression of *vtg* (Griffin et al., 2013; Nelson and Habibi, 2010). Other than these studies, both on cyprinids, functional information on teleost Esr subtypes pertaining to the regulation of hepatic expression of estrogen-responsive genes important for oogenesis (i.e., *chg*, *vtg* and *esr1* etc.) has been quite limited.

Widespread multiplicity of *chg*/Chg and *vtg*/Vtg has become evident in teleosts. Chgs are categorized into high (ChgH) and low type (ChgL) based on their molecular weight (Hara et al., 2016). These ChgH and L typically belong to the ZPB and C subfamilies, respectively, based on the unified nomenclature system for the ZP gene family (Goudet et al., 2008). ChgH in salmonids can be further classified into ChgHα and

ChgHβ (Fujita et al., 2008; Hyllner et al., 2001; Westerlund et al., 2001). Meanwhile, highly evolved acanthomorph species are likely to express three Vtg subtypes, VtgAa (previously termed VtgA), VtgAb (previously termed VtgB), and VtgC (Hiramatsu et al., 2005). Salmonids, on the other hand, express multiple copies of salmonid-type A Vtg (VtgAs) alongside a VtgC orthologue (Buisine et al., 2002; Mushirobira et al., 2018; Trichet et al., 2000).

As described above, E2 regulates the expression of oogenesis-related genes, such as *chg*, *vtg* and *esr* subtypes, in the liver of teleosts during vitellogenesis. However, it is unclear how E2 can differentially regulate the expression profiles of these genes. Resolving this issue leads to further our understanding of oocyte development, and of the molecular mechanisms underlying the transcriptional regulation of multiple genes by E2. To date, many studies have demonstrated responses of *chg*, *vtg* and *esr1* genes to E2 stimulation. Because each of these studies used different species and/or did not consider the multiplicity of *chg*, *vtg* and *esr*, it has been difficult to obtain an integrated view of transcriptional regulation of these genes in response to E2 stimulation; it is therefore needed to perform such a study in a single species and include all subtypes of the genes of interest. Recently, the two promoters of *vtgAs* (1 and 2) and one promoter of *vtgC* for cutthroat trout (*Oncorhynchus clarki*) have been cloned and analyzed (Mushirobira et al., 2018).

The objective of the present study was *i*) to reveal the properties of estrogen-induced transcription of nine hepatic genes (i.e., *chg*: *chgHα*, *chgHβ*, *chgL*; *vtg*: *vtgAs*, *vtgC*; *esr*: *esr1a*, *esr1b*, *esr2a*, *esr2b*), and *ii*) to evaluate the involvement of each of the four Esr subtypes in the transcriptional regulation of these estrogen-responsive genes, using our model salmonid, the cutthroat trout. To achieve this objective, the present study utilized primary hepatocyte culture to observe and compare the estrogen-induced transcriptional properties of the nine genes. In addition, the functional property of four Esrs in driving the expression of their putative target (*chg*, *vtg* and *esr*) genes was investigated by reporter gene assays using reporter vectors containing promoter regions of the target genes.

2. Materials and methods

2.1. Experimental fish and tissue sampling

Cutthroat trout used in this study were obtained from a breeding stock held in flow-through fresh water under ambient conditions at Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido University (Nanae, Japan). Fish were reared in outdoor tanks at the Faculty of Fisheries Sciences, Hokkaido University (Hakodate, Japan), receiving a continuous flow of well water under natural photothermal conditions. The fish were anesthetized by 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan) before sampling. All experimental procedures involving live fish followed the policies and guidelines of the Hokkaido University Animal Care and Use Committee.

For cloning of *esr* cDNAs (section 2.2), livers were collected from females with the following characteristics: for *esr1b* and *esr2a*, body weight (BW): 355.3 g, total length (TL): 320 mm, gonadosomatic index (GSI: gonad weight/body weight × 100): 5.8; for *esr2b*, BW: 129 g, TL: 222 mm, GSI: 0.3. Tissue samples were immediately immersed in ice-cold RNA later (Thermo Fisher Scientific, Waltham, MA, USA), incubated overnight at 4°C, and stored at −30°C until used for RNA extraction.

A two-year-old female cutthroat trout was used in order to clone the promoters of each gene (section 2.3). Blood was collected and immediately mixed with ethylenediaminetetraacetic acid (EDTA) to a final concentration of 17 mM. This whole blood sample was stored at −80°C until used as a source for genomic DNA extraction.

2.2. Molecular cloning of cutthroat trout esr1b, esr2a and esr2b

Total RNA was extracted from the liver samples with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Concentration of total RNA was measured with NanoDrop ND-1000 (Thermo Fisher Scientific). One μg total RNA was reverse-transcribed by PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. The resulting cDNA templates were stored at −30°C. Primer sets (Table 1) were designed using sequence data from rainbow trout *esr1b* (NM_001124558), *esr2a* (NM_001124753) and *esr2b* (NM_001124570.1) to amplify the respective open reading frames. PCR for each *esr* subtype was performed using PrimeSTAR® Max DNA Polymerase (Takara Bio) in a volume of 10 μl, which contained 1 μl 1st-strand cDNA, 5 μl PrimeSTAR® Max premix, 1 μl of each 1 μM forward and reverse primer and 2 μl nuclease-free water. The PCR amplification was carried out using the following thermal parameters: 40 (*esr1b* and *esr2a*) or 35 (*esr2b*) cycles at 98°C for 10 s, 55°C for 5 s and 72°C for 3 min. The PCR products were separated by electrophoresis on 1% agarose gels, excised from the gels, and purified by GENECLEAN Turbo Kit (MP-Biochemicals, Santa Ana, CA, USA) according to the kit manual. The PCR products were subjected to an A-tailing reaction (Knoche and Kephart, 1999) followed by ligation into pGEM-T Easy Vector (Promega, Madison, WI, USA) according to the manufacturer's instruction. The ligated products were transformed into XL1-Blue competent cells (Stratagene, La Jolla, CA, USA). Recombinant clones were cultured overnight at 37°C on an agar plate containing ampicillin and tetracycline, followed by selection of colonies of interest by blue-white screening. Selected clones were grown in culture medium, and then used to extract and purify plasmid DNA by Wizard Plus SV Minipreps DNA Purification System (Promega). Purified plasmid DNAs were sequenced using BigDye terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and a 3130xl Genetic Analyzer (Thermo Fisher Scientific) according to the manufacturer's protocol.

2.3. Molecular cloning of chg, vtg and esr1a gene promoters

Genomic DNA was extracted from whole blood as described in Mushirobira et al. (2018). A genome walking library was used for amplification of *chgHβ*, *chgL* and *esr1a* gene promoters. Four genome walking libraries were made from genomic DNA using the GenomeWalker Universal Kit (Takara Bio) according to manufacturer’s protocol. Gene specific primers (GSP, sense primer) were designed within the coding region of each gene (Table 1). Primary PCR was carried out after mixing 5 µl of PrimeSTAR Max Premix (Takara Bio), 0.2 µl of adaptor primer (AP, antisense primer) 1, 0.2 µl of 10 µM GSP (for *chgHβ*: chgHβ-GW(Genome Walker)-R1, for *chgL*: chgL-GW-R1, for *esr1a*: esr1a-GW-R1), and 0.2 µl of genomic DNA template; 7 cycles of 98°C for 10 s and of 72°C for 3 min were then run, followed by 32 cycles of 98°C for 10 s and of 68°C for 3 min. Nested PCR was carried out using 5 µl of PrimeSTAR Max Premix, 0.2 µl of AP1, 0.2 µl of 10 µM GSP-2 (for *chgHβ*: chgHβ-GW-R2, for *chgL*: chgL-GW-R2, for *esr1a*: esr1a-GW-R2), and 0.2 µl of the PCR product from the first round of amplification; after running 5 cycles at 98°C for 10 s and at 72°C for 3 min, a further 20 cycles were run at 98°C for 10 s and at 68°C for 3 min.

Primers (Table 1) for amplification of *chgHα*, *vtgAs* and *vtgC* promoters were designed from rainbow trout whole genome (RefSeq assembly accession:

GCF_002163495.1). PCR was carried out as follows: 5 µl of PrimeSTAR Max Premix, 1 µl of each 2 µM forward and reverse primer, and 0.2 µl of genomic DNA template were mixed and DNA amplified at 35 cycles of 98°C for 10 s, 55°C for 10 s and 72°C for 3 min.

The PCR products were ligated into cloning vectors (pGEM-T Easy Vector), transformed into XL1-Blue competent cells, and sequenced as described above.

2.4. Computational search for putative transcription factor binding sites

Two online algorithms (ConSite; http://asp.ii.uib.no:8090/cgi-bin/CONSITE/ and NUBIScan; http://www.nubiscan.unibas.ch/) were used for prediction of transcription factor binding sites, including both basal promoter elements and putative EREs. The specifics of ConSite and NUBIScan, were described previously by Sandelin et al. (2004) and Podvinec et al. (2002), respectively.

2.5. Primary culture of hepatocytes

For Experiments 1 and 2 described below, hepatocytes were isolated from one male cutthroat trout for each experiment, using a two-step collagenase perfusion technique as described previously (Klaunig et al., 1985) with modifications; briefly, trout (body weight, BL: 420 ~ 480 g; total length, TL: 314 ~ 410 mm) were anesthetized in 2-phenoxyethanol). The liver was perfused with 50 ml of Ca-free modified Hanks solution (137 mM NaCl, 5.4 mM KCl, 0.5 mM NaH_2PO_4, 0.42 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 5 mM glucose, 0.5 mM EGTA, 10 mM HEPES, pH 7.4) to remove the blood from the liver. The liver was then perfused with 100 ml of the same solution without EGTA and glucose and with added $CaCl_2$ (5 mM), 0.05% collagenase (Wako, Tokyo, Japan) and 0.005% trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Perfusion was performed at room temperature using a peristaltic pump (Iwaki, Tokyo, Japan) at a flow rate of 10 ml/min. After perfusion, the hepatocytes were dispersed in ice-cold L-15 medium (Thermo Fisher Scientific) supplemented with 10 mM HEPES (pH 7.4), 1% of an antibiotic + antimycotic solution (final concentration: 100 units/ml penicillin, 100 μg/ml streptomycin sulfate and 250 ng/ml amphotericin B; Wako) and 5% fetal bovine serum (FBS, Thermo Fisher Scientific). The cell suspension was filtered through a cell strainer (Ikemoto Rika, Tokyo, Japan) and the filtrate centrifuged at 100 g for 90 s at 4°C. The pellet was resuspended in fresh medium and re-spun at 100 g for 2 min at 4°C; altogether, the cell pellet was washed three times. Cell viability was about 95% as determined by the trypan blue exclusion test. Cells were plated at a density of 3×10^5 cells/well on 24-well Falcon Primaria Multiwell plates (Corning, New York, NY, USA) that were coated with Matrigel (basement membrane matrix, Corning) as described by Schreer et al. (2005). Cells were cultured at 15°C in 0.5 ml of L-15 supplemented with 10 mM HEPES (pH 7.4), 1% antibiotic + antimycotic solution, 5% FBS and 10 μg/ml bovine insulin (Sigma-Aldrich). The cultured cells were settled for 24 h in order to adhere to the culture plate.

2.6. Hormone treatment

2.6.1. Experiment 1: effects of continuous treatment with E2

Cells in each well were washed once with 0.5 ml of FBS- and insulin-free L-15. Then, cells were treated ('initiation') with 1 μM E2 or with solvent (ethanol) only; the amount of ethanol in the medium did not exceed 0.1%. Half the volume (0.25 ml) of the culture medium was changed every 24 h. At 0, 24 and 72 h post initiation (hpi), the medium was removed and 200 μl ISOGEN was added to each well. Following 5 min incubation, the samples were stored at −80°C until use. All incubations were run in triplicate.

2.6.2. Experiment 2: effects of various doses of E2

Cells were treated with various concentrations of E2 (10^{-11} ~ 10^{-6} M). Treatments and sampling were performed as described in Experiment 1 with the following modifications; culture medium was not changed after treatment, and all incubations were replicated 4 times.

2.7. Quantitative real-time PCR (qPCR)

Primers used in qPCR for *chg*s, *vtg*s, *esr1a* and elongation factor 1-α (*ef1-α*; reference gene) were designed in our previous studies (Luo et al., 2013; Mushirobira et al., 2013; Nagata et al., 2018). Primer sets for *esr1b*, *esr2a* and *esr2b* (Table 1) were designed in Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) to cover intron/exon boundaries which were predicted from the rainbow trout genome database (Accession No. GCA_900005705.1). Predicted amplicon sizes for each gene were as follows: *esr1b*: 141 bp, *esr2a*: 111 bp and *esr2b*: 118 bp.

Total RNA was extracted from hepatocytes in ISOGEN following manufacturer's instructions. The RNA (200 ng) was reverse-transcribed by SuperScript® IV VILO cDNA Synthesis kit (Thermo Fisher Scientific). Some aliquots of total RNA from all E2-treated groups were pooled, reverse-transcribed and used as an inter-assay control (IAC) to normalize between plates.

All qPCR reactions were performed as described in Nagata et al. (2018) using FastStart Universal SYBR Green Master (Rox) (Roche, Basel, Switzerland) and

StepOnePlus (Thermo Fisher Scientific) with the following modification. Primers were added to the reaction at a final concentration of 150 nM except for *chgHα* and *esr2b* (50 nM). No PCR amplification was observed from no-reverse-transcription control templates. Primer specificity was confirmed by dissociation curve analysis of PCR products. The efficiencies of the standard curves were within the range of 81–104%, with R^2 values > 0.99.

2.8. Reporter gene assays

The reporter plasmid containing either one of six promoters (*chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *esr1a*) and the expression plasmid containing either one of four *esr* subtypes (*esr1a*, *esr1b*, *esr2a*, *esr2b*) were co-transfected into HeLa cells, and the transactivation of the reporter gene was induced in the presence or absence of 1 µM E2 (10^{-6} M).

Construction of plasmid vectors for reporter gene assays was done using In-Fusion HD Cloning Kit (Takara Bio) according to the manufacturer's instructions. Primer sets (Table 1) for In-Fusion cloning were designed using the Primer Design tool for In-Fusion® HD Cloning Kit (http://www.takara-bio.co.jp/infusion_primer/infusion_primer_form.php). The promoter regions (2 kb in size) of *chg*Hα, *chgHβ*, *chgL*, *vtgAs,* *vtgC* and *esr1a* in pGEM T-Easy vector were subcloned into pGL4.10[*luc2*] Vector (Promega). The open reading frames of esr*1b*, *esr2a* and *esr2b* were subcloned into pcDNA3.1(+) Vector (Thermo Fisher Scientific). The Esr1a expression plasmid, pcDNA3.1-Esr1a, has been described previously (Mushirobira et al., 2018). The subcloned plasmids were purified for transfection using PureLink HiPure Plasmid Filter Midiprep Kit (Thermo Fisher Scientific).

Reporter gene assays were carried out as described previously (Mushirobira et al., 2018) with some modifications. HeLa cells were seeded in 24-well Falcon Primaria Multiwell plates at 3×10^4 cells/well in phenol-red free Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10% charcoal/dextran treated FBS (Hyclone, Logan, UT, USA). The cells were pre-incubated for 24 h at 37°C under 95% air, 5% CO_2 and 100% humidity. After pre-incubation, the cells were transfected with 400 ng of either the promoter-harboring pGL4.10 (pGL4.10-*chgHα*, pGL4.10-*chgHβ*, pGL4.10-*chgL*, pGL4.10-*vtgAs,* pGL4.10-*vtgC* and pGL4.10-*esr1a*) or the empty pGL4.10 (negative promoter-construct control), 200 ng of the *esr*s-harboring pcDNA3.1 (pcDNA3.1-*esr1a*, pcDNA3.1-*esr1b*, pcDNA3.1-*esr2a* and pcDNA3.1-*esr2b*), and 100 ng of pRL-TK Vector (internal control to normalize for variation in transfection efficiency between wells, Promega) using X-tremeGENE HP DNA Transfection reagent (Roche) according to the manufacturer's instructions. Four h after commencement of transfection at 37°C, the cells were treated with 1 μM E2 or vehicle (ethanol). The amount of ethanol in the medium did not exceed 0.1%. After a further 40 h of incubation at 37°C, the cells were collected to measure luciferase activities using Dual-Luciferase Reporter Gene Assay System (Promega) by the Luminescaner-JNR (ATTO, Tokyo, Japan). All incubations were run in quadruplicate.

2.9. Statistics

All data analyses were carried out using JMP Pro 14 Software program (SAS Institute, Cary, NC, USA). Data were analyzed by two-way ANOVA with interaction, using time × dose as factors for the primary hepatocyte cultures, and promoter type × dose for the reporter gene assay. When significant effects were found ($P < 0.05$), comparisons between groups were conducted by Student's t-test, and multiple comparisons were done with Tukey Kramer HSD test. The difference between groups was considered significant at $P < 0.05$. All the results are expressed as means ± SE. For statistical analyses, samples with values below the detection limit (100 copies/reaction mix) in qPCR were assigned this minimum detectable level.

3. Results

3.1. Molecular cloning of esr1b, esr2a and esr2b

Cutthroat trout *esr1b*, *esr2a* and *esr2b* cDNAs (*esr1b*: 1782 bp, *esr2a*: 2324 bp, *esr2b*: 2354 bp) encoding the cutthroat trout Esr1b, Esr2a and Esr2b were isolated and sequenced (GenBank accession no. *esr1b*: LC577088, *esr2a*: LC577089, *esr2b*: LC577090). The open reading frames of *esr1b*, *esr2a* and *esr2b* encoded 556, 594 and 606 amino acids, respectively. The deduced cutthroat trout Esr1b, Esr2a and Esr2b proteins exhibited domain features typical of the estrogen receptor (A/B, C, D, E, F domain). The Esr1b, Esr2a and Esr2b sequences shared high similarity (Esr1b: 99.3%, Esr2a: 99.3% Esr2b: 98.8%) with the homologous sequences of rainbow trout (GenBank Accession No. Esr1b: NP_001118030.1, Esr2a: NP_001118225.1, Esr2b: NP_001118042.1).

3.2. Hormone Treatment

3.2.1. Experiment 1: effects of continuous treatment with E2

In *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *esr1a* mRNA levels, significant interaction effects of time × dose were observed ($P < 0.001$), as well as the significant main effects of time ($P < 0.001$) and dose ($P < 0.001$), respectively. In both *esr2* subtypes, interaction effects of time × dose were not significant while significant main effect of time was observed ($P < 0.01$); main effect of E2 was significant only in *esr2b* ($P < 0.01$).

Levels of *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *esr1a* mRNA in E2-treated hepatocytes were significantly higher than those in the corresponding control group, both at 24 hpi and 72 hpi (Fig. 1). In the E2 culture, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *esr1a* mRNA levels significantly increased from 24 hpi to 72 hpi, while *chgHα* mRNA levels did not. Levels of *esr1b* mRNA were undetectable in all groups at 24 hpi and 72 hpi. Treatment with E2 did not affect mRNA levels of *esr2a* at 24 hpi and 72 hpi when compared to levels in the corresponding control groups. Levels of *esr2b* mRNA in E2 exposure groups were significantly lower than those in the corresponding control groups at 24 and 72 hpi. In cultures supplemented with E2, *esr2b* mRNA levels decreased from 24 to 72 hpi.

3.2.2. Experiment 2: effects of various doses of E2

Exposure of hepatocytes to various doses of E2 yielded significant interaction effects of time × dose in all mRNAs ($P <0.001$: *chgHα*, *chgHβ*, *chgL*, *vtgC*, *esr1a*; $P < 0.01$: *vtgAs*), in addition to the main effect of each factor ($P < 0.001$).

In hepatocytes cultured for 24 and 72 h, *chgHα*, *chgHβ*, *chgL*, *vtgAs* and *vtgC* mRNAs levels were upregulated by E2 treatments in a dose-dependent manner (Fig. 2). The lowest doses that induced a significant increase in the expression level (LOEC) for *chg*s and *vtg*s were 10^{-10} M at both sampling points, except for those of *chgHα* at 24 hpi (10^{-11} M) and *vtgC* at 72 hpi (10^{-9} M). The *chgHα*, *chgHβ* and *chgL* mRNAs levels in the high E2 concentration groups (*chgHα*, *chgHβ*, *chgL* and *vtgAs*: 10^{-7}, 10^{-6} M E2; *vtgC*: 10^{-6} M E2) increased from 24 hpi to 72 hpi; treatment means of all target gene mRNAs, excluding that encoding *vtgAs*, exhibited significant differences. At the 24 hpi sample collection, *esr1a* mRNA levels were upregulated by E2 treatments in a dose-dependent manner and yielded an LOEC *esr1a* of 10^{-9} M E2. Unlike other target genes, *esr1a* mRNA levels in cultures exposed to 10^{-9} ~ 10^{-6} M E2 significantly decreased from 24 hpi to 72 hpi; moreover, significant differences between the E2-treated and the control group were found only in high concentration groups (10^{-7}, 10^{-6} M E2) at 72 hpi, and the LOEC was 10^{-7} M..

3.3. Molecular cloning and sequence analysis of cutthroat trout chgHα, chgHβ, chgL, vtgAs, vtgC and esr1a promoters

The *chgHa* DNA promoter sequence consisted of 2851 bp located upstream of the translation initiation site and 113 bp in the transcribed region (Fig. A. 1, Accession No. LC577091). A complete palindromic ERE (GGTCAnnnTGACC) was not identified in the analyzed promoter sequences. One ERE-like palindrome sequence differing from the consensus ERE and six ERE half sites (1/2 ERE: GGTCA or TGACC) were predicted in *chgHα* at the following positions: ERE-like: -198 to -184 (AGATCTatgTGACCT); 1/2ERE: -2594 to -2590, -2468 to -2464, -1014 to -1010, -237 to -233, -215 to -211 and -103 to -99. Other putative regulatory elements such as activator protein-1 (AP-1) and specificity protein 1 (Sp1) were predicted at the following positions: AP-1: -2077 to -2070 (AGACTCAC), -1198 to -1191 (ATGATTCA), -1197 to -1190 (TGATTCAT) and -565 to -558 (GTGACTGA); Sp1: -985 to -976 (ACCCTCCCTA) and -69 to -60 (ACACACCCCA). The conserved TATA box and CAAT box were found at positions -55 to -41 (GTATAAAAGCAGCAA) and -103 to -88 (GTCAGCCAAGGAGGTG), respectively.

The *chgHβ* promoter sequence was 2320 bp in length and was followed by the translation initiation site and of DNA transcribed into 101 bp of exon (Fig. A. 2, Accession No. LC577092). No complete palindrome ERE was found in the analyzed promoter sequences. Two ERE-like sequences and eight 1/2 ERE were predicted in *chgHβ* at the following positions: ERE-like: -2246 to -2228 (GGACAcactaagtcTGATC) and -1139 to -1129 (GGTTAcTGACC); 1/2ERE: -2124 to -2120, -1245 to -1241, -1071 to -1067, -1057 to -1053, -260 to -256, -234 to -230, -137 to -133 and -97 to –93. The *chgβ* promoter contained other putative regulatory elements at the following positions: AP-1: -1354 to -1347 (GTGACTGA), -1088 to -1081 (TTACTGAC) and -728 to -721 (GTGACTAA). The TATA box and CAAT box were predicted at -17 to -3 (TTATAAAGGTGGCCG) and -234 to -219 (AGCCTCCAATGACATG), respectively.

The *chgL* DNA promoter was 3636 bp in length. The promoter was upstream of the translation initiation site and of 111 bp of transcribable DNA (Fig. A. 3, Accession No. LC577093). No complete palindrome ERE was identified in the analyzed promoter sequences. One ERE-like sequences and seven 1/2 ERE were predicted at the following positions: ERE-like: -703 to -692 (GGTCAtTCACC); 1/2ERE: -3544 to -3540, -2303 to -2299, -2268 to -2264, -1732 to -1728, -1616 to -1612, -1395 to -1391 and -760 to -756. The AP-1 and Sp1 were at the following positions: AP-1: -3333 to -3326 (TTAATCAC), -3179 to -3172 (TTAATCAC), -2538 to -2531 (ATGAGTCA), -2537 to -2530 (TGAGTCAC), -2480 to -2473 (ATACTCAC), -1972 to -1965 (GTGAATAA), -1719 to -1712 (TGGGTCAC), -1144 to -1137 (TCAGTCAC), -765 to -758 (TTACTGAC), -703 to -696 (GTCATTCA) and -386 to -379 (GTGACACA); Sp1: -3559 to -3550 (GAGGGGTGGT), -3514 to -3505 (GAGGCAGTGA), -2109 to -2100 (ACACAGCCCC), -1642 to -1633 (GGGGCATGGA), -1517 to -1508 (TCCATGCCTC), -95 to -86 (GGGGGCGGGT) and -94 to -85 (GGGGCGGGTT). The TATA box and CAAT box were found at positions -57 to -43 (TTATAAAACTGGCCA) and -153 to -138 (TGTGCCCAATGGGCAG), respectively.

The *vtgAs* promoter sequence (2872 bp) was located upstream of the translation initiation site and 225 bp of the transcribed gene (Fig. A. 4, Accession No. LC577095). Unlike the other tested promoters, *vtgAs* promoter contained the consensus palindrome ERE at position -690 to -678 (GGTCAagcTGACC). Putative sequences of seven ERE-like sequences and six 1/2 ERE were found at the following positions: ERE-like: -2767 to -2754 (GGTGAaatcTGACCT), -2758 to -2749 (TGACCTCTCC), -2168 to -2156 (GGTCAgttTGTAC), -1027 to -1014 (GGTCAagttTGATG), -612 to -599 (GGACAagctTGGAC), -509 to -496 (GATCAtacaTGTAC) and -233 to -224 (TGACCTCTCC); 1/2ERE: -2713 to -2709, -2058 to -2054, -2005 to -2001, -1987 to -1983, -1197 to -1193 and -1175 to -1171. The putative AP-1 and Sp1 sites were identified at the following positions: AP-1: -2057 to -2050 (GTCAGTAA), -1760 to -1753 (TGAATGAC), -1746 to -1739 (TGTCTCAC), -1582 to -1575 (GTGTGTCA), -1434 to -1427 (GTGAGTGA), -1167 to -1160 (CTGAGTAA), -948 to -941 (TTACTCAT), -830 to -823 (TGTCTCAC), -721 to -714 (TGACTGAC) and -274 to -267 (GTGATTCT); Sp1: -2774 to -2765 (GAGGCGAGGT), -2349 to -2340 (GGGGCTGGGA), -2209 to -2200 (ACGGCGTGGT), -2092 to -2083 (GGGGCAGGCA), -1092 to -1083 (ACCCTGCCCA), -1009 to -1000 (GGGGGGTGGT) and -201 to -192 (GGGGCAGGTT). The predicted TATA box and CAAT box were found at -47 to -33 (CTTTAAAAGGCGGAC) and -189 to -174 (CCTAACCTATGGGTGT), respectively.

The *vtgC* DNA promoter sequence consisted of 3025 bp located upstream of the translation initiation site and of 39 bp of transcribable DNA (Fig. A. 5, Accession No. LC577096). No complete palindrome ERE was observed in the analyzed promoter sequences. Six ERE-like sequences and seven 1/2 ERE were predicted at the following positions: ERE-like: 1611 to -1601 (GGTCAgAGACC), -1474 to -1461

(GTTCAttggTGTCCA), -1324 to -1315 (GAGCAtccTGACC), -586 to -575 (GTTCAaaTGCAC), -312 to -309 (GGTCAaagaTGTTG) and -87 to -78 (GGTCATGTAC); 1/2ERE: -2193 to -2189, -2087 to -2083, -1273 to -1269, -1118 to -1114, -712 to -708, -660 to -656 and -189 to -185. Other gene regulatory elements were predicted at the following positions: AP-1: -2570 to -2563 (TTACTCAT), -2351 to -2344 (ATACTCAC), -1633 to -1626 (ATGAGACA), -1562 to -1555 (GTGAGCAA), -862 to -855 (GTGAGTAA) and -73 to -66 (TTAGTCAT); Sp1: -2221 to -2212 (ATACTGCCCC). The conserved TATA box was identified at position -48 to -34 (TTATAAAACTGGCCA).

A total of 6155 bp of sequence upstream of the translation initiation site and a 1041 bp transcribable region were isolated and squenced for the *esr1a* DNA promoter (Fig. A. 6, Accession No. LC577094). The promoter contained no complete palindrome ERE. The presence of three ERE-like sequences and eight 1/2 ERE was predicted at the following positions: ERE-like: -1107 to -1090 (GAGCAaccgaggcTTGAC), -525 to -511 (GGTCAagagtTGTCC) and -79 to 67(TGTCAtgtTGACC); 1/2ERE: -5609 to -5605, -5509 to -5505, -5095 to -5091, -4948 to -4944, -4865 to -4861, -4086 to -4082, -3761 to -3757, -3698 to -3694, -3645 to -3641, -3600 to -3596, -3287 to -3283, -3057 to -3053, -2191 to -2187, -2091 to -2087, -1482 to -1478, -899 to -895, -285 to -281, -100 to -96 and -70 to -66. Other putative gene regulatory elements were confirmed at the following positions: AP-1: -5754 to -5747 (GTGAGCCA), -5493 to -5486 (TTACTCAC), -5368 to -5361 (TGTGTCAC), -5050 to -5043 (GTGACTGA), -5049 to -5042 (TGACTGAT), -4213 to -4206 (TGAATGAC), -3815 to -3808 (GTGAGTTA), -3711 to -3704 (TGACTCCC), -2213 to -2206 (GTGAGAAA), -1126 to -1119 (TGAATGAC) and -267 to -260 (TTAGTCAG); Sp1: -5855 to -5846 (AGGGCAGTGT), -5808 to -5799 (GGGGCGGTAT), -4409 to -4400 (ACCCAGCCAG), -3604 to -3595

(CAGGGAGGGT), -3329 to -3320 (ACAATGCCTC), -3099 to -3090 (GGGGCAGGGG), -2201 to -2192 (AAGGGAGGGT), -2099 to -2090 (GGGGCTGTGA) and -396 to -387 (TTGGCGGGAT). The putative TATA box and CAAT box were found at positions, -203 to -189 (CTATGAAAAGGGGGA) and -336 to -321 (AAGGCCCAATGATAGC), respectively.

Numbers of ERE, ERE-like, 1/2 ERE, AP-1 and Sp1 sites in *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *esr1a* promoters are presented in Table 2.

The transcriptional response of *esr1b*, *esr2a* and *esr2b* genes to E2 stimulation were weak in salmonids, unlike those of *chg, vtg* and *esr1a* genes described above. In a preliminary analysis that was based on the whole genome database of rainbow trout, the presence of many predicted sites involved in the E2 responsiveness of genes (ERE-like sequences, etc.) was observed in *esr1b*, *esr2a* and *esr2b* promoters, as well as the *chg, vtg* and *esr1a* promoters (supplemental Table: Table A. 1).

3.4. Transactivation of estrogen-responsive gene promoters by E2 via Esr1a, Esr1b, Esr2a and Esr2b

Significant interaction effects of promoter type × dose were observed in transactivation *via* each Esr subtype ($P < 0.001$: *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC*, *esr1a*), as well as the main effect of each factor ($P < 0.001$).

In all promoter-containing constructs, Esr1a-mediated reporter activity in the presence of E2 was significantly higher than that in the solvent control group (non-E2 controls) and the empty-vector control group (Fig. 3). The reporter activities in E2-treated HeLa cells transfected with *chgHα* and *vtgAs* promoters were significantly higher than those in HeLa cells provided with the *chgHβ* promoter. The reporter activities of the remaining gene promoters in E2 culture were significantly lower than those of *chgHβ* promoter, and higher in the order of *chgL* and *esr1a*.

All promoters in HeLa cells were significantly transactivated by Esr1b in the presence of E2 compared to the corresponding solvent control groups or empty vector construct. Reporter activity in E2-supplemented cultures was highest for the *chgHβ* promoter, followed, in descending order, by *chgHα, chgL, vtgAs*, *vtgC* and *esr1a*. Significant effects on reporter activity were found among *chgHβ, chgHα, chgL, vtgAs* and *vtgC* promoters in the E2-treated groups. No significant difference was detected between *vtgC* and *esr1a* promoters in E2-treated groups.

In the presence of E2, Esr2a significantly transactivated all promoters relative to the solvent control groups and empty-vector groups. Again, as for the Esr1b-expressing HeLa cells, the *chgHβ* promoter showed the highest Esr2a-mediated activity when exposed to E2. The reporter activities in E2-supplemented cultures ranked highest in HeLa cells transfected with *chgHβ*, followed by *chgL*, *chgHα*, *vtgAs*, *vtgC* and *esr1a*. E2-induced reporter activities differed significantly between the following promoter pairs: *chgL* and *chgHα*, *vtgAs* and *vtgC* and *vtgC* and *esr1a*.

Esr2b-mediated transactivation of the different target gene promoters followed a pattern essentially the same as seen for Esr1b and Esr2a; thus, the *chgHβ* promoter displayed the strongest, and the *esr1a* promoter the weakest activity when Esr2b was evaluated as receptor for E2. The reporter activities in E2 culture decreased in the order of *chgHβ*, *chgHα*, *vtgAs*, *chgL*, *vtgC* and *esr1a*.

4. Discussion

The predicted cutthroat trout Esr1b, Esr2a and Esr2b polypeptide sequences showed high similarities with homologous sequences of rainbow trout (Esr1b: 99.3%, Esr2a: 99.3%, Esr2b: 98.8%). Primary structures of cutthroat trout Esr1b, Esr2a and Esr2b exhibited typical functional domains reported for vertebrate ESRs/Esrs and the motifs required for DNA and ligand binding were highly conserved (data not shown). These structural similarities suggest that cutthroat trout Esr1b, Esr2a and Esr2b have the basic functions of vertebrate ESRs/Esrs, such as ligand (estrogen) binding and transactivation of target genes (Davis et al., 2010; Menuet et al., 2002).

To reveal the responsiveness to E2 stimulation, effects of continuous treatment with a high E2 dose (10^{-6} M) on expression of *chg*, *vtg* and *esr* genes were examined in Experiment 1. With replenishment of E2 every 24 hpi, the level of *chg*, *vtg* and *esr1a* mRNAs increased by 72 hpi. Levels of these mRNAs were also upregulated by E2 treatment *in vivo* in male and immature cutthroat trout (Mushirobira et at., 2018), as were mRNA/protein levels in the liver of other salmonids (Amano et al., 2010; Boyce-Derricott et al., 2009; Hiramatsu et al., 1997; Thomas-Jones et al., 2003; Westerlund et al., 2001). Thus, these results indicate that E2-induction of hepatic expressions of *chg*s, *vtg*s and *esr1a* is a common feature in salmonid species.

In Experiment 1, levels of only *esr1a* mRNA were upregulated by E2 treatment – those of the other *esr*s (i.e., esr*1b*, *esr2a* and *esr2b*) were not. In rainbow trout fed with E2-containing pellets for five days, Casanova-Nakayama et al. (2018) confirmed that E2 could stimulate hepatic *esr1b* gene expression. Osachoff et al. (2013) confirmed that 7-day exposure to E2 upregulated hepatic *esr1b* mRNA levels in rainbow trout, but that 2-day exposure did not. Similarly, Boyce-Derricott et al. (2009) observed no significant effect of E2 on hepatic *esr1b* mRNA expression in rainbow trout by 24 h following injection. These results suggest that it takes a relatively long time for E2 to upregulate *esr1b* gene expression. Meanwhile, E2 did not show any significant effect on hepatic *esr2a* expression in rainbow trout, regardless of the duration of the exposure period (Boyce-Derricott et al., 2009; Casanova-Nakayama et al., 2018; Cleveland and Weber, 2015; Osachoff et al., 2013), which is in keeping with our results on primary cultured hepatocytes of cutthroat trout in this study. As for *esr2b* in rainbow trout, Cleveland and Weber (2015) reported downregulation of the mRNA levels by E2 treatment, similar to the results for cutthroat trout in the present study, but other studies (Boyce-Derricott et al., 2009; Casanova-Nakayama et al., 2018; Osachoff et al., 2013) did not document such a response of *esr2b* mRNA to E2 treatment. Collectively, *esr1a* gene alone appears to exhibit strong and acute upregulation by E2 stimulation, suggesting that *esr1a* is the major transcriptional factor among *esr* subtypes involved in active (i.e., strong and acute) synthesis of hepatic *chg* and *vtg* mRNAs in salmonids.

Because expression of six gene transcripts (i.e., *chgHα*, *chgHβ*, *chgL, vtgAs*, *vtgC* and *esr1a*) appeared to be strongly and acutely upregulated by E2 treatments, a follow-up experiment (Experiment 2) sought to examine the effects of E2 dose (10^{-11} ~ 10^{-6} M) on the expression of these six genes. Transcript levels of *chg*s and *vtg*s at 24 and 72 hpi increased in a E2-dose-dependent manner. In addition, *esr1a* mRNA levels in E2-supplemented incubations also showed a dose-dependent increase at 24 hpi. These patterns suggest that the expression of these genes is under strict regulation by E2. The LOECs of *chg* and *vtg* mRNAs for E2 were 10^{-11} M for *chgHα* at 24 hpi and 10^{-9} M for *vtgC* at 72 hpi, which differed from the LOEC (10^{-10} M) for other target gene mRNAs at both time points, indicating that the *chgHα* and *vtgC* exhibit differential sensitivity to E2 from the other *chg* and *vtg* subtypes in primary cultured hepatocytes of cutthroat trout.

From 24 to 72 hpi, *chgHα*, *chgHβ*, *chgL*, *vtgAs* and *vtgC* mRNA levels increased or were maintained at high values in high-dose E2 cultures, both in Experiments 1 (culture with replacement of medium) and 2 (culture without replacement of medium). Meanwhile, *esr1a* mRNA levels in high-dose E2 incubations increased in Experiment 1 but decreased in Experiment 2 from 24 hpi to 72 hpi. Thus a difference in the way of E2 supplementation possibly caused the opposite *esr1a* response to E2 between the two experiments, similar to a study using primary cultured hepatocytes of rainbow trout (Flouriot et al., 1996). In said study, high *esr1a* mRNA levels were maintained from 24 to 72 h following supplementation with 10^{-6} M E2 and replacement of E2-containing culture medium every 24 h, but levels decreased when the medium was not replaced – those findings are in good agreement with the results of Experiment 1 and Experiment 2 in our study on cutthroat trout. It has been shown that hepatocytes of *Xenopus laevis* in primary culture rapidly metabolize E2 in culture medium (Tenniswood et al., 1983). Although such analysis was not performed in the present study, rapid metabolism of E2 in the medium could thus be a cause of the time-dependent decrease of *esr1a* mRNA expression. Adhesion of E2 to the wall of the plate could be another potential cause for this decrease.

Numbers of ERE, ERE-like, 1/2 ERE, AP-1 and Sp1 sites in *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *esr1a* promoters were different, suggesting these promoters have differential transcriptional properties. An ERE consensus sequence was identified only in the *vtgAs* promoter while all promoters had ERE-like and 1/2 ERE sequences. In teleosts, ERE-like and 1/2 ERE sites elicit a significant increase in estrogen-dependent synthesis of reporter protein (Le Drean et al., 1995; Menuet et al., 2004; Teo et al., 1998). Thus, aside from the consensus ERE, ERE-like and 1/2 ERE sites are likely to be responsible for the E2-induced expression of *chg*, *vtg* and *esr1a* genes. This was also supported by the results of this study; cutthroat trout *chg*, *vtg* and *esr1a* promoters were transactivated in the presence of E2 and Esrs. In addition, AP-1 and Sp1 binding sites, which are known to interact with Esr, were predicted in promoters of cutthroat trout *chg*, *vtg* and *esr1a*. These binding sites possibly contribute to the transactivation of *chg*, *vtg* and *esr1a*.

In silico analysis predicted the binding sites for transcription factors; it is unclear if the predicted sites are functional *in vivo* and *in vitro*. Therefore, binding sites with low or no functionality for E2 responsiveness of genes were possibly identified in *esr1b*, *esr2a* and *esr2b* promoters. To verify the transcriptional response of the targeted genes, *in vitro* experiments, such as promoter assays, will thus be needed.

In reporter gene assays with teleost Esrs, several mammalian cell lines (CHO-K1, HepG2, HeLa, HEK-293, CHO, etc.) have been used (Davis et al., 2010; Le Drean et al., 1995; Lee Pow et al., 2016; Menuet et al., 2004; Mushirobira et al., 2018). It has been shown that these cells have endogenous ESRs and other estrogen-related proteins. For example, CHO-K1 expresses functional endogenous Esr2 (Thomas et al., 2003), whereas HepG2 expresses the gene encoding G protein-coupled estrogen receptor 1 (*GPER1*) at high levels (Transcripts Per Kilobase Million: TPM: 15.3, calculated by next-generation sequences) compared to levels in other human cell lines (TPM; HeLa: 0.9, HEK-293: 1.9; see The Human Protein Atlas, https://www.proteinatlas.org/). The GPER has been shown to bind estrogens and initiate subsequent signaling cascades *in vitro* (Langer et al., 2010). Because of high expression of endogenous estrogen receptors, the CHO derivative cells (CHO and CHO-K1) and HepG2 are perhaps not best-suitable for the Esr/ESR-based reporter gene assay. HeLa cells have been widely used for reporter gene assay for Esrs of teleosts (Lee Pow et al., 2016; Menuet et al., 2004; Sumida and Saito, 2008). In addition, HeLa expresses low levels of Esr1 (TPM: 0.1) and Esr2 (TPM: 0.4). Thus, the HeLa cell line was selected for the reporter gene assays in the present study. To eliminate the influence of promoter length on the transfection efficiency, reporter gene assay in this study was performed using 2000 bp promoter regions of all genes.

Reporter gene assay with four different trout Esr subtypes revealed that all of them are functional and can transactivate *chg*, *vtg* and *esr1a* promoters in the presence of E2. E2-induced transactivation of *esr1a* promoters mediated by the different Esr subtypes was lowest for *esr1a*. In rainbow trout, reporter assay using *esr1a* cDNA and *esr1a* promoter, E2 treatment increased activities up to 10-fold only in the presence of different Esr subtypes (Le Drean et al., 1995); such weak induction of reporter activities was also seen in zebrafish when using *esr1* cDNA and *esr1* promoter (Menuet et al., 2004). The fold-activation of teleost *esr1* seems generally low, findings that are reinforced for the *esr1a* promoter of cutthroat trout in the present study.

Each Esr subtype differentially transactivated *chg*, *vtg* and *esr1a* promoters in the presence of E2. For example, E2-induced transactivation of the promoters through Esr1a was *chgHα* ≥ *vtgAs* > *chgHβ* > *chgL* > *vtgC* > *esr1a*, while that through *esr1b* was *chgHβ* > *chgHα* > *chgL* > *vtgAs* > *vtgC* ≥ *esr1a*. These results suggest that the different Esr subtype vary in their transactivation properties, conceivably explaining the different transcriptional profiles of *chg*, *vtg* and *esr1a* genes in primary cultured hepatocytes. The selectivity of Esr subtypes in transactivation of such E2-responsive genes remains unclear. Further investigations are required to address the differential role of the Esr subtypes in the transactivation of *chg*, *vtg* and *esr1a* genes.

In conclusion, the present study documented i) *in vitro* hepatic responses of *chg*, *vtg* and *esr* gene expression to E2 stimulation within a single teleost species, and ii) transactivation properties of *chg*, *vtg* and *esr* promoters by four Esr subtypes. To our knowledge, this is the first report to show the different responsiveness to E2 among hepatic estrogen-responsive genes in a single teleost species alongside the differential roles of Esr subtypes in the transactivation of promoters for those genes. We demonstrated that there were clear differences in E2-induced transactivation of *chg*, *vtg* and *esr1a* gene promoters by four discrete Esr subtypes. Differences in *chg*, *vtg* and *esr1a* gene expression in trout hepatocytes following E2 exposure possibly reflect differential transactivation properties among four Esr subtypes for these gene promoters.

Acknowledgements

We are grateful to Dr. M. Lokman (University of Otago, New Zealand) for critical reading of the manuscript. We thank Dr. E. Yamaha, Mr. S. Kimura and Dr. E. Takahashi, Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, for rearing experimental fish in this study. We acknowledge Dr. M. Shimizu, Faculty of Fisheries sciences, Hokkaido University for helpful discussions.

Funding

This work was supported in part by the JSPS KAKENHI [grant number 18H02272 to N. H.]

Declarations of interest

None

Appendix A. Supplementary data

References

Amano, H., Mochizuki, M., Fujita, T., Hiramatsu, N., Todo, T., Hara, A., 2010. Purification and characterization of a novel incomplete-type vitellogenin protein (VgC) in Sakhalin taimen (*Hucho perryi*). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 157, 41–48. https://doi.org/10.1016/j.cbpa.2010.05.006.

Boyce-Derricott, J., Nagler, J.J., Cloud, J.G., 2009. Regulation of hepatic estrogen receptor isoform mRNA expression in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 161, 73–78. https://doi.org/10.1016/j.ygcen.2008.11.022.

Buisine, N., Trichet, V., Wolff, J., 2002. Complex evolution of vitellogenin genes in salmonid fishes. Mol. Genet. Genomics 268, 535–542. https://doi.org/10.1007/s00438-002-0771-5.

Casanova-Nakayama, A., Von Siebenthal, E.W., Kropf, C., Oldenberg, E., Segner, H., 2018. Immune-specific expression and estrogenic regulation of the four estrogen receptor isoforms in female rainbow trout (*Oncorhynchus mykiss*). Int. J. Mol. Sci. 19. https://doi.org/10.3390/ijms19040932.

Cleveland B.M., Weber G.M, 2015. Effects of sex steroids on expression of genes regulating growth-related mechanisms in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 216, 103–115. https://doi.org/ 10.1016/j.ygcen.2014.11.018.

Choi, C.Y., Habibi, H.R., 2003. Molecular cloning of estrogen receptor α and expression pattern of estrogen receptor subtypes in male and female goldfish. Mol. Cell. Endocrinol. 204, 169–177. https://doi.org/10.1016/S0303-7207(02)00182-X.

Davis, L.K., Katsu, Y., Iguchi, T., Lerner, D.T., Hirano, T., Grau, E.G., 2010. Transcriptional activity and biological effects of mammalian estrogen receptor ligands on three hepatic estrogen receptors in Mozambique tilapia. J. Steroid Biochem. Mol. Biol. 122, 272–278. https://doi.org/10.1016/j.jsbmb.2010.05.009.

Filby, A.L., Tyler, C.R., 2005. Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). Biol. Reprod. 662, 648–662. https://doi.org/10.1095/biolreprod.105.039701.

Flouriot, G., Pakdel, F., Valotaire, Y., 1996. Transcriptional and post-transcriptional regulation of rainbow trout estrogen receptor and vitellogenin gene expression. Mol. Cell. Endocrinol. 124, 173–183. https://doi.org/10.1016/S0303-7207(96)03960-3.

Fujita, T., Fukada, H., Shimizu, M., Hiramatsu, N., Hara, A., 2008. Molecular cloning and characterization of three distinct choriogenins in masu salmon, *Oncorhynchus masou*. Mol. Reprod. Dev. 75, 1217–1228. https://doi.org/10.1002/mrd.20857.

Goudet, G., Mugnier, S., Callebaut, I., Monget, P., 2008. Phylogenetic analysis and identification of pseudogenes reveal a progressive loss of zona pellucida genes during evolution of vertebrates. Biol. Reprod. 78, 796–806. https://doi.org/10.1095/biolreprod.107.064568.

Grierson, J.P., Neville, A.C., 1981. Helicoidal architecture of fish eggshell. Tissue Cell 13, 819–830. https://doi.org/10.1016/S0040-8166(81)80016-X.

Griffin, L.B., January, K.E., Ho, K.W., Cotter, K.A., Callard, G. V., 2013. Morpholino-mediated knockdown of ERα, ERβa, and ERβb mRNAs in zebrafish (*Danio rerio*) embryos reveals differential regulation of estrogen-inducible genes. Endocrinology 154, 4158–4169. https://doi.org/10.1210/en.2013-1446.

Halm, S., Martínez-Rodríguez, G., Rodríguez, L., Prat, F., Mylonas, C.C., Carrillo, M., Zanuy, S., 2004. Cloning, characterisation, and expression of three oestrogen receptors (ERα, ERβ1 and ERβ2) in the European sea bass, *Dicentrarchus labrax*. Mol. Cell. Endocrinol. 223, 63–75. https://doi.org/10.1016/J.MCE.2004.05.009.

Hara, A., Hiramatsu, N., Fujita, T., 2016. Vitellogenesis and choriogenesis in fishes. Fish. Sci. 82, 187–202. https://doi.org/10.1007/s12562-015-0957-5.

Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., Thomas, P., 2000.

Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc. Natl. Acad. Sci. 97, 10751–10756. https://doi.org/10.1073/pnas.97.20.10751.

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Treuter, E., Warner, M., Hartman, J., Tujague, M., Stro, A., 2007. Estrogen receptors : How do they signal and what are their targets. Physiol. Rev. 87, 905–931. https://doi.org/10.1152/physrev.00026.2006.

Hiramatsu, N., Shimizu, M., Fukada, H., Kitamura, M., Ura, K., Fuda, H., Hara, A., 1997. Transition of serum vitellogenin cycle in Sakhalin taimen (*Hucho perryi*). Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol. 118, 149–157. https://doi.org/10.1016/S0742-8413(97)00084-4.

Hiramatsu, N., Cheek, A.O., Sullivan, C. V., Matsubara, T., Hara, A., 2005. In: Biochemistry and Molecular Biology of Fishes. Elsevier Science BV, Amsterdam, pp 431–471. http://dx.doi.org/10.1016/S1873-0140(05)80019-0.

Hyllner, S.J., Westerlund, L., Olsson, P.E., Schopen, A., 2001. Cloning of rainbow trout egg envelope proteins: members of a unique group of structural proteins. Biol. Reprod. 64, 805–811. https://doi.org/10.1095/biolreprod64.3.805.

Klaunig, J.E., Ruch, R.J., Goldblatt, P.J., 1985. Trout hepatocyte culture: Isolation and primary culture. Vitr. Cell. Dev. Biol. 21, 221–228. https://doi.org/10.1007/BF02620933.

Knoche, K., Kephart, D., 1999. Cloning Blunt-End Pfu DNA Polymerase- Generated PCR Fragments into pGEM-T Vector Systems. Promega Notes 71, 10.

Langer, G., Bader, B., Meoli, L., Isensee, J., Delbeck, M., Noppinger, P.R., Otto, C., 2010. A critical review of fundamental controversies in the field of GPR30 research. Steroids 75, 603–610. https://doi.org/10.1016/J.STEROIDS.2009.12.006.

Le Drean, Y., 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. https://doi.org/10.1677/jme.0.0150037.

Lee Pow, C.S.D., Yost, E.E., Aday, D.D., Kullman, S.W., 2016. Sharing the toles: An assessment of japanese medaka estrogen receptors in vitellogenin induction. Environ. Sci. Technol. 50, 8886–8895. https://doi.org/10.1021/acs.est.6b01968.

Luo, W., Ito, Y., Mizuta, H., Massaki, K., Hiramatsu, N., Todo, T., Reading, B.J., Sullivan, C. V, Hara, A., 2013. Molecular cloning and partial characterization of an ovarian receptor with seven ligand binding repeats, an orthologue of low-density lipoprotein receptor, in the cutthroat trout (*Oncorhynchus clarki*). Comp. Biochem. Physiol. Part A 166, 263–271. https://doi.org/10.1016/j.cbpa.2013.06.026.

Ma, C.H., Dong, K.W., Yu, K.L., 2000. cDNA cloning and expression of a novel estrogen receptor β-subtype in goldfish (*Carassius auratus*). Biochim. Biophys. Acta 1490, 145–152. https://doi.org/10.1016/S0167-4781(99)00235-3.

Menuet, A., Le Page, Y., Torres, O., Kern, L., Kah, O., Pakdel, F., 2004. Analysis of the estrogen regulation of the zebrafish estrogen receptor (ESR) reveals distinct effects of ERalpha, ERbeta1 and ERbeta2. J. Mol. Endocrinol. 32, 975–86. https://doi.org/10.1677/jme.0.0320975.

Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., Pakdel, F., 2002. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol. Reprod. 66, 1881–1892. https://doi.org/10.1095/biolreprod66.6.1881.

Mushirobira, Y., Mizuta, H., Luo, W., Morita, Y., Sawaguchi, S., Matsubara, T., Hiramatsu, N., Todo, T., Hara, A., 2013. Changes in levels of dual vitellogenin transcripts and proteins in cutthroat trout *Oncorhynchus clarki* during ovarian development. Nippon Suisan Gakkaishi 79, 175–189. https://doi.org/10.2331/suisan.79.175.

Mushirobira, Y., Nishimiya, O., Nagata, J., Todo, T., Hara, A., Reading, B.J., Hiramatsu, N., 2018. Molecular cloning of vitellogenin gene promoters and in *vitro* and in *vivo* transcription profiles following estradiol-17β administration in the cutthroat trout. Gen. Comp. Endocrinol. 267, 157–166. https://doi.org/10.1016/j.ygcen.2018.06.017.

Nagata, J., Mushirobira, Y., Nishimiya, O., Fujita, T., Hiramatsu, N., Hara, A., Todo, T., 2018. Expression analysis of estradiol-17β responsive genes in the liver of female cutthroat trout (*Oncorhynchus clarki*) during a reproductive cycle. Aquac. Sci. 66, 91–101. https://doi.org/10.11233/aquaculturesci.66.91.

Nagler, J.J., Cavileer, T., Sullivan, J., Cyr, D.G., Rexroad, C., 2007. The complete nuclear estrogen receptor family in the rainbow trout: Discovery of the novel ERα2 and both ERβ isoforms. Gene 392, 164–173. https://doi.org/10.1016/j.gene.2006.12.030.

Nagler, J.J., Cavileer, T.D., Verducci, J.S., Schultz, I.R., Hook, S.E., Hayton, W.L., 2012. Estrogen receptor mRNA expression patterns in the liver and ovary of female rainbow trout over a complete reproductive cycle. Gen. Comp. Endocrinol. 178, 556–561. https://doi.org/10.1016/j.ygcen.2012.06.010.

Nelson, E.R., Habibi, H.R., 2010. Functional significance of nuclear estrogen receptor subtypes in the liver of goldfish. Endocrinology 151, 1668–1676. https://doi.org/10.1210/en.2009-1447.

Osachoff, H.L., Shelley, L.K., Furtula, V., Van Aggelen, G.C., Kennedy, C.J., 2013. Induction and recovery of estrogenic effects after short-term 17β-estradiol exposure in juvenile rainbow trout (*Oncorhynchus mykiss*). Arch. Environ. Contam. Toxicol. 65, 276–285. https://doi.org/10.1007/s00244-013-9890-8.

Podvinec, M., Kaufmann, M.R., Handschin, C., Meyer, U., 2002. NUBIScan, an in silico approach for prediction of nuclear receptor response elements. Mol. Endocrinol. 16, 1269–1279. https://doi.org/10.1210/mend.16.6.0851.

Sabo-Attwood, T., Kroll, K.J., Denslow, N.D., 2004. Differential expression of largemouth bass (*Micropterus salmoides*) estrogen receptor isotypes alpha, beta, and gamma by estradiol. Mol. Cell. Endocrinol. 218, 107–118. https://doi.org/10.1016/j.mce.2003.12.007.

Sandelin, A., Wasserman, W.W., Lenhard, B., 2004. ConSite: Web-based prediction of regulatory elements using cross-species comparison. Nucleic Acids Res. 32, 249–252. https://doi.org/10.1093/nar/gkh372.

Schreer, A., Tinson, C., Sherry, J.P., Schirmer, K., 2005. Application of Alamar blue/5-carboxyfluorescein diacetate acetoxymethyl ester as a noninvasive cell viability assay in primary hepatocytes from rainbow trout. Anal. Biochem. 344, 76–85. https://doi.org/10.1016/j.ab.2005.06.009.

Sladek, F.M., 2011. What are nuclear receptor ligands? Mol. Cell. Endocrinol. 334, 3–13. https://doi.org/10.1016/J.MCE.2010.06.018.

Songe, M.M., Willems, A., Sarowar, M.N., Rajan, K., Evensen, Drynan, K., Skaar, I., van West, P., 2016. A thicker chorion gives ova of Atlantic salmon (*Salmo salar L.*) the upper hand against Saprolegnia infections. J. Fish Dis. 39, 879–888. https://doi.org/10.1111/jfd.12421.

Sumida, K., Saito, K., 2008. Molecular cloning of estrogen receptors from fathead minnow (*Pimephales promelas*) and bluegill (*Lepomis macrochirus*) fish: Limited piscine variation in estrogen receptor–mediated reporter gene transactivation by xenoestrogens. Environ. Toxicol. Chem. 27, 489. https://doi.org/10.1897/07-250R1.1.

Tenniswood, M.P.R., Searle, P.F., Wolffe, A.P., Tata, J.R., 1983. Rapid estrogen metabolism and vitellogenin gene expression in xenopus hepatocyte cultures. Mol. Cell. Endocrinol. 30, 329–345. https://doi.org/10.1016/0303-7207(83)90068-0.

Teo, B.Y., Tan, N.S., Lim, E.H., Lam, T.J., Ding, J.L., 1998. A novel piscine vitellogenin gene: Structural and functional analyses of estrogen-inducible promoter. Mol. Cell. Endocrinol. 146, 103–120. https://doi.org/10.1016/S0303-

7207(98)00191-9.

Thomas-Jones, E., Thorpe, K., Harrison, N., Thomas, G., Morris, C., Hutchinson, T., Woodhead, S., Tyler, C., 2003. Dynamics of estrogen biomarker responses in rainbow trout exposed to 17beta-estradiol and 17alpha-ethinylestradiol. Environ. Toxicol. Chem. 22, 3001–3008. https://doi.org/10.1897/03-31.

Thomas, P.B., Risinger, K.E., Klinge, C.M., 2003. Identification of estrogen receptor beta expression in Chinese hamster ovary (CHO) cells and comparison of estrogen-responsive gene transcription in cells adapted to serum-free media. J. Steroid Biochem. Mol. Biol. 86, 41–55. https://doi.org/10.1016/S0960-0760(03)00250-4.

Trichet, V., Buisine, N., Mouchel, N., Morán, P., Pendás, A.M., Le Pennec, J.P., Wolff, J., 2000. Genomic analysis of the vitellogenin locus in rainbow trout (*Oncorhynchus mykiss*) reveals a complex history of gene amplification and retroposon activity. Mol. Gen. Genet. 263, 828–37. https://doi.org/10.1007/s004380000247.

Walker, P., Germond, J.-E., Brown-Luedi, M., Givel, F., Wahli, W., 1984. Sequence homologies in the region preceding the transcription initiation site of the liver estrogen-responsive vitellogenin and apo-VLDLI genes. Nucleic Acids Res. 12, 8611–8626. https://doi.org/10.1093/nar/12.22.8611.

Westerlund, L., Hyllner, S.J., Schopen, A., Olsson, P.E., 2001. Expression of three vitelline envelope protein genes in arctic char. Gen. Comp. Endocrinol. 122, 78–87. https://doi.org/10.1006/gcen.2001.7614.

Figure captions

Fig. 1 Effects of continuous treatment of primary cultured hepatocytes of male cutthroat trout with estradiol-17β (E2, black columns) or a control solvent (C, white columns) on the transcript levels of choriogenin (*chg*: *chgHα*, *chgHβ* and *chgL*), vitellogenin (*vtg*: *vtgAs* and *vtgC*) and estrogen receptor (*esr1a*, *esr1b*, *esr2a* and *esr2b*) subtypes.

Hepatocytes from one trout were treated with 10^{-6} M E2. Half of the culture medium was replaced by fresh medium every 24 h post initiation (hpi) of E2 treatment. At 0, 24 and 72 hpi, cells were harvested and mRNA levels quantified by quantitative real-time reverse transcription PCR. Columns indicate mean values and vertical lines indicate standard errors. Different letters denote that values are significantly different ($P < 0.05$).

Fig. 2 Effect of treatment of hepatocytes of male cutthroat trout in primary culture with single doses of estradiol-17β (E2, black columns) or a control solvent (C, white columns) on the transcript levels of choriogenin (*chg*: *chgHα*, *chgHβ* and *chgL*), vitellogenin (*vtg*: *vtgAs* and *vtgC*) and estrogen receptor (*esr1a*). Hepatocytes from one trout were treated with 10^{-11} ~ 10^{-6} M (-11 ~ -6 in horizontal axis) E2 for 72 h without replacement of the medium. At 24 and 72 h post initiation (hpi) of E2 treatment, cells were harvested and mRNA levels quantified by quantitative real-time reverse transcription PCR. Columns indicate mean values and vertical lines indicate standard errors. Different letters denote that values are significantly different ($P < 0.05$). Arrow heads exhibit the lowest dose that induced a significant increase in the expression levels of each mRNA (LOEC) for E2.

Fig. 3 Fold-induction change in transactivation of choriogenin (*chg*), vitellogenin (*vtg)* and estrogen receptor 1a (*esr1a*) promoters mediated by four Esr subtypes (Esr1a, Esr1b, Esr2a and Esr2b) in the presence or absence of estradiol-17β (E2). Hela cells were co-transfected with two constructs: one *esr* expression vector (*esr1a*, *esr1b, esr2a* or *esr2b*) and one gene promoter (*chgHα*, *chgHβ*, *chgL*, *vtgAs, vtgC* or *esr1a*) in a reporter vector. Reporter vector without a promoter (empty vector, 'vector') was used as negative control. The transfected cells were treated with 1 μM E2 (closed columns) or control solvent (C, open columns). The fold-induction was initially normalized to the luminescence from Renilla luciferase; data were averaged from 4 replicate wells. Fold-activation was represented after setting the empty vector (vehicle control) set to 1. Columns indicate mean values and vertical brackets standard errors. Different letters denote significant differences ($P < 0.05$).

Highlights

- Choriogenin, vitellogenin and estrogen receptor-1a genes were estrogen-responsive
- These estrogen responsive genes responded differentially to estradiol-17β (E2)
- E2 transactivated these gene promoters in the presence of four estrogen receptors

Table 1 Name, nucleotide sequence, direction, and use of primers

Name	Sequence	Direction	Use
esr1b-F	GCCATCTCACCCCAGAAACT	Forward	Cloning for *esr1b*
esr1b-R	ACTCTCACCTCCACAAATGTCA	Reverse	Cloning for *esr1b*
esr2a-F	CACGGATGGATTGCTACTCC	Forward	Cloning for *esr2a*
esr2a-R	GAACAGTGCTAATGCCTGAAAGG	Reverse	Cloning for *esr2a*
esr2b-F	CACTGATGACAGGCTTGGCAG	Forward	Cloning for *esr2b*
esr2b-R	AATGGTCACAGACACTGATAAAG	Reverse	Cloning for *esr2b*
esr1b-F-qPCR	AGCCTCCCCAGCCAGTCTATC	Forward	qPCR for *esr1b*
esr1b-R-qPCR	TGAGCCTGACCCTGACTCCAC	Reverse	qPCR for *esr1b*
esr2a-F-qPCR	TCCATTGTCTCTGCACCATCG	Forward	qPCR for *esr2a*
esr2a-R-qPCR	TTCCTCAGAGGCTTACTGCTCTC	Reverse	qPCR for *esr2a*
esr2b-F-qPCR	TCCAAACGAGGCCTGTCATTC	Forward	qPCR for *esr2b*
esr2b-R-qPCR	TCTTCATGCTAGAGAGGTGCTG	Reverse	qPCR for *esr2b*
chgHα-promoter-F	ACTCCCCCAACCTTCCTCTT	Forward	Cloning of *chgHα* promoter
chgHα-promoter-R	GTTCTGAGGGGGTTGGTAAGG	Reverse	Cloning of *chgHα* promoter
chgHβ- GW-R1	CCCTGGTTTTTCCAAGTAAATCTGAG	Reverse	Genome walking for *chgHβ*
chgHβ- GW-R2	GAACAGTGCTAATGCCTGAAAGG	Reverse	Genome walking for *chgHβ*
chgL-GW-R1	CTGACGATTGGGTCTGAAGGGTTGCTG	Reverse	Genome walking for *chgL*
chgL-GW-R2	TTTACTGAAGGGTGGCCAATTCTGAG	Reverse	Genome walking for *chgL*
esr1a-GW-R1	CTGACGATTGGGTCTGAAGGGTTGCTG	Reverse	Genome walking for *esr1a*
esr1a-GW-R2	TTTACTGAAGGGTGGCCAATTCTGAG	Reverse	Genome walking for *esr1a*
vtgAs-promoter-1-F	AAGATGCAATTCGTCAGACTTCG	Forward	Cloning of *vtgAs* promoter 1
vtgAs-promoter-1-R	AAAGTTAACAGATTGACTCGCTACA	Reverse	Cloning of *vtgAs* promoter 1
vtgC-promoter-F	ATCAAACCATGCAATAATCTGAGTC	Forward	Cloning of *vtgC*
vtgC-promoter-R	GGCCAAGGCCACAAGGT	Reverse	Cloning of *vtgC*
pcDNA3.1-linealize-F	GTTTAAACCCGCTGATCA	Forward	Subcloning of *esr1b* , *esr2a* and *esr2b*
pcDNA3.1-linealize-R	GCTAGCCAGCTTGGGTCT	Reverse	Subcloning of *esr1b* , *esr2a* and *esr2b*
esr1b-pGL3.1-infusion-F	CCCAAGCTGGCTAGCACCATGTACCCTG	Forward	Subcloning of *esr1b*
esr1b-pGL3.1-infusion-R	TCAGCGGGTTTAAACTCATGGAATGGG	Reverse	Subcloning of *esr1b*
esr2a-pGL3.1-infusion-F	CCCAAGCTGGCTAGCACCATGTCACAAT	Forward	Subcloning of *esr2a*
esr2a-pGL3.1-infusion-R	TCAGCGGGTTTAAACTCACCACTGTCTT	Reverse	Subcloning of *esr2a*
esr2b-pGL3.1-infusion-F	CCCAAGCTGGCTAGCACCATGGCATGTT	Forward	Subcloning of *esr2b*
esr2b-pGL3.1-infusion-R	TCAGCGGGTTTAAACTTACTGAGGTACA	Reverse	Subcloning of *esr2b*

Table 1 (Continued)

pGL4.10-linearize-F	CTGTTGGTAAAGCCACCATGGAAG	Forward	Subcloning of *chgHα*, *chgHβ*, *chgL*
pGL4.10-linearize-R	GCGAGCTCAGGTACCGGC	Reverse	Subcloning of *chgHα*, *chgHβ*, *chgL*
chgHα-pGL4.10-infusion-F	GGTACCTGAGCTCGCGATATTTCTGTGTC	Forward	Subcloning of *chgH α* promoter
chgHα-pGL4.10-infusion-R	TGGCTTTACCAACAGAGACAATCCGAGG	Reverse	Subcloning of *chgH α* promoter
chgHβ-pGL4.10-infusion-F	GGTACCTGAGCTCGCTCATAATGCATCAA	Forward	Subcloning of *chgH β* promoter
chgHβ-pGL4.10-infusion-R	TGGCTTTACCAACAGAAGAACTAATCCG	Reverse	Subcloning of *chgH β* promoter
chgL-pGL4.10-infusion-F	GGTACCTGAGCTCGCATCCGGGTTTGCAG	Forward	Subcloning of *chgL* promoter
chgL-pGL4.10-infusion-R	TGGCTTTACCAACAGCAGCAATGTTCAC	Reverse	Subcloning of *chgL* promoter
vtgAs-promoter-1-pGL4.10	GGTACCTGAGCTCGCGGGCAGTCAGAATG	Forward	Subcloning of *vtgAs* promoter 1
vtgAs-promoter-1-pGL4.10	TGGCTTTACCAACAGGGCCAGTGTGATGT	Reverse	Subcloning of *vtgAs* promoter 1
vtgC-promoter-pGL4.10	GGTACCTGAGCTCGCCCGCACCATAGCAC	Forward	Subcloning of *vtgC* promoter
vtgC-promoter-pGL4.10	TGGCTTTACCAACAGGGTGAAATCCAG G	Reverse	Subcloning of *vtgC* promoter
esr1a-pGL4.10-infusion-F	GGTACCTGAGCTCGCCTTCTCCCATTTAAC	Forward	Subcloning of *esr1a* promoter
esr1a-pGL4.10-infusion-R	TGGCTTTACCAACAGAGATTTAAAAAAG	Reverse	Subcloning of *esr1a* promoter

Table 2 The numbers of putative transcription factor binding sites in choriogenin, vitellogenin and estrogen receptor 1a promoters of cutthroat trout

Promoter	Transcription factor binding site				
	ERE	ERE-like	1/2 ERE	Sp1	AP-1
chgH α	–	1	6	2	4
chgH β	–	2	8	–	3
chgL	–	1	7	7	11
vtgAs	1	7	6	7	10
vtgC	–	6	7	1	6
esr1a	–	3	19	9	11

ERE: Estrogen responsive element; ERE-like: Incomplete ERE; 1/2 ERE: ERE half-site; Sp1: Specificity Protein-1;
AP-1: binding sites for activator protein 1

Table A. 1 The numbers of putative transcription factor binding sites in choriogenin, vitellogenin and estrogen receptor 1a promoters of rainbow trout. The 2000 bp promoter sequences upstream of each gene (Accession No., *chgH α*: NM_001124273.1; *chgH β*: NM_001124600.1; *chgL*: NM_001124274.1; *vtgAs*: XM_036969074.1; *vtgC*: XM_021599921.2; *esr1a*: AJ242741; *esr1b*: NM_001124558.1; *esr2a*: NM_001124753.1; *esr2b*: NM_001124570.2) were obtained from whole genome database (Accession No.: GCF_013265735.2). Two online algorithms (ConSite; http://asp.ii.uib.no:8090/cgi-bin/CONSITE/ and NUBIScan; http://www.nubiscan.unibas.ch/) were used for prediction of transcription factor binding sites.

Promoter	Transcription factor binding site				
	ERE	ERE-like	1/2 ERE	Sp1	AP-1
chgH α	-	4	4	-	2
chgH β	-	2	7	-	2
chgL	-	2	4	2	3
vtgAs	1	4	4	2	4
vtgC	-	7	5	-	3
esr1a	-	2	5	1	-
ese1b	-	2	3	-	3
esr2a	-	4	7	1	2
esr2b	-	5	3	2	3

ERE: Estrogen responsive element; ERE-like: Incomplete ERE; 1/2 ERE: ERE half-site; Sp1: Specificity Protein-1; AP-1: binding sites for activator protein 1

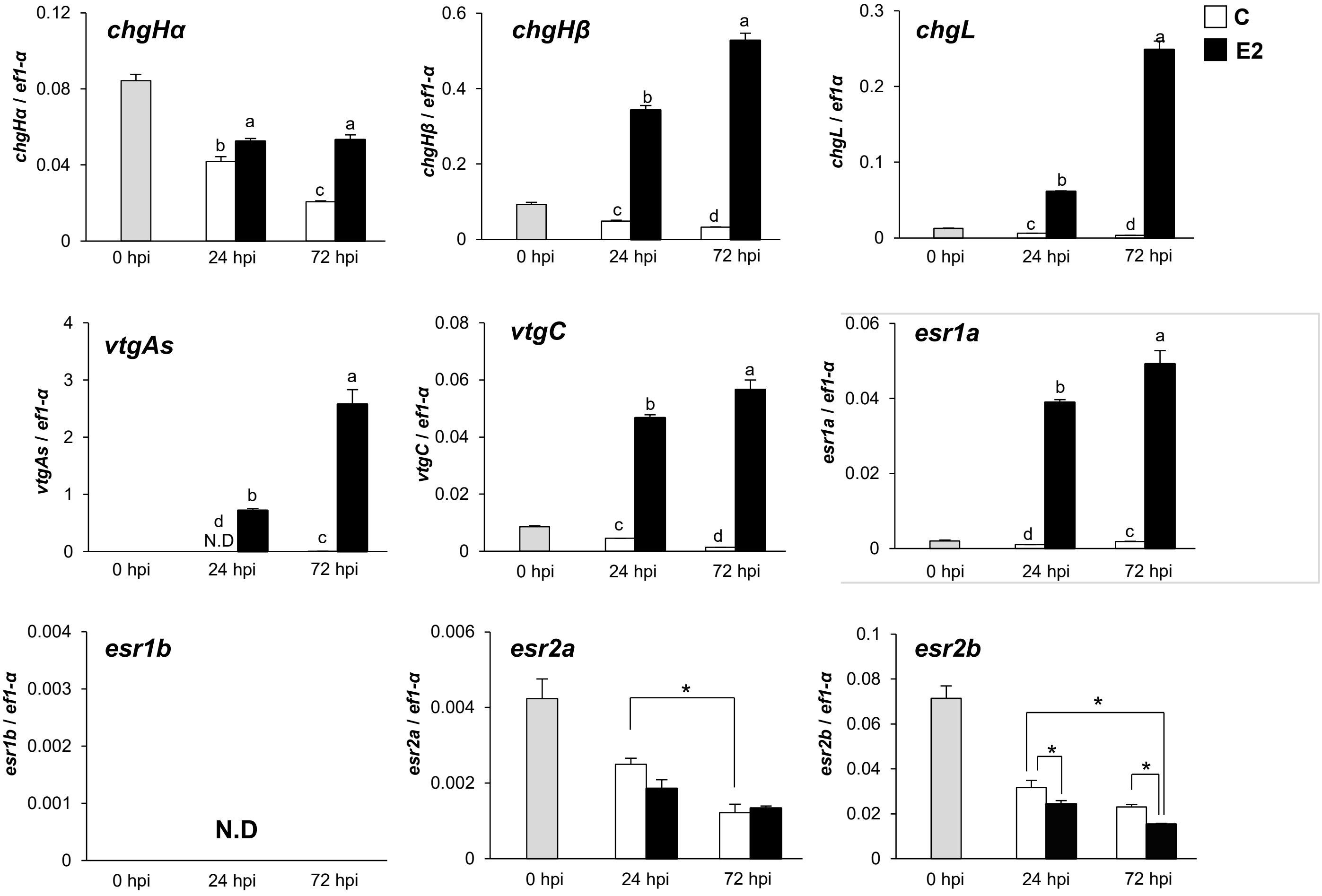

chgHα
chgHα / ef1-α
0.12
0.08
0.04
0
b
a
c
a
0 hpi
24 hpi
72 hpi
chgHβ
chgHβ / ef1-α
0.6
0.4
0.2
0
b
a
c
d
0 hpi
24 hpi
72 hpi
chgL
chgL / ef1α
0.3
0.2
0.1
0
a
b
c
d
0 hpi
24 hpi
72 hpi
C
E2
vtgAs
vtgAs / ef1-α
4
3
2
1
0
a
b
d
N.D
c
0 hpi
24 hpi
72 hpi
vtgC
vtgC / ef1-α
0.08
0.06
0.04
0.02
0
b
a
c
d
0 hpi
24 hpi
72 hpi
esr1a
esr1a / ef1-α
0.06
0.04
0.02
0
a
b
d
c
0 hpi
24 hpi
72 hpi
esr1b
esr1b / ef1-α
0.004
0.003
0.002
0.001
0
N.D
0 hpi
24 hpi
72 hpi
esr2a
esr2a / ef1-α
0.006
0.004
0.002
0
*
0 hpi
24 hpi
72 hpi
esr2b
esr2b / ef1-α
0.1
0.08
0.06
0.04
0.02
0
*
*
*
0 hpi
24 hpi
72 hpi

Fig. 1

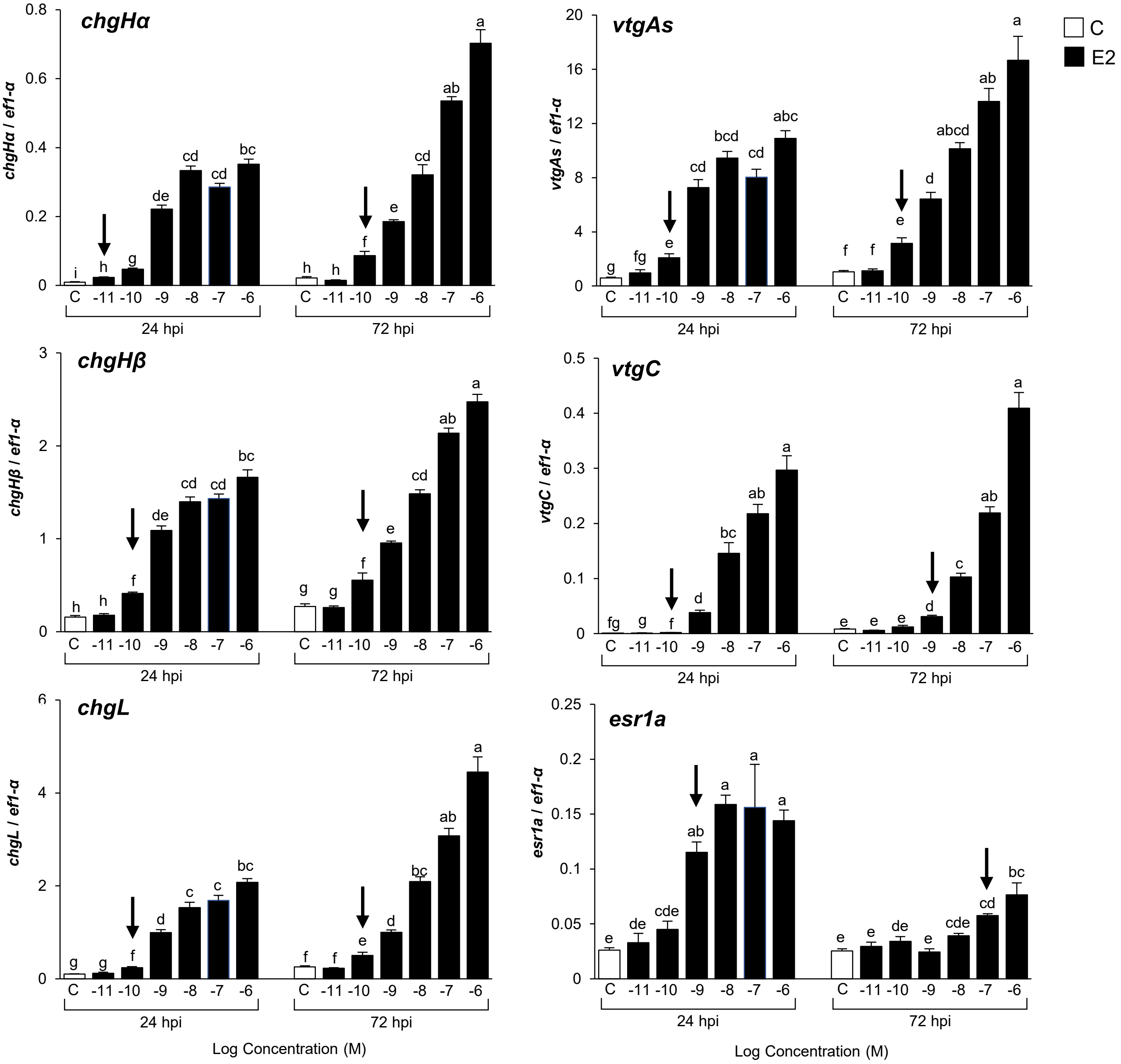

chgHα
chgHα / ef1-α
0.8
0.6
0.4
0.2
0
i
h
g
de
cd
cd
bc
h
h
f
e
cd
ab
a
vtgAs
vtgAs / ef1-α
20
16
12
8
4
0
g
fg
e
cd
bcd
cd
abc
f
f
e
d
abcd
ab
a
C
E2
chgHβ
chgHβ / ef1-α
3
2
1
0
h
h
f
de
cd
cd
bc
g
g
f
e
cd
ab
a
vtgC
vtgC / ef1-α
0.5
0.4
0.3
0.2
0.1
0
fg
g
f
d
bc
ab
a
e
e
e
d
c
ab
a
chgL
chgL / ef1-α
6
4
2
0
g
g
f
d
c
c
bc
f
f
e
d
bc
ab
a
esr1a
esr1a / ef1-α
0.25
0.2
0.15
0.1
0.05
0
e
de
cde
ab
a
a
a
e
e
de
e
cde
cd
bc
C -11 -10 -9 -8 -7 -6
C -11 -10 -9 -8 -7 -6
24 hpi
72 hpi
Log Concentration (M)

Fig. 2

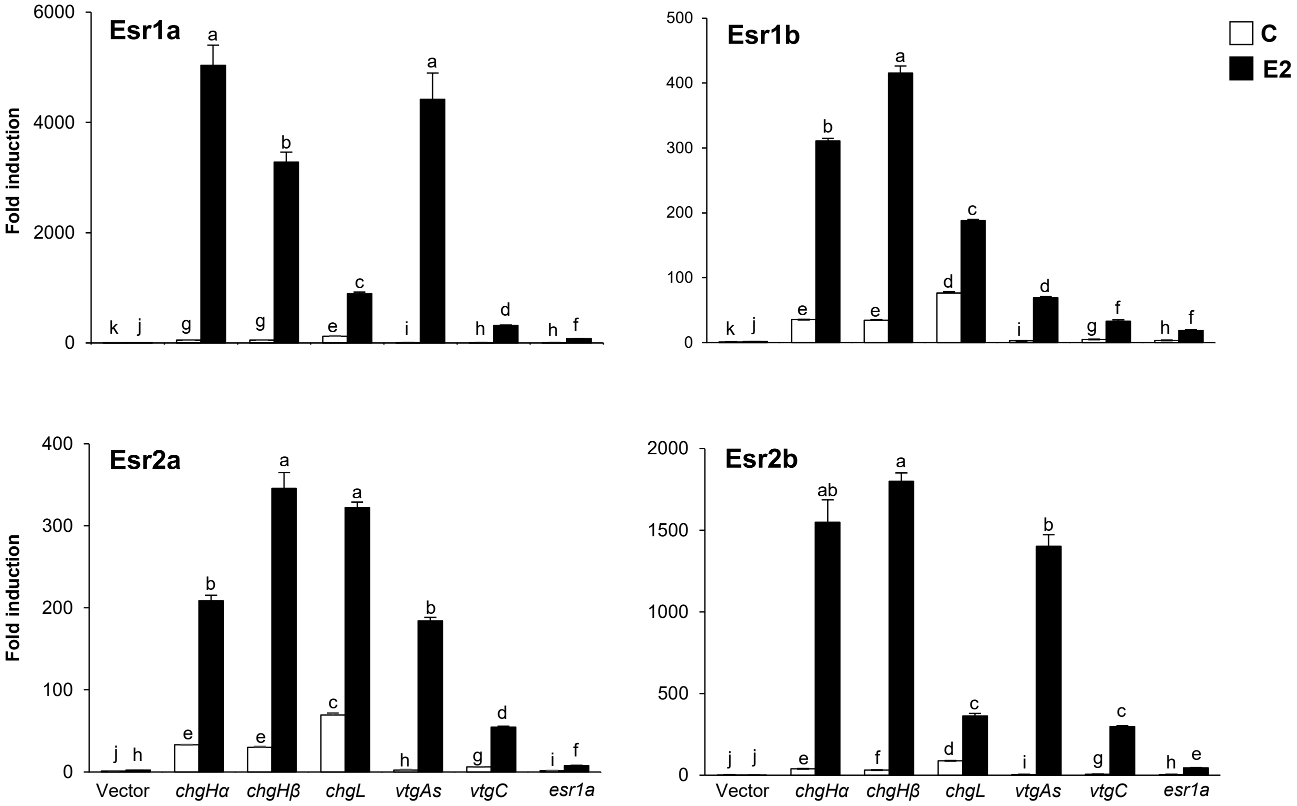
Esr1a
6000
4000
2000
0
Fold induction
k j g a g b e c i a h d h f
Esr1b
500
400
300
200
100
0
k j e b e a d c i d g f h f
C
E2
Esr2a
400
300
200
100
0
Fold induction
j h e b e a c a h b g d i f
Vector
chgHα
chgHβ
chgL
vtgAs
vtgC
esr1a
Esr2b
2000
1500
1000
500
0
j j e ab f a d c i b g c h e
Vector
chgHα
chgHβ
chgL
vtgAs
vtgC
esr1a

Fig. 3

```
-2851    ACTCCCCCAACCTTCCTCTTCCTGGAGAGGTGTTTATCTCTGTCGGCGCT

-2801    ATGCATGGAGAAGCCTGGTGGCTGAACAGATTCTGACAACATATCCCGAG

-2751    AGAGCCCTGTTTCTGTGAAAAAGATAATGTTACAATTGTTGATGTCTCTC

-2701    TGGAAGGCCACCCTTGCTTGAATTTCATCTACCTTGTTGTCAAGAGACTG

-2651    GACATTGCCGAGTAGTATACTCGGGAGCGGTGGGCAATGTGCATGTCTAC
                  1/2ERE
-2601    GAAGCCTGACCACGAGGCCACTCTGTCTGCCCCTTCTGCGGCGCCGTTAT

-2551    CTTGGGTCGAATTCTGGGATTAGATCCATTGTCCTGGGTGGTGGTGCAAA
                                             1/2ERE
-2501    CAGAGGATTCGCTTTGGGAAAGTCATATTCCTGGTCATAATGTTGACAAG

-2451    TTGACGTCGCTCTTATATTCAATAGTTCTTCCCAGCCGTATGTAATAAGA

-2401    CTTAAGATTTCCTGGGGTAACAAAGTAAGAAATAATTCATTAAGAAAACA

-2351    AAATACTGCATAGTTTCCTAAGGACTCGAAGCGAGCCGACCATCTCTGTC

-2301    GGTGCCATCTCCATAGGAGATCTACACAGGAGTTGCTTACCAAGAAGACA

-2251    GTGAATGTTCCTGAGTGGCCAAGTCAGTTTTAACTTAAATCTACTTTAAA

-2201    AGATATGGCAAGACCTGAAAATGGTTGTCTAGCATTGATCAACAACTAAT

-2151    TTGGCAGAGCTTGAAGAATTTTGAACAGAATAATGGGCAAATTTTGTACA
                                        AP-1
-2101    ATCCAGGTGTAGTAAACTCCAGAAAGACTCACAGCTGTAATCGCTGCCAA

-2051    AGCTACTTCTAAAAATTATTGACTTGGGGGTGTGAATACTTATGTAAATT

-2001    AGATATTTCTGTGTCTTATTTTCAATACATTTGCAAAAATGTCTAAAAAC
```

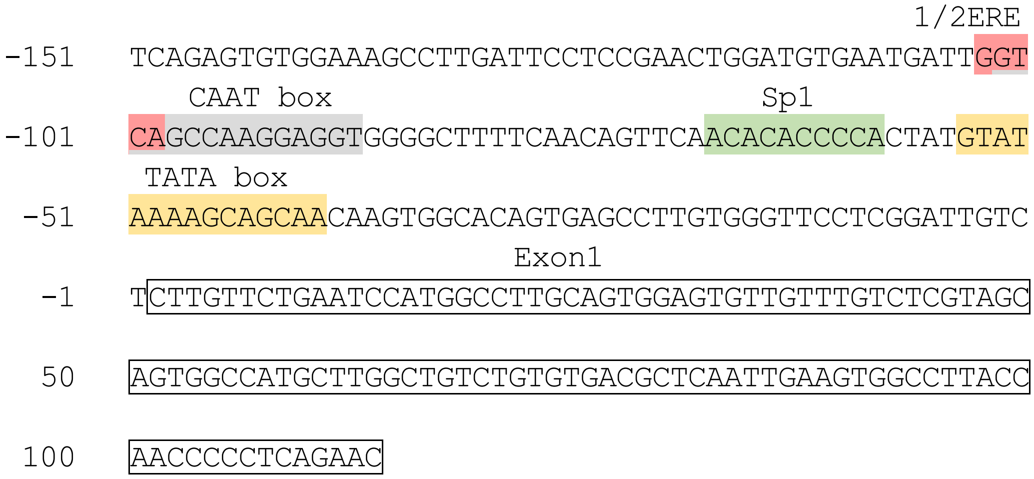
Fig. A. 1 Putative promoter sequence of the cutthroat trout choriogenin Hα gene including 5'flanking region and exon 1. The exons are indicated by open boxes. Nucleotides are numbered from the 5'-end of exon 1, negative numbers representing the 5'-flanking region. Estrogen responsive element (ERE)-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein-1; AP-1: activator protein-1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

```
-1951   ATGTTTCCACTTTGTCATTATGGGGTATTGTGTGTAGATTAATAAAAACT

-1901   GTAACACAACAAAATGTGGAATTAGTCCATGGGTATGAATACTTTCTGAA

-1851   GGCACTGTATGTACTTGTCCCTGCTTACAAATATCTGGGCATCTGGATAG

-1801   ATGAAAAGCTGTCTTTAAAAAAGCATATTGATGAGTTAGTTAAGAATCTG

-1751   AGAATAAAAAATGGGCTTTACTATAGAAATACAGTAAGTCCTGCCTCTCG

-1701   CTAAATAGTAGAAAGCAGATTATTCAGTCGACATTACTATCGGTCCTAGA

-1651   CCATGGCGAAATCATATATATGAACGCAGCTGACACTTCATTAAAGCCGG

-1601   TAGATGCAGGTGATCATAATGCACTGTGCTTTATTACGGGCAACAATTTT

-1551   AATACTCATCACTGCATTTTCTAGTAGAAAGTTGGTTGGTCCTCTTTGAT

-1501   GTCACCTAGGTTGATACATTACTATGTTTTCATTTATAAAGCCATTTTAC

-1451   AAAAAGTCCCACTGTACCTAACATCATTACTGAACTTTTGACATGAGTTA

-1401   CCAAACCTGGTCTCATGAATGGCTAACTCTGGAAATTCCTTTTGTCTCTA

-1351   ATTAGTTAGGTAAATCAGTTTTTTTATGATTGTGTTTTTCTTTCTGCTTG

-1301   CATTTTGTGTTTAGATTTGTGTATTTCCTGTAAAATTATCCCTGATAACA

-1251   TAAAGGTTAAATAGTATAAAAAAGTTTTGTAGGAATGCTGTTATTTTCTT
          AP-1   AP-1
-1201   TGAATGATTCATTTGGTCGTTGGTAAACATTATAGGAAGAACCTACTACT

-1151   GAAAGAGACACGAGAAACCACATTGAACTTTGCAAAAGCCCACCTAAACA

-1101   AGTCTAAATCCTGTGAAAATGTTCTGTGGACCACTGAAACAAAATTAGAG
```

Fig. A. 1 (Continued)

```
                                            1/2ERE
-1051    CTATTTGGCAATACAGATCAGTGCTATGTTTACAGATGACCAACTGAAGC
                            Sp1
-1001    TTTAAAAGAAAAGAACACCCTCCCTACAATCAAACATGGGGAGGTTTGAT

 -951    AATGCTGTGGGGTTGTTTTGCTTCCTCTGGTACTGAGGACCTTGAACATG

 -901    TGAAAGACGTAGTAGATTATCAATGTGTTTTTGGTAAACTAGTGGGTGTC

 -851    TGTTGAAGGTTGTGGGTCTTCCATCAGGACAAAAACACAAACACACATCA

 -801    ATAAACAACAGGAATGGTTAAAAATGAACACTGGACTGTTCTGGAGCGGC

 -751    CAGCGATGAGTTCAGATCAGAATCCCATCAAAAACCTATGGTGAGATATT

 -701    TTTCACAGATAACTCCAGTACCTGAAAATAAGTACCTGGATGTTCATATG

 -651    TTCTTCAGATTTTGTGTATGAGATCTGAAAACAGTAGTTAGTGGAAGGCA
                                             AP-1
 -601    CCACTCAAACATTGAAGAATTAGAGCAGTTTGAAGAGTGACTGAAGAGTG

 -551    GGACAAATTGCCATTAGAAAGGTGCAGCAAGCTCATTGATGGCAACAAGA

 -501    AGCATTTTCTGGTGGTTCTGCAATGCTGACAATCCAATAACAACATGTCT

 -451    AGACTGAAGTTTTTTTTAAATGTCAACTTATTTTTGAAGAAATAGGGAAT

 -401    TATTTAAGAAAAGTGAAAGGCAATATATTTATCTATATTAGAAATAGAAT

 -351    ACAATAGATACATTTGTGTCTATTATTATTTATAAAATGTTTACTTCTTG

 -301    AGTGTAGTTCTGCACACCGTGTGTACCTGCAAAATAAACATTTGTTAACA
                     1/2ERE                1/2ERE
 -251    AATACAATGTTACGGTCACAAAGACCTTCAACTCTGGTCAAAGGCATGTC
              ERE-like
 -201    AGAGATCTATGTGACCTGAACATTGCGTTTGTCAATAAATGTTTGGGACT
```

Fig. A. 1 (Continued)



```
                                                     1/2ERE
-151    TCAGAGTGTGGAAAGCCTTGATTCCTCCGAACTGGATGTGAATGATTGGT
           CAAT box                               Sp1
-101    CAGCCAAGGAGGTGGGGCTTTTCAACAGTTCAACACACCCCACTATGTAT
         TATA box
 -51    AAAAGCAGCAACAAGTGGCACAGTGAGCCTTGTGGGTTCCTCGGATTGTC
                             Exon1
  -1    TCTTGTTCTGAATCCATGGCCTTGCAGTGGAGTGTTGTTTGTCTCGTAGC

  50    AGTGGCCATGCTTGGCTGTCTGTGTGACGCTCAATTGAAGTGGCCTTACC

 100    AACCCCCTCAGAAC
```

Fig. A. 1 (Continued)

```
-2320   ATCACGATCGTGAAATACGTTGCGCACATTGGCACTCAAACAAAATAATA
                                    ERE-like
-2270   TCCCACATCGCAGGTGGGCAAAAGGACACACTAAGTCTGATCCCCAATTA

-2220   GAGGTAACGATCATCAGCTGCCTCCAATTGGGAACCATGCACACACACAC
                                                    1/2ERE
-2170   ACCAACATAGAAATATAATACCTAGAAACCCCCCTAGTCACGCCCTGACC

-2120   TAAAACACAGGGCGTGACATGGTGTGAATACTGTCCTAACTTATTCTGCA

-2070   AGTGCTGGATTTGCTAGTTAACGTATATGGTTTGATAACTAATGAACTCT

-2020   GTAAATGTCTATCTTTTACCTCATAATGCATCAATTTGACATCAATTTAT

-1970   GTTGCAACCAAATAATATATGGATGTGGCTGTAAATACTGCATCATCATT

-1920   CTCCAATGGAAAGAGTCATTGGAAAAAAGAGTCATCTGTGCTATATTTTG

-1870   GAAATGTAGTTTAGTGTACAATGCACTACTTACCTGGGTTGTATATAACA

-1820   ATATAATCCACTCATTATTTAAATTACAGTAATATATAAATAATTTCAAT

-1770   TAGAGAGCCAGGCAAAACTGTATGATTTGACAAGTCACATAGAAAAGGTG

-1720   ATCTTGTACAATGTTGCATCGGGCAGAAATGGCATACAACAACATGCTAT

-1670   GTTTTCACAGTAGCCACCTGCTTTACAATACACCTATTTCTGGTTCTGTC

-1620   CTATATATGTAGTGTATACGGCAGGGGTTCCCAAACTGTTTTTTTGGCCG

-1570   CAACCCCATTTTGAAATTAAAATGTTTTTGTAATCCCGCCATGTGAAAAA

-1520   TGTTATGTTATAAACGGCCAATGTTTACTTTTTTTATTTGGCTATGACA

-1470   GTTTATTACAAATCACTTTGACAGTACCTTTGAAAGTATTTCAATCTGAT
```

Fig. A. 2 Putative promoter sequence of the cutthroat trout choriogenin Hβ gene including 5’flanking region and exon 1. The exons are indicated by open boxes. Nucleotides are numbered from 5’-end of exon 1, with negative numbers representing the 5’flanking region. Estrogen responsive element (ERE)-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein 1; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

```
                           AP-1
-1420    GGAAGTATGATTTTAAGTGACTGAAATGCATCAGAACATTTTGAGTGCTC

-1370    TTGATGATGATCTTTTCCCCCCAGTCTGATTTAGTACCTGGTCTTGTCCA

-1320    TGAGTCCTGTGTGGCTTGTAGAGGGAAACAGAAGACACATACGTATTCCT
                                 1/2ERE
-1270    GAGAGTATTATCTTTCAATGATTGGGTCATAATATTTTGTAGCTTAAACG

-1220    GTTTAAAAGATAGAGCCACATTTGTAGGAAGAAAACAGAAACCGCTCTGT
                                      ERE-like AP-1
-1170    TTATTTCAAACACATCTAGCGGCAACCAGAGGTTACTGACCTAATGTCGG

-1120    TTCTATCAACCAAGCTCAATTTGATACATTTGATTTCAGTTTCTCTCGTG
         1/2ERE      1/2ERE
-1070    ACCCCCACATCGGGTCACAACCCCTAGTTTGGGACACGATGGTATAAGGA

-1020    TAATGAAACTGGAAGACTGACATATTTGTCAACATACTAACAACCTGCCG

 -970    TTTGACACAAGTTATTTTAAAAAGTGTGCATGTCAAGTTATAGTCAAATA

 -920    CTGTATAAAAATGATGTCTACTATTCTTTCTATTCAGAACTTCAAAAATA

 -870    TGTTTTGAAATGTGTATCCTGTCATTTATTCTATTGGATTAAATGGTAGT
                                                     AP-1
 -820    TAAAAGGACTAAATGGTGAGCCTATCATTATCTACTGTAGCGGTGACTAA

 -770    TCAAAAGATGCACATGGTATTTCAAGGAAAACTTTGATTTTGCATAAACG

 -720    ACTAAATGTGGAGGTGTGTGAAACCCCAATGAATGTACAATCAAAGCTTC

 -670    TGAACATAAGATAGGGGACATTAGAAGTGTTATCAGTTTAGAGTTTTTTA

 -620    CTTAGAGTTTTGAAAATATACCACTTCTGAAAAATGTGCCAAAGTGGTTG

 -570    AAAAAACAGTATATACAACCCAGGTATTTCAGAAATGCATTCTACACTAC
```

Fig. A. 2 (Continued)

```
-520    ACTATACACTGAGTATACAAAACATTAGAACACCTTGCTACTATTGAGTT

-470    GCACCCTCTTTTGCCCTCAGAAAAGAAAAAAACTGTAATATATACAAAAT

-420    TGTCTAATTATCTATTTATAATGCGTAAATCATTTTGAATTGTTTCTGAC

-370    TATCAAAATAAATATATAAATAAATATATATGTATATTGTCACATACAAC

-320    AACTAGGAGCAAATTAGATACTGACAGAACTGGCAAAGCCTCCAATGACA
                1/2ERE                    1/2ERE
-270    TGACGTGTCGGTCACAAAGACCTTTCTCAAGGCCATGACCGAAATGTTGG

-220    ATGTTCGCAAATGTTACACAACTGTTTTGAATAGTGTGCTACTAAAGGAA
                       CAAT box       1/2ERE
-170    CATTAGCCAGGGCATTTGATTGGCTAAAACTTTGACCCCTCAGAAAAGGA
          1/2ERE
-120    ACGGTCAGATAAAGAGGGGGCGGATTTCCAATAAGTAAACAACGCTCCAT
             TATA box
 -70    CTGTTATAAAGGTGGCCGCAATTCGTTCATCCTCATCACAGCATCCAGTG
                                      Exon 1
 -20    AACATTGCGGATTAGTTCTTGTAGCGAAGCCATTGCGATGAAGTGGAGTG

 +81    CAGTTTGTCTAGTGGCAGTGGCCACGCTTGGCTGGCTGTGTGATGCTCAG

+131    ATTTACTTGGAAAAACCAGGG
```

Fig. A. 2 (Continued)

```
-3636    ACTATAGGGCACGCGTGGTCGACGCGCCCGGGCTGGTCCTGTTCACAGAA
                                       Sp1       1/2ERE
-3586    GTGTTTTAGGGAGCGTGTGACAGTGATGAGGGGTGGTCGTTTGACCGCAG
                                  Sp1
-3536    ACCCATTACGGATGCAGGCAATGAGGCAGTGATCGCTGAGATCTTGGTTG

-3486    AAAACAGCAGAGGTGTATTTGGAGGGCGAGTTAGTTAGGATGATATCTAT

-3436    GAGGGTGCCCGTGTTTACGGATTTGGTGTTGTACCTGGTAGGTTCATTGA

-3386    TAATTTGTGTGAGATTGAGGGCATTAAGCTTAGATTGTAGGATGGCTGGG
              AP-1
-3336    GTGTTAATCACAACTTGGAACTACCCATCCCGGATCCAGGAGAATTGTCA

-3286    TCAACTACACTAATTAGCATAGCGCAACGGCCAAATAATCTTACTAGAAA

-3236    ATATTAATATTCATGAAATCACAAGTGAAATATAATGAAACACATTTTAG
                  AP-1
-3186    CCTTTTGTTAATCACCCTGTAGTCTCAGATTTTGAAATGATGCTTTACAG

-3136    CCAAAGCAAGACAAGCGTTTGTGTCAGTTTATCGATAGCCTAGCATAGCA

-3086    TTATGTCCAGCTAGCAGCAGGAAGCTCTGTCACAAAAATCAGAAAAGCAA

-3036    TCAAATTAACCGTTTACCTTTGATGATCTTCGGATGTTTTCACTGACGAG

-2986    ACTCCCAGTTAGACAGCAAATGTTCCTTTTGTTCCATAAAGATTATTTTT

-2936    ATACCCAAAATACCTCAGTTTGTTTGTCACGTTATGTTGAGAAATCTACC

-2886    GGAAATAGCTGTCACAACAACACCAAAAAAAATAATCTAATTATATCAAT

-2836    AATATCGACAGAAACATTGCAAACGTTTTTTTATAATCAATCCTCAAGGT

-2786    GTTTTTCAAGTATCTATTCGATAATATATCAACCGGGACAATTGGCTTTT
```

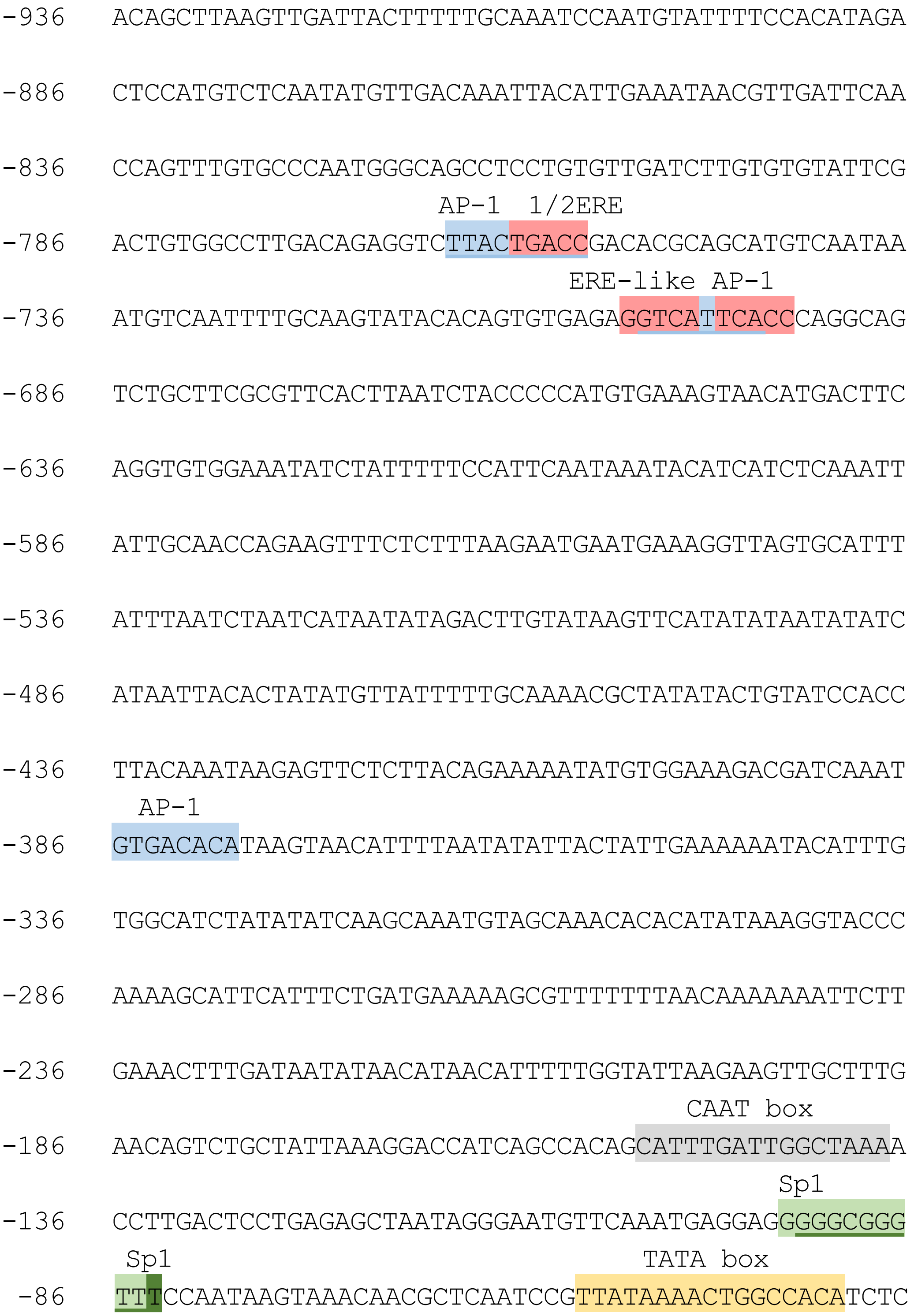
Fig. A. 3 Putative promoter sequence of the cutthroat trout choriogenin L gene including 5'flanking region and exon 1. The exons are indicated by open boxes. Nucleotides are numbered from 5'-end of exon 1, with negative numbers representing the 5'flanking region. Estrogen responsive element (ERE)-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein 1; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

```
-2736   CAGTAGGACCGAGAGGAAAAATGGCTACCTTTGTCTTTTACGCAATAATC

-2686   ACTCTGAGAGCGCTCATTCTTCAACATAAAGGCGTGAAACTACGTCTAAA

-2636   GGCTGTAGACACCTTAGGGAATACGTAGAAAAAGGAATCTGGTTGATATC
                                                      AP-1
-2586   CCTTTCAATGGCCAATAGGGATGCATAGGAACACAACGGTTTCAAAATAT
            AP-1
-2536   GAGTCACTTCCTGATTGGATTTTTCTTAGGCTTTCGCCTGCAATATCAGT
               AP-1
-2486   TATGTTATACTCACAGACAATATTTTGACAGTTTTGGAAACTTTAGAGTG

-2436   TTTTCTATCCTAAGCTGTCAATTATATGCATATTCTAGCATCTGGTCCTG

-2386   AGAAATAGGCTGTTTACTTTGGGAACGTTATTTTTCCAAAAATAAAAATA
                                       1/2ERE
-2336   GTGCCCCCTAGCTTCAAGCATGTCCCAGTTTAGGTCACCTAGTAGCACGA
                        1/2ERE
-2286   GCTCATAAGATAGATGGGGTCAATCAATTCACATATGATGTCCAGGGCAC

-2236   TTGTTTCTGGAAATAAATAGACTTGTTTCTGGAAAGGTGAATTTTTAGAA

-2186   GTAGAATCTCGAATTGTTTTGGTGCAGACCGGGATGGTAAGACAGAATTT
                                      Sp1
-2136   TGCAGGCTATCTCTGCAGAAGATTACAACACAGCCCCTTTGGCAGTTCTA

-2086   TCTTGTCGGAAAATGTTATAGTTAGGGATGGAAATTTCAGGGTTTTTGGT

-2036   GGTTTTCCTAAGCCAGGATTCAGACGTGGCTAAGACATCCGGGTTTGCAG
                        AP-1
-1986   AATATGCTAAACCAGTGAATAAAGCAAACTTAGGGAGTAGGCTTCTAATG

-1936   TTAACATGCATGAAACCAAGGCTTTTACGGTTACAGAAGTCAACAAATGA

-1886   GAGCACCTGGGGAGAAGGAGTAGAGCTGGGCACTGCAGGTCCTGGATTAA
```

Fig. A. 3 (Continued)

```
-1836    CCTCTACATCACCAGAGGAACAAAGGAGAAGTAGGACTTGTGGTTTCTAG

-1786    CTAACATTACATCAATCAAAGTATTAGAGCATGTCGTGAAGCAAAACTCA
            1/2ERE     AP-1 1/2ERE
-1736    AGTGGTCAAAACCATCTTGGGTCACCGGGAGGAGCGGGTTTGCAAAACAC
                                                      Sp1
-1686    CTTCCCAATATTGAGTTTTGCCCTCAGAACAGCCTCAATTCGTCGGGGCA

-1636    TGGACTCTTCAAGGTGTCAAAAGCGTTCCACAGGGATGCTGGCCCAAGTT

-1586    GGCTCGATGTCAAAGGCACTTCAATATTTTGCTTTGTCCATTCACACTCT
                              Sp1
-1536    GAATGGCACAGATACACAATCCATGCCTCAATTGTCTCAAGGCTTAACAA

-1486    TCCTTCTTTAACCTGTATCCTCCTCTTTATCTACACGGATTGAAGTCAAT

-1436    TTAATAAGTGACATCAGTAAGGGGTTGAAGATTTCACCTGGATTCACCTG
         1/2ERE
-1386    GTCAGTCTTTGTCATGGAAAGAGCAGGTGTTCTTAATGTTTTGTACACTC

-1336    AGTGTATATTTTCTTTATACAGACTAGTAAAAGATAAGTGAAAATTGACA

-1286    AATTATCCAATGGATTATGATAATTTTCTGGTAAAAAAAAGCTTGGGTAG

-1236    TCTGCCTGAGAATTGAGGGTAGTTTGCACCCTCTATTTGTATTTGCTCCA
                                                     AP-1
-1186    TGGCTCAGATGGTTAAATGGAGACAAGGCTGTTTGGCTTATCTCAGTCAC

-1136    GAAGAGGAATACATTTTCATCTGTTTCTAATCAATAAACAATGCCATTCT

-1086    GGTGGTTGGTAACATTCAAATAAAACCTAAACCTGAAATGAAACGTAGGC

-1036    TGAATTTAAAAAAAAAATTGGGTATGATGATGACATTTTTTAAATACATT

 -986    TTTTATTTAACGAGGCATAATCCTGTGGTTTAAATTTAACCCTCAAAACA
```

Fig. A. 3 (Continued)



```
-936    ACAGCTTAAGTTGATTACTTTTTGCAAATCCAATGTATTTTCCACATAGA

-886    CTCCATGTCTCAATATGTTGACAAATTACATTGAAATAACGTTGATTCAA

-836    CCAGTTTGTGCCCAATGGGCAGCCTCCTGTGTTGATCTTGTGTGTATTCG
                            AP-1  1/2ERE
-786    ACTGTGGCCTTGACAGAGGTCTTACTGACCGACACGCAGCATGTCAATAA
                                     ERE-like AP-1
-736    ATGTCAATTTTGCAAGTATACACAGTGTGAGAGGTCATTCACCCAGGCAG

-686    TCTGCTTCGCGTTCACTTAATCTACCCCCATGTGAAAGTAACATGACTTC

-636    AGGTGTGGAAATATCTATTTTTCCATTCAATAAATACATCATCTCAAATT

-586    ATTGCAACCAGAAGTTTCTCTTTAAGAATGAATGAAAGGTTAGTGCATTT

-536    ATTTAATCTAATCATAATATAGACTTGTATAAGTTCATATATAATATATC

-486    ATAATTACACTATATGTTATTTTTGCAAAACGCTATATACTGTATCCACC

-436    TTACAAATAAGAGTTCTCTTACAGAAAAATATGTGGAAAGACGATCAAAT
          AP-1
-386    GTGACACATAAGTAACATTTTAATATATTACTATTGAAAAAATACATTTG

-336    TGGCATCTATATATCAAGCAAATGTAGCAAACACACATATAAAGGTACCC

-286    AAAAGCATTCATTTCTGATGAAAAAGCGTTTTTTTAACAAAAAAATTCTT

-236    GAAACTTTGATAATATAACATAACATTTTTGGTATTAAGAAGTTGCTTTG
                                            CAAT box
-186    AACAGTCTGCTATTAAAGGACCATCAGCCACAGCATTTGATTGGCTAAAA
                                                  Sp1
-136    CCTTGACTCCTGAGAGCTAATAGGGAATGTTCAAATGAGGAGGGGGCGGG
         Sp1                             TATA box
 -86    TTTCCAATAAGTAAACAACGCTCAATCCGTTATAAAACTGGCCACATCTC
```

Fig. A. 3 (Continued)

```
                                                     Exon 1
-36    CTTCATCCTCTTCACAGCATCCAGTGAACATTGCTGATCAATTCTTATTG

+15    TGAAGCCATGGCGATGAAGTGGAGTGTAGTTTGTCTCGTGGCAGTGGCCA

+65    TGCTTGGCTGTCTGTGTGTTGCTCAGAATTGGCCACCCTTCAGTAAA
```

Fig. A. 3 (Continued)

```
-2872    AAGATGCAATTCGTCAGACTTCGGAGACTCTCTGGAAATCTCGGGTAACC
                                                        Sp1
-2822    TCGGATTCTCTAGGAAACGGAGACGTCAGAGATTTCCGGGATGCCTCAGA
             ERE-like  ERE-like
-2772    GGCGAGGTAAAATCTGACCTCTCCTTCCTACGTTACCGTAGCCACCCATC
                  1/2ERE
-2722    GATCCAGAAGGTCAAGGCGATGAGCAAGTCGAGATCTGTTGGTAGTTCCC

-2672    GGGCTGCTAGCTTATCTTTAACGTCCTCCGATAATCCATGCAGGAACGTG

-2622    GCGAACAGCGATTCCGGGTTCCAGGCACTCTCAGCCACAAATGTACGGAA

-2572    ATCCACTGCATATGTCCAACGGACATCAGCGTGGTGAGGTACGCTTTCTT

-2522    CGAGCAGTCCGAGGGAAAGGAGGAGGGCTGCAGCTCGAAGATGAGGGAAT

-2472    ACTGAGCGAGAAACGCCTGACAGGTTCCCGACTTTCCAGCGAAGCATTCC

-2422    AGAGGAGGTAAGCAGGGTTCTCAGGAAGCCGGGGTGGCCGGGAGAGACGC
                                   Sp1
-2372    GCTGCTGACAGTCGGGTTACTGAGGGGCTGGGAAGTTATATTCGTGGTAG

-2322    GCTGCCTAACAGACAACCCACGGAATTGCTCCAGCAATGTATCCAATGCA

-2272    AAACCTTCCATAAGACCACGAAGCAACTCCTCGTGCCTTCCAATGGTGGC
                         Sp1
-2222    TCCTTGGGAGAAGACGGCGTGGTGGAGCTGGTCCAAGTCTGCTGCGTCCG
               ERE-like
-2172    TCATGGTCAGTTTGTACTATCACGACTCAGGATAAGACCCAGATGGAGTT
                                          Sp1
-2122    CAAAATATCAAATGTATATTTACAAAACAGGGGGCAGGCAAACGACAGGT
                     1/2ERE  AP-1
-2072    CCAGGGCAGGCAGAGGTCAGTAATCCAGAGCAGAGTCCGAGAGGTACAGA
                         1/2ERE                1/2ERE
-2022    ACGGCAGGCAGGCTCAGGGTCAGGGCAGTCAGAATGGTCAAAACCGGGAA
```

Fig. A. 4 Putative promoter sequence of the cutthroat trout vitellogenin As gene including 5'flanking region, exon 1, intron 1 and exon 2. The exons are indicated by open boxes. Nucleotides are numbered from 5'-end of exon 1, intron 1 and exon 2, with negative numbers representing the 5'flanking region. Estrogen responsive element (ERE), ERE-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein 1; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

-1972 AACAGGGACTAGAGTGAAAACAGGAGTACGTGAAAACCACTAGTAGGCTT

-1922 GACGAGACAAGACGAACTGGTAACAGACAAACAGAGAACACAGGTATAAA

-1872 TGCACCAGGGATAATGGGGAAGATGGGCGACACCTGGAAGGGGGTAGAGA

-1822 CAAGCACAAAGACAGGAGAAACAGATCAGGGTGTGACGGATAGGGTATTT

AP-1 AP-1

-1772 ATGGATGTGTTATGAATGACTGAAGGTGTCTCACTTCAAACAAAGTATTA

-1722 CAGTATTACATGAGGTGTCAATAAATAGTTTTTTAATATTCTGCTAATTT

-1672 ATGAAGGTTCTCTCATGATCCACAGGTTATTGTAGGGTATGGGAGATATT

AP-1

-1622 TAAATGGAGAGATGGACCTCCAAAGCTTGTGTTTGGGTGTGTGTGTCAGA

-1572 GCCAAGATGATTTGGAGTAGTCCAACCTAAGACGACCTCACCAGGTATTC

-1522 CTTTTCTTTTCCCATGCTGGAGACCAGGGCTTGTTCACAACCTGTTATAA

AP-1

-1472 CGGTGCCAACATTTTGTGGCTGATCTTTCAGCGGGGGAGTGAGTGAATGT

-1422 GTCAAAGACCTGACTGGGCCCAGCACTGCACGGTATGGCTCGTTATGTTG

-1372 TTGTGTGTGGGTTTGTGTACTGAGGAACATGTAACTTGGTTGCAGTAAAA

-1322 GCCACTTTCAATTTCCTTATGTTGGGAGGTTTCATCAGGGTAAGTGTTTC

-1272 TTGGTGTAAAGTCGTCCCGAACACAGCACATATCAGCCTGGGATATCAGC

1/2ERE 1/2ERE

-1222 CATTCATAGTTAGCCTTAGTTGGAGTGACCATAGAATTCTATGGGAGTGA

AP-1

-1172 CCTTACTGAGTAAAGTATTTGCCATCATTCCCCCTGGTCGTCACTAGTTA

Sp1

-1122 ACGCTGCCACAAAGTCATAATTATGGCTAAACCCTGCCCATTTCCACAAT

Fig. A. 4 (Continued)

```
                                                  ERE-like
-1072   TTCTCTTCTAGAAAATAGATTTTAAATCTAACCCTAACTAATGAAGGTCA
                        Sp1
-1022   AGTTTGATGCGTTGGGGGGTGGTATGTGAAAAGTGCTGTAATTTCTAAAT
                                 AP-1
 -972   GGTTTACTCAAATTTCTAAACGGTTTACTCATTATGGATGAAAATACCCT

 -922   CAGATTAAAGCTGACAGTCTGCACTTTAACCTCCGTCATAGCATCATTTC
                                                   AP-1
 -872   AAATCAAAATTGCTGGAGTACAGAGCCAAAAACAACAACAAATGTCTCAC

 -822   TGTCCAATTTTTTTTTTTAAATTGACACATTAATATTTCAGTTGAATTTA

 -772   ACATCTGGATCGAACAACAAACCTGTTGTAGGCCTGCATATTCAGTGTCA
          AP-1                               ERE
 -722   TTGACTGACAAGATGGCAGAGTCTTATCTCTAGGTCAAGCTGACCATTGT

 -672   TGACACATGGTTGGTTCATCTGAATTACAAATACTTGTTCAAGGACTTCA
                    ERE-like
 -622   ATAGTGTTGAGGACAAGCTTGGACCAATGGCTGATCCATGTTCAAGTGCC

 -572   AAAACTCGTTGGGTTTTGACATTACTAGTATTCTAATTTATCACATTCTC
                         ERE-like
 -522   TAACAAATTAGAATCGATCATACATGTACTGATCCAAACCTAGACAATAT

 -472   ATTTTATTTAGCATTGTGTTGCTTCATTAATTTAGGCACCAACTGCCTAC

 -422   TTATCTCTACTGGGTTCTACTTCTAAAGTACAAGGGCCTTTCTGTACATT

 -372   TGTATTTAGTGGTTAGATAAATAGAGAAAAATAACTAATGTAGTATTTAA

 -322   AGAAAAATGAAGGTCGTAGTATACATAACTTGTTGGATAAAACAACACGT
         AP-1                                   ERE-like
 -272   GATTCTCCAGATAATTTTCATTAAAGAACTGACTTAGATGGACATTGATC
                                Sp1
 -222   TGTAAAGGGCTAAATGGCAGTGGGGCAGGTTAACCTAACCTATGGGTGTA
```

Fig. A. 4 (Continued)

```
-172    TGTGGCCACCTCAGAACTAGTGATAACCCACTCAACAGGCTTTATTATGA

-122    GCACATACTAATCTCAACTTATGTAACGTTAGTTAATTATATATTTTGTT

 -72    ACGATTGTTTTGTCGACAGAACATTTCTTTAAAAGGCGGACTGGGACTGT
                                      Exon 1
 -22    TGTTCCTCACATCACACTGGCCATGAGAGCAGTAGTACTTGCACTGACTC
                                 Intron 1
  29    TAGCCCTTGTGGGTAAGTACAGTTTTTCTGTCTTATTCTAGTGCAGATAT

  79    TATAACTAACTTCATGTAAATATAACTAAGTTATGTAAATATAGAAATGT

 129    ACAATTCTGTATCTATGACATTTTTTGAAAAACATTCAGTAACGTATTGT
                                      Exon 2
 179    GACAATAATTTTTGTTTTATCTTGTAGCGAGTCAATCTGTTAACTTT
```

Fig. A. 4 (Continued)

```
-3025    ATCAAACCATGCAATAATCTGAGTCGGCGCTCAGAGCCCAATCAAGACAC

-2975    AAATATATCTGCCATATTATGCAGTCAACAAAAGTCATAAATAGCATAAT

-2925    AAATATTCACTTACCTTTGCTGATCTTCGTCGGAATGCACTCCCAGGAAT

-2875    CCCACTTCCACAAGAAATTTTGTTTTGTTCGGTAATGTCCATCATTTATG

-2825    TCCAAATAATTACTTTTGTTAGCGCATTTGGTAAACAAATCAAGTCACGA

-2775    AGTGCGTTCACTAAAAGCAGACGAAATGTCAAAAAGTTCCGTAACAGTCA

-2725    GTAGAAACATGTCAAACGATTTATTGAATCAATCTTTAGGATGTTTTTAA

-2675    CATAAATCTTCAATAATATTCCAACCGGAGAATTCCATTGTCTTCAGAAG

-2625    TGTGATGGAACAGAGCTCCCTCTCATGTGAACGCGCATGGTTCACATGGC
                AP-1
-2575    AGACCTTACTCATTCCCTTCTCCTTCGGCCCCACTTCACAGTAGAATCAT

-2525    CAGACAAGGTTCTCAAGACTGTTGACATCTAGCGGAAGCCTTAGGAAGTG

-2475    CAACATTAATATATCCCACTGTATCGTCAATTGGAGCTCAGTTGAAAATC

-2425    AACCAACCTCAGATTTCCCAGATTTCCTGGTTGGATTTTTTTCTCAGGTT
                                  AP-1
-2375    TTTGCCTGCCGTATGAGTTCTGTTATACTCACAGGCATCATTCAAACAGT

-2325    TTTAGAAACTTCAGAGTGTTTTCTATCCAAATCTACTTATAATATGCTTC

-2275    ATGGCTTTATAGCAGGCCATTTACTCTGGGCATGCTTTTCATCCGGACGT
                Sp1                      1/2ERE
-2225    GAAAATACTGCCCCCTACCCCAAAGTTAAACAGGTCAACATCACAAAGCC

-2175    GCAGGGCCAGACAGATTACCAGGACGTGTACTCAAAGCATGTACGCACCA
```

Fig. A. 5 Putative promoter sequence of the cutthroat trout vitellogenin C gene including 5’flanking region and exon 1. The exons are indicated by open boxes. Nucleotides are numbered from 5’-end of exon 1, with negative numbers representing the 5’flanking region. Estrogen responsive element (ERE)-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein 1; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by

```
                                                     1/2ERE
-2125    ACTGGCATGCGTATTCACTGAGATTTTTAACCTCTCGCTGACCGAGTCTG

-2075    CAATACGTACAGGTTTAAGCAGACCACCATAGTCCCTGTGGCTAAGGAAG

-2025    CGAAGGTAACCTACCAAAATAATTACCGCACCATAGCACTCACGTATGTA

-1975    GCCATGAAGTGCTTTGAAAAGCTGATCATGGATCATATCAATACCATTAT

-1925    CCCAGAAACCCCAGACCCAGCGTTCAACACCATAGGGCCCACAAAGCTAG

-1875    TCACTAAGCTAAGTACCATGGAACTAAACACCTCCCTCTGCAACTGGATC

-1825    CTGGATTTCCTGACGGACACCCCCAGGTGGTAAGGGTAGGCAACAACAC

-1775    GTCTGCCACGCTGATCCTCAACTGTGGGGACCTCAGGGGTGTGTACTTAG

-1725    TCCCCTCCTGTACTCCCTGATCACCCATGACTGCGCGGCCAAACACGACT
                                                    AP-1
-1675    CAAACACCATCATTAAGTTGGTAAACCTGATCACCAACAATGATGAGACA
                         ERE-like
-1625    GCTTATAGGGAGGAGGTCAGAGACCTGGCAGTGTGGTGCCAGGACAACAA
                        AP-1
-1575    CCTCTTCCTCAATGTGAGCAAGACAAATGAGCTTATTGTGGACTACAGGA

-1525    AAAGGCAGGCTGAACATGCCCCCATTAACATTGACGGGGATGTAGTTTCA
            ERE-like
-1475    AGTTCATTGGTGTCCACATCTCCAACAAACGATCACAGTTGAAACACACC

-1425    AAGACAGTCGTAAAGAGAGCACGACACCACCACCTCAGGAGACTGAAAAG

-1375    ATTTGACATAGGTTCCCAGATCCTCAAAACCTTCTACAGCTGCAACATCG
            ERE-like
-1325    AGAGCATCCTGACCGGTTGCATCACTGCCTGGTATGGCAACTGCTCGGCA
          1/2ERE
-1275    TCTGACCATAAGGCGTTACAGAGGGTAGTGCGTACGGCTCTGCTACCACA
```

Fig. A. 5 (Continued)

```
-1225    CAGCAAGTGGTACCAGAGCGCCAAGTCTTGGACCAAAAGGCTCCTTAACA

-1175    GCTTCTACCCCCAAGACTGCTTAACAATTAAACAAATGGCCACTGGACTA
                1/2ERE
-1125    TTTACATTGACCCCCCTCCATTTGTTTTGTACACTGCTGTTACTCTATGT

-1075    TTATTATCTACGCATAGTCACTTCACCCCCACCTACATGTATAAATTACC

-1025    TCGACTAACCTGTACCCCCGCATATTGACTCGGTACCGGTACCCCTTGTA

 -975    TATAGGCTCATTATTGTTATTTAATTTTGTTACTTTTTATTTATTTTTTA

 -925    CTTTAGTTTATTTGGTAAATATTTTCTTAACTCTTCTTCAACTGCACTGT
                       AP-1
 -875    TGGTTAAGGGCTTGTGAGTAAGCATGTAAGGTCTACACTTGTTGTATTTG

 -825    GTGCATGTGACAAATAGAGTTTGATTTGATTTGATTTTGACTGTCAGTTG

 -775    AGTAACTGTAGCCCGTCTGTTGCTCTGCAAAATTTGTGTCAGTCCTTTAT
                      1/2ERE
 -725    CCTCTTTCATCAATGACCCGTTTTCGACAACTGGCCTGAAATTGTCTGGA
                        1/2ERE
 -675    TGTCCTTTGGATGGTTGACCATTCTTGATACAAAGAGGAAACTGTTGAGC
                                                 ERE-like
 -625    GTGAAAAAACGCTGCACCTGGTATCTACCAGCATACCCTGTTCAAATGCA

 -575    CTTAAATATTTAGTCGTACCCATCCACCCTCTGAATTGCACACAAGCACA

 -525    ATCCATGTCTCAATTTTATCAAGGCTTAAAAAGCCTCCTTTAAACCTGTC

 -475    TCCTCCCCTTCATTACACTGATTGAAGTGGAATTAATATCAATAAGGGAT

 -425    CATAGCTTTCACCTGGTTAGTCTATGTCATGGAAAGCACAGGTGTTCCTA

 -375    ATGTTGTGTACATCCTGTGAATATATTATTTGTGCAAGCAGCACACTTTG
```

Fig. A. 5 (Continued)

```
                       ERE-like
-325    TGCATTCAAATAAGGTCAAAGATGTTGCATTTTAAACAATTCTTTGTAAT

-275    GTGAGTTTCACAAAGCACTGATTATTAGTGTCCATTTTAGATCACTGTTT
                                           1/2ERE
-225    ATTTTTCCTGAAATGTCCTCCTTTTCCATGTCATACTGACCACTGTCAAC

-175    GTCACTGGTTACACTCGTCTGACATACCTTTGCCCCCTTGTTACTTTATG
                                              ERE-like
-125    CCCCTCATTGATGCCAAAGATGTGCTGTGCAGACACTGGGTCATGTACTC
           AP-1                           TATA box
 -75    GTTTAGTCATTGAGAACACAGCATTGCATATAAAAGGAAACGTTGAGCTG
                                          Exon1
 -25    CAGGATCCCACTCCTGGATTTCACCATGTGGGGGTTCCTTCTTTGTCACC

  25    TTGTGGCCTTGGCC
```

Fig. A. 5 (Continued)

```
-6155    CCTCGGCAAACTTAATGATGGTGTTGGAGTTAAGCTTAGCCATTCAGGTA

-6105    TGGGTGAGCAGGGAGTTCAGGAGGGGACTGAGCATGCACCCCTGAGGGGC

-6055    CCCTGTGTTGAGGATCAGCGAGGCAGATGTGTTGTTACCTACCCTTACCA

-6005    CCTGGGGGCGGCCCATCAGGAAGTCCACGATCCAGTTGCAGTGGGAGGTG

-5955    TTTAGTCCCAGGGTTCTTAGCTTGATGATGAGCTTTGAGGGTACTATGGT

-5905    GTTGAATGCTGAGCTGCAGTCAATGAATAGCAAAAGGTGTTCCTTTTGAA
            Sp1
-5855    AGGGCAGTGTGGAGTGCAATAGAGATTGCAACATCTGTGGATCTGTTGGG
           Sp1
-5805    GCGGTATGCAAATTGGAGTGGGTCTAGGGTTTCTGGGATAATGGTGTTGA
           AP-1
-5755    TGTGAGCCATTATCAGCCTTTCAAAGCACTTCATGGCTACATATGTGAGT

-5705    TCTACGGGTTGGTAGTCATTTAAGCGTTGTTACCTTGGTGTTCTTGGGCA
                                                     1/2ERE
-5655    CAGTTCTATGGTGGTCTACTTGAAACATGTTGGTGTTACAGACTCGGTCA

-5605    GGGACAGGTTGAAAACGTCAGTGAAGACACTCGCCAATTGCATGCTCAGA
                                                     1/2ERE
-5555    GTACAAGTTCTGGTAATCCGTCTGGCCCTCTGGCCTTGTGAATGTTGACC
                      AP-1
-5505    TATTTAAAGGTCTTACTCACATCGGCTATGGAGAGCATGATCATACAGTC

-5455    GTTCGGAACAGCTGATGCTCTCATGCATGCTACAGTGTTGCTTGCCTCAA
                                               AP-1
-5405    AGCAAGCATGGAAGTTATTTAGCTTGTCTGGTAGGCTTGTGTCACTGGGC

-5355    AGCTCGTGGCTGTGCTTCCCTTTTGTAGTCCGTAATAGTTTGCAGGCCCT

-5305    GCCACATCCAATGAGCGTCGGAGCCGGTGTAGTACAATTCAATCTCAGTC
```

Fig. A. 6 Putative promoter sequence of the cutthroat trout estrogen receptor 1a gene including 5’flanking region and exon 1, intron 1 and exon 2 . The exons are indicated by open boxes. Nucleotides are numbered from 5’-end of exon 1, with negative numbers representing the 5’flanking region. Estrogen responsive element (ERE)-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein 1; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

```
-5255   CTGTATTGACGCTTTGCCTGTTTGATGGTTCATCAGAGGACATAGCGGGA

-5205   TTTCTTATGTCCGGGTTAGAGTCCCGCTCCTTGAAAGCGGCAGCTCTAGC

-5155   CTTTAGCTCAGTGGGGAGGTTGCCTGTAATCCATGACTGCTGTTGGAGTA
                1/2ERE
-5105   TGTATGTACGGTCACTGTGGGGACGACGTCATCAATGCACTTATTGGCGA
             AP-1  AP-1
-5055   AGCCAGTGACTGATGTGGTGTACTCCTTAATGCATTCGGAAGAATCCCGG

-5005   AACATATTCCAGTCTGTGCTAGCAAAACAGTCCTGTAGCTTAACATCTGT
             1/2ERE
-4955   GTCAACTGACCACTTCCTTATTGAGCAAGTCACTGGTACTTCCTGCTTTA
                                              1/2ERE
-4905   GTTTTTGCTTGTAAGCAGGAATCAGGAGGATAGCGTTATGGTCAGATTTG

-4855   TCAAATGGAGGGCGATGGAGAGCTTTGTACACGTCTCTGTGTGTGGAGTA

-4805   AAGGTCGTCTAGAGTTTTTTTCCCCCTCTGGCTGCACATTTAACAAGCT

-4755   GCTAGAAATGAGGTAAAACTGATTTAAGTTTCCCTGTATTAAAGTCCCCG

-4705   GCCACTAGGAGCGCCGCCTCTGGATTAGCATTTTCTTGTTTGCTTATGGC

-4655   AGAATACAGCTCATTGAGAGCCGTCTTAGTGCCAGCATCGGTTTGTAGTG

-4605   GTAAATAGACAGCTACGAAAAATATAGATGTAATTATCTTGGTAAATAGT

-4555   GTGGTCTACAGCTTATCATGAGATACTTTACCTCTGGACTTCCTTAATAT

-4505   TAGATTTCATGCATCAGCTGTTATTTTCAAATAGACACAGACCGCCACCC

-4455   CTTGTCTTACCGGAGGCAGCTGTTCTATCTTGCCGATGGACAGAAAACCC
         Sp1
-4405   AGCCAGTTATCCATGTTGTTGTTCAGCCACGACTAAGTGAAACATAAGAT
```

Fig. A. 6 (Continued)

-4355 ATTACATTTGTCCTTTTGGTAGCATAGTCTTGATCGGAATTTGTTATCCA

-4305 TTGATTGCACGTTGGCTAATAGTACTGATGGTAGAGCAATCCTTACAAGG

AP-1
-4255 CACCCCAACCTACGTCCCCGATATCTCCGTCTCTTCTTCATGTGAATGAC

-4205 GGGGATGTGGGCCTTGTCGGGTGTCCGAAGTAAATCCTTTGCGTCGACTC

-4155 GTTAATGAAAAAGTCTTCGTCCAGTTCGAAGTGTGTAGTCACTGTCCTGA

1/2ERE
-4105 TATCCAGAAGCTCTTTTCGGTCAGAGGCGGTGGCAGAAACATTATGTATA

-4055 AAATAAGTTCAAAATAACGCGAAACACGCACAATAGTACAATTGGTTAGG

-4005 AGCCCGTAAAACGGCAGCCATCTCCTCCGGCGCCATCTCACAGGTGAAGC

-3955 TGTGCAAACTTCAGGGGTTTATTATAAGAGGCCAGCTAAATGTTGATTGT

-3905 TGAAAATGAAAGTGAAGTCGCATTACCAAAGATAATATGATGTTATGGCT

AP-1
-3855 GTTTTGGCAATTAATCCCAAATGTTACGATCACAGTTTCTGTGAGTTAAT

1/2ERE
-3805 GTCTCTGTTCCTGCTCTTAGGTGTCTCTGTGGGGTAACAGGTGTGACCTG

AP-1
-3755 TCCATCTCTGCTGGCATGGAGAACTCTCAGAAGGCCAGTCCCATTGACTC

1/2ERE
-3705 CCTGTCTGACCTGAAGTCTTTTATCCTGGTGGACGACTCCAACATGGTGT

1/2ERE
-3655 GGTCTGCTCTGACCTCTGCCCAGAGACCAGGGGAGGATGGCAAAATCACC

Sp1 1/2ERE
-3605 CCAGGGAGGGTGGACATCGTCCTGGACAACGCTGGCTTCGAGCTGGTCAC

-3555 AGACCTGGTCCTCGCTAACTTCCTGGTGTCCGCTGGTCTGGCCAGGGAGG

-3505 TCCGCTTCCATGGCAAGTCCATCCCCTGGTTTGTCTCCGACGTCACCTCG

Fig. A. 6 (Continued)

```
-3455    CACGACTTCCAGTGGACCATCCGCCAGACCCTGGCGGCCAATCACAAGTG

-3405    GATGTCCAAGAGCGGTGTCCAGTGGCAGAGCTACGTACGGGAGGGCGTGT
                                      Sp1
-3355    GGTCCTATCACGACCACCCGTTCTGGACAATGCCTCATGAGTACTGTGAC
                         1/2ERE
-3305    ATGGCGGGCGACGCGCCTGACCTCTACGCGACACTGCAGGGAGCCGACCT

-3255    GATTCTGTTTAAAGGAGACCTGAACTACAGGAAGCTGACTGGGGACAGGG

-3205    AGTGGGATCACATGGTGCCGTTTGATAGGGCACTGAGGGGATTCGGCCCA

-3155    GCTCCCCTGTGCAGTCTGAGGACTCTGAAGGCTAACATCCAGGTGGGGCT
                  Sp1                                 1/2ERE
-3105    CCAGCCGGGGCAGGGGGAGAACCTCAACACCCAGGACCCCAGCTGGATGA

-3055    CCAGCGGGAAGTACGCTGTGGTCCAGTTCTCCAGCCCTCACAGGGAACAG

-3005    TAGACCTGTCTGGGACAGGAAATGACGTCAATATGTTCATAATAAGTGGC

-2955    GTCATCATAATCAGAGGAAGTAAGGGCGTGAGCAGGGCCAGTGTACTTGT

-2905    AACCATATGCCAGGTTAGAGGTGTAGTTTGTTTTGTCGTAGAACTACCTA

-2855    ATGTATTTAAATGAGGAGAGTGGTTTAAAGGAGGATGATAGAGGATACTT

-2805    TAAAAAGAGGAGGGTAGTTAAATAGGGAGTGGCTGCAGATCCGACTTCTC

-2755    TGGACCCGTCGTCCCGACAGCTAAAGGTTCACAGCTTCTGTGAATGTGTG

-2705    GTAATCTCTACTTTACACACCATCTCTGTTCTATTTGTTCAGCTGCCCAG

-2655    AGAAGAAGCTACTCTGGCGAAAGGTGCTTGTTTAATAAAGTTAGATCAAA

-2605    CTCGAATCATTGTAAGATTACATAAAGATAATATTCTATAGAACCAAATA
```

Fig. A. 6 (Continued)

```
-2555   AAATAGCATAGTATAACATTACTGTATGACAAAAAACACCTAATTTCTTA

-2505   TATTTTAGGATGATTGTGCTCATCGTCACATTTTTCTCTCATGCAGTCTA

-2455   AACTCAACAGTGCACGCCACTCAGCAAAGGTATGCTACAGTTTAATACCA

-2405   TCTGCAAAAAAATGTGCTAAGGTACATAACTAATTCAAAACACTGTAGCC

-2355   TACTTCAAAACAACACTGTAGCCTACACTCAATAATATTTAAATGCATAT

-2305   GCCCATGAAACTGATTGCAAATGGTTGGCGTGCAGATTCTGCATGTATGT
                                                   AP-1
-2255   TTCACCGGTAAAACTTGGAAGAATTATCATTCACGATTGACAGTGAGAAA
             Sp1     1/2ERE
-2205   TGCGAAGGGAGGGTGACCAGAAGTTTGGGTGTGCGTAGAGTTAAAGAAAC

-2155   ACATGCACAGAACGTGAGTGGGATCTTCAGCTCTGGGGAAAGGATCCAG
              Sp1    1/2ERE
-2105   TGGGATGGGGCTGTGACCTCCGGATCCCCAGCCTCTGCCATTTAAATAAT

-2055   GATCAACTGCTGGTTAGCAATCCATCCTGGCATAACTACAAAACAATGAC

-2005   GTTTACTTCTCCCATTTAACAAATGTGTTGACGGTTGTTTACCATCATTC

-1955   AGTACTAGGGTCGGGCTCTACCTCGATTCCAGTTCAAATCAGTTAATTTA

-1905   GAAACATTGAAATTCCAAGTCATGAATTGAAACATGCTTATCTGATCTTA

-1855   AGTGACTTTTTTCCAGACATATTATGTAATTTATGTAATGTTTTTGTCTC

-1805   CCCAACAGCGACTACACAAAATATAGATTTGAGACACATTTCATTCATTA

-1755   TCAGAGGCAATAAGATGCAAGGTGCAATTTGAACATTTACATTTTAAGTT

-1705   GGCCTGTCGTAAATAGTTTAGAATTGTTATTTTAAGTTGAGCCCTTATGT
```

Fig. A. 6 (Continued)

```
-1655    AACACCCAAAGCCAGCGGCTATTTTTTAGCCATTTATTTTGCATGAGCTT

-1605    GGCAAGACATTACAGAGTTAACTTGTGGTTGCTGGTATTTTCTGGGTCTT

-1555    AATCAACGTCATCAGTTTGTCTTGTCAATTTATTGCATGCGTTTTTGTTT
                              1/2ERE
-1505    GCATACATCGGGGTTTGCACAGTGACCACAGTCCCGGAGTTTACATTTTC

-1455    ATAGTTAAATACCTGAAGTTGAACGACAAGGTAATGTTCCCACTCTGGGA

-1405    AACTATAGATAAATCATAGTCTTTGGGGCAGTGCAATCACACGTACAAGG

-1355    TGTGTGTTGTTAAACACCTGTCTATGATGTATTTCTGTTTGTTTGATAGT

-1305    ACAAAATGTTATTTCCGACTCATTAGAATGCCTCTGTAACCAGTGCAGGT

-1255    GCCGTTGTGATCCTAAATGACTGGATTGTTCTCTTTGCAGAAATCTGGAA

-1205    CAAAAATTGTTATATTATGATGGACTTGGTTTAAATAAAGAACTTCAATG
                                       AP-1
-1155    ACTGGTCTTATCTCATACTGTTGTATAAATGAATGACATTAAGAAAAGAG
           ERE-like
-1105    CAACCGAGGCTTGACCTAGAAAGATGATTCCCTTTCACTCATTCTCCCCA

-1055    TACTCTTATTACGCTCTTCAATTTATCCTGGGAACATGTATAAAAAATAG

-1005    AGTTATAATCAGACTGCTTGTTTAGCACTTGACATCACATGTCTGAATCT

 -955    GGGCTGTTTACTGAGTGAAGAGACGTTGTAGAGAATGGAGCTTCTCTAGG
             1/2ERE
 -905    CAGCAGGTCAAACAGGAGGCCATTTTCTCCCAGTTTGCAGAGATAGAGGT

 -855    TAATGAACCATCACTTTAATAAGCAAAAAGTTAACTTTCCCCCGTAAAAT

 -805    CCAGCTGTCAATTTGTCAAGAAAGTTTGGAAAAATCAGAATCCCCAGTAT
```

Fig. A. 6 (Continued)

```
-755   GATCTCACTGAATTGTTTGTGGAAATGAAGACACTCATCTTCATACTGTA

-705   ATATGGGGAGCTGATCTGTCCATTTTTATGTACTGAGAATTGTTGCACAC

-655   GTTGAGGACTGGAAATACTAGTAGTCTTAAATTAGTAATAACAGTGTTGT

-605   GCTAGGCACACCCTGCATTTCAACAAGTGACACCGTTATTCCTTTAGAGA
                                       ERE-like
-555   AACAGAGTTATCTTCCTGTGTAGGTTGAAGGTCAAGAGTTGTCCTTGTAC

-505   GCCCTTGAAACTTACCTAAAGGAATGGGGAAGACATTAAATGACTTTTCA

-455   AACATTGGTTGAATTTCATCATGATGCAGCATGTACTTGAAACCCCTTTG
                Sp1
-405   TTTGCTGCTTTGGCGGGATAATAGTTTATATTTGATCAGTGAATATCGTT
                         CAAT box
-355   GATAAGGTATTCCTCTTGAAAGGCCCAATGATAGCAAGACAATGTACTTT
                       1/2ERE              AP-1
-305   AAATTAGTAGCTATTCCTTGGTCACAGTAGAGAGTGATTTAGTCAGTGTA

-255   GTTTGGTGATCTTACTGCAATGCTGTATTCTGTTTTCCTGAATAGATTTA

-205   GGCTATGAAAAGGGGGAACATGTAAGAACATGCATTGCCTGATGTCTGAA

-155   TCCTCTCATCTTTTCCCCACTGGGAGTTACATAATGTTGGCATCAGAAGG
          1/2ERE                         ERE-like
-105   GTGTTGACCTCACATACTGTTTGCTGTGTCATGTTGACCTGCTCTAGAGA
                                                TATA box
 -55   TACACTATCAATATCGATCCGGCTGCGTTCATTTGTTTCTACCTTTTTTA
                     Exon1
  -5   AATCTCTTTTTTTTCTCATTGTGATGCGAAGCCAGATCTCAGAAAGAGGGA

 +46   TGAGAGCGAGAGGACAGGGAGAAAGAGGAACCACTCAACACAACAATGCT

 +96   CATGATTAGACCCAAAGAGCTGAATATTGATCCATAACGTCATTAAGGTA
```

Fig. A. 6 (Continued)

```
                    Intron1
+146    AGTACTGTATATATGACGTCTTTGTGTGTATTTCATGTAATGGGTTCCTA

+196    TTTTATTGCTTCCTTCTCGTGGAATTGTTTTGTATAATTTTTCCATATTT

+246    GCACATAAATCCACTGATAGACACATTTAATAATGATTTAACTTCTTAGA

+296    AGAGTGAGTCAGAAAGGAGTGATATTCTGGGCCACGGCAGTACATGTAAC

+346    TAAATGACTGATAAGCATTCAGAGAGGAAGTCACACCAGGGTGTAACTCT

+396    CCTTGGAGCCTAGCAGCGTCCTCTAAAAGCTTTTCAGGGAAGAGAAAAAT

+446    AAGCTGCATTTTAGAAAAAAATGTAAGGGGACAGGGGAGTTGTTGGAGTG

+496    GTTGCCTCTTTCTTTCGTTCTCTCACTCTCCTTCTCTCTTGCTCTCTATC

+546    TCGAAATATCTGTCACTTGTTGTCCTTTCCTCCACCCCTCTCTCCCACAG

+596    CAGTGTCGATAGTTAAGGGAAAAGAGAAAAGCTCTGCCTCTCAAAAATAA

+646    GTGGGAGGGAGGAATGTTTTTGGGCTGTCTCCAGGCGGAACCTGGGAGGG

+696    AAAGAGAGAGCAAGGAGGGACGAGAAAAGAGAGAGAGAGAACCTAGTGAA

+746    TGCCTCTTCCCTTCCTCTTCCCAACAGCCAGTATTGAGTTGCTTAGCACG

+796    GGCTGTTAAGGAAGAAACAGAGCAAGAGAGGGACGAGAGAAAAGAGAGAG

+846    AGAAGACAGAACAGAGCCCTTCTCCCCTCCCACCCCTTAGTGAGCCAGTC
                                                    1/2ERE
+896    TAAACCAAGCTGCTTGTCACTGCTGTTGTTCTGTGAATGTGATGCTGGTC

+946    AGACAGTCCCATACGCAGATTTCCAAACCTCTCGGAGCTCCTCTCAGATC
                   Exon2
+996    CCGAACGACCCTGGAGAGCCACGTCATCTCCACCCCAAAACTCTCACCAC
```

Fig. A. 6 (Continued)

+1046 AGCAGCCGACCACCCCCAACAGCAACATGTACCCTGAGGAGACACGCGGA

+1096 GGTGGTGGGGCGGCCGCCTTTAACTACCTGGACGGAGGGTATGACTACAC

+1146 AGCCCCTGCCCAA

Fig. A. 6 (Continued)