Studies on molecular mechanisms underlying regulation of hepatic estrogen-responsive genes in cutthroat trout, *Oncorhynchus clarki*

カットスロートトラウト肝臓におけるエストロジェン応答性遺伝子の
発現調節機構に関する研究

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2019（平成 **31** 年）

Chapter I. An introduction to studies on molecular mechanisms underlying regulation of hepatic estrogen-responsive genes in cutthroat trout, *Oncorhynchus clarki*

Aquaculture has been responsible for the supply of fish for human consumption. Recently, aquaculture has been encouraged in many fish species, but often met serious concern: one of the major concerns is the production of large numbers of viable eggs with high survival during early developmental stages. To date, the most of study for aquaculture has attempted to improve the efficiency of egg production and viability of progeny. However, in some cases, the resulting offspring in captivity has serious problems. For example, in Japanese eel (*Anguilla japonica*), European seabass (*Dicentrarchus labrax*) and yellowtail flounder (*Limanda ferruginea*), treatment with hormones to induce oocyte development and/or ovulation leads to high mortality of their embryo and larvae (Forniés et al., 2001; Avery et al., 2004; Kagawa et al., 2005). Efficient production of a large number of eggs with high survival during early developmental stages entails not only the programmed production of large numbers of eggs, but also high-quality eggs with the potential to support normal development and high survival of offspring to juvenile and later stages of development and growth. To achieve the efficient production of a large number of high-quality eggs, it is required to clarify the physiological mechanisms underlying oocyte growth in fishes, leading to the application of the mechanisms to egg production.

A teleost egg is surrounded by chorion and contains a substantial yolk mass. The chorion consists of an extremely thin, high-density outer layer and a thick, low-density inner layer making up most of the structure. The chorion protects the egg and the

embryo from physical and environmental stressors (Grierson and Neville, 1981; Songe et al., 2016). On the other hand, the yolk mass contains yolk proteins as a major component. The yolk proteins serve as the primary source of nutrients for embryonic development and larval growth. Thus, the chorion and yolk proteins are essential factors for the embryonic development and larval growth.

Choriogenin (Chg) and vitellogenin (Vtg) are precursors of the chorion and the yolk proteins, respectively (Hara et al., 2016). The Chg is a glycoprotein belonging zona pellucida (ZP) superfamily (Goudet et al., 2008). The Chg is produced in the livers of many teleosts including salmonids (Hara et al., 2016). The Vtg is lipoglycophosphoprotein and produced in the livers of oviparous vertebrates. The mechanisms underlying Chg and Vtg synthesis in the liver and their incorporation into the oocyte have been well studied in teleost, especially salmonid species (Hara et al., 2016; summarized in Fig. 1). Follicle-stimulating hormone secreted into bloodstream from the pituitary gland acts on the follicle cells surrounding developing oocytes to induce synthesis of estrogen, estradiol-17β (E2) in most case. E2 binds to sex steroid hormone-binding globulin in the blood and is transferred to hepatocytes through circulation. The E2 induces the synthesis of both Chg and Vtg in hepatocytes, and the produced Chg and Vtg are secreted into the blood and transported to the growing oocytes. The Chg accumulates in chorion as chorion proteins. The Vtg binds to the Vtg receptor that is present in the oocyte plasma membrane and is incorporated into the oocyte by the receptor-mediated endocytosis. The incorporated Vtg is cleaved proteolytically into four major yolk proteins, such as lipovitellin (Lv), phosvitin (Pv) β'-component (β'-c) and C-terminal peptide.

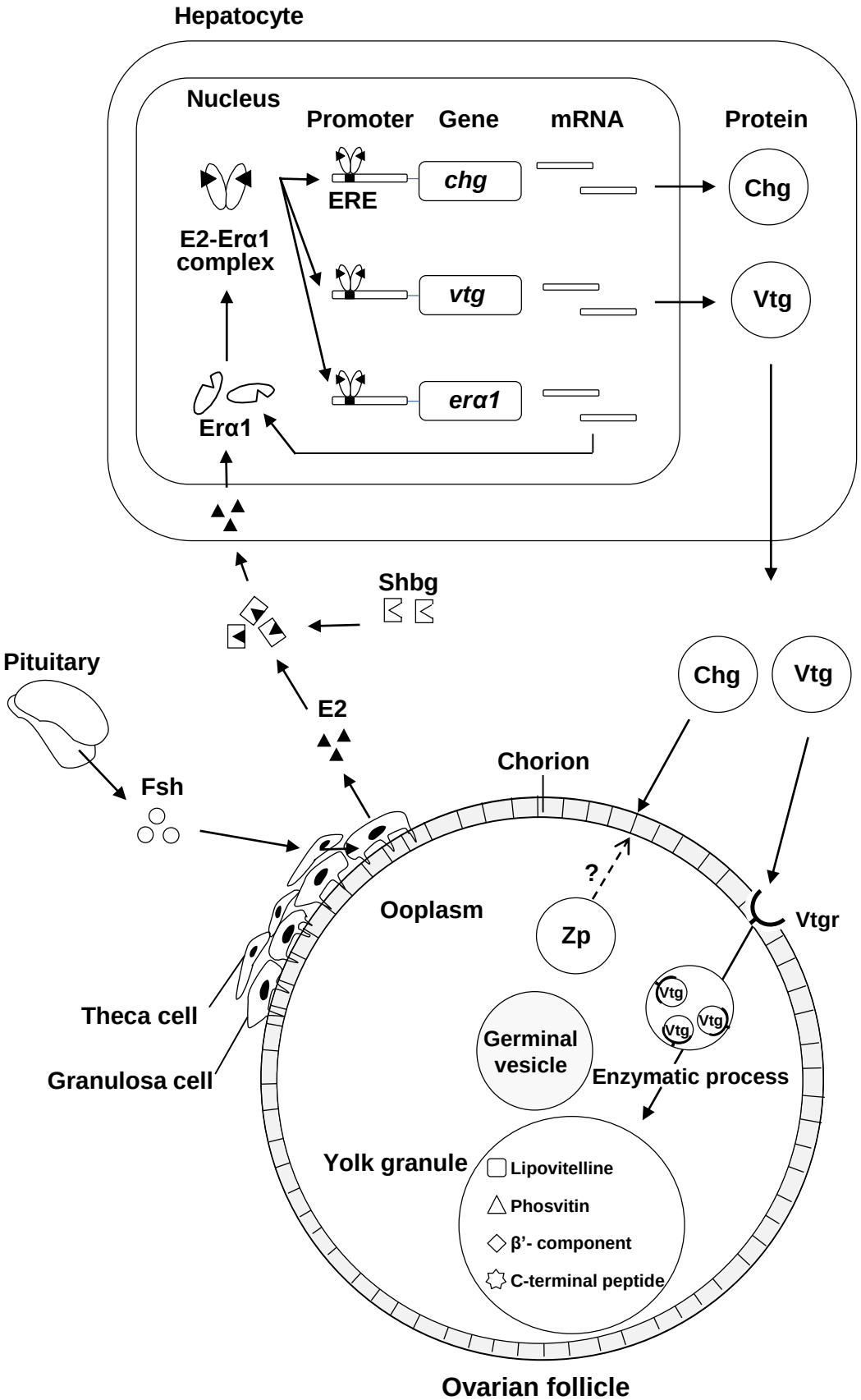

Fig. 1. A model for formation of chorion and yolk granule in salmonids. Chg: choriogenin, E2: estradiol-17β, Erα1: estrogen receptor α1, ERE: estrogen responsive element, Fsh: follicle-stimulating hormone, Shbp: steroid hormone binding protein, Vtg: vitellogenin, Vtgr: vitellogenin receptor, Zp: zona pellucida

The E2 generally acts on target cells through nuclear estrogen receptor (Er/ER). The Er/ER belongs to a superfamily of nuclear receptors that include receptors for various lipophilic ligands, such as other classes of steroids (progestin, androgen, glucocorticoid and mineralocorticoid), thyroid hormone, vitamin D, and retinoic acids (Bridgham et al., 2010; Baker, 2011; Sladek, 2011). In addition, numerous orphan receptors whose ligands have not been identified are also present in the family. Based on structural and functional similarities, the nuclear receptor proteins are divided into five or six domains (designated as domains A-F). Two domains (C and E domains) are well conserved among nuclear receptors. The cysteine-rich C domain is also known as the DNA binding domain (DBD), and the E domain is also termed as the ligand binding domain (LBD). The DBD recognizes and binds to specific response elements of DNA and contains two zinc binding motifs that are essential for binding to DNA. The LBD is essential for both ligand binding and for ligand-dependent transactivation function.

The molecular mechanism underlying E2-Er/ER dependent transactivation of the target gene has been generally accepted as follows. After E2 binds to Er/ER in the cell, the complex of E2 and Er/ER forms a homodimer that binds to estrogen responsive elements (ERE), the most typical of which is composed two head-to-head GGTCA half sites separated by three nucleotides (5'-GGTCAnnnTGACC-3', Walker et al., 1984), present in the promoter regions of targeted gene. Then, expression of the target gene is induced. In addition to ERE, the binding sites of other transcription factors such as activator protein-1 (AP-1) and specificity protein 1 (Sp1), have been known to interact with Er and transactivate an estrogen-responsive gene (Björnström and Sjöberg, 2005).

So far, cDNAs encoding ER orthologs have been cloned and characterized in

various vertebrates including teleosts. The presence of two forms of ERs designated as ERα and ERβ has been confirmed in most vertebrates, while most teleosts exhibit at least three distinct subtypes of Ers, including Erα, Erβ1 (also known as Erγ) and Erβ2 (also known as Erβ) (Hawkins et al., 2000; Ma et al., 2000; Choi and Habibi, 2003; Halm et al., 2004; Menuet, 2004; Nagler et al., 2007). Of the *er* subtypes in teleosts, hepatic *erα* (*erα*1 in salmonids) expression is up-regulated in the liver during vitellogenesis (Sabo-Attwood et al., 2004; Nagler et al., 2012) and up-regulated by E2 treatment (Sabo-Attwood et al., 2004; Filby and Tyler, 2005; Boyce-Derricott et al., 2009). Thus, Erα/Erα1 is thought to be the major regulator of E2 signaling in the liver during vitellogenesis. In rainbow trout (*Oncorhynchus mykiss*), an additional *erα* subtype (*erα2*) has been identified (Nagler et al., 2007). However, *erα2* is considered to be a minor *er* subtype, because its hepatic mRNA levels are relatively low during vitellogenesis and after E2-treatment (Boyce-Derricott et al., 2009; Nagler et al., 2012). In addition, the functionality of Erα2 protein (abilities of ligand-binding and estrogen-dependent transactivation of target gene) has not been confirmed yet. Recent studies have shown that Erβ is also involved in the expression of *vtg* (Nelson and Habibi, 2010; Griffin et al., 2013). However, the information on involvement of Er subtypes in the regulation of hepatic estrogen-responsive genes (*chg*, *vtg* and *erα* etc.) is quite limited.

It has been shown that genes encoding proteins consisting of fish chorion are expressed not only in the liver (hepatic type: *chg*) but also in the ovary (ovarian type: *zp*). Cyprinidae fishes, such as carp (*Cyprimus carpio*) and zebrafish (*Danio rerio*), exclusively express *zp* genes in the ovary (Chang et al., 1996, 1997; Wang and Gong,

1999). In zebrafish, ovarian *zpB* (*zp2*) and *zpC* (*zp3*) mRNAs do not show the response to E2 (Liu, 2006). No *chg* gene has been found in the liver of Cyprinidae. Therefore, in Cyprinidae, chorion appears to be made from ovary-derived Zp proteins alone. Meanwhile, medaka (*Oryzias lapites*), pacific herring (*Clupea pallasii*), masu salmon (*O. masou*) and cutthroat trout (*O. clarki*), which express *chg* genes in the liver, also express *zp* genes in the ovary (Kanamori, 2000; Fujita et al., 2003; Kawakita, 2013; Sano et al., 2013). In these species, it has been considered that both Chg and Zp proteins are involved in the chorion formation.

Widespread multiplicity of *chg*/Chg has become evident in teleosts. Chgs are categorized into high type (ChgH) and low type (ChgL) from their molecular weight (Hara et al., 2016). These proteins are typically classified into the zona pellucida B (ZPB) or C (ZPC) subfamilies, respectively, based on the unified nomenclature system for the ZP gene family (Goudet et al., 2008). ChgH and ChgL contain N-terminal signal peptide, ZP domain and consensus furin-like cleavage site. ChgH also has trefoil domain just upstream of its ZP domain. It has been shown that salmonids have three Chg subtypes; ChgHα ChgHβ and ChgL (Hyllner et al., 2001; Westerlund et al., 2001; Fujita et al., 2008a).

It has been confirmed that multiple subtypes of Vtg exist in fish (Hiramatsu et al., 2006; Finn and Kristoffersen, 2007). The multiple Vtg subtypes are simply classified into either a complete type or an incomplete type based on following features. The complete Vtg composed of the five egg yolk protein regions: Lv heavy chain (LvH), Pv, Lv light chain (LvL), β'-c, C-terminal coding domain. As a result of homology analysis, the complete Vtg is further divided into two subgroups, type A (called VtgA or VtgAa)

and type B (VtgB or VtgAb). On the other hand, the incomplete type of Vtg consists only of LvH and LvL and is classified into type C (VtgC). In salmonids, besides a *vtgC* gene, multiple copies of salmonid A-type *vtg* genes (designated as *vtgAs*) are evident (Trichet et al., 2000; Buisine et al., 2002).

As described above, E2 regulates the expression of oogenesis-related genes such as *chg*, *vtg* and *er*α (*er*α*1* in salmonids) in the liver during vitellogenesis. In addition, these estrogen-responsive genes are up-regulated by E2 treatment not only in female fish but also in juvenile or male fish (Hyllner et al., 2001; Westerlund et al., 2001; Boyce-Derricott et al., 2009; Amano et al., 2010; Mushirobira et al., 2018). However, there is still a room to elucidate how E2 can regulate the expressions of these multiple genes. The elucidation of this mechanism leads to the further understandings of oocyte development, as well as the molecular mechanisms underlying transcriptional the regulation of multiple genes by E2. To date, many studies for the E2 responses of *chg*, *vtg* and *er*α genes have been conducted. However, these studies were not conducted in a single species nor consider the multiplicity of *chg*, *vtg* and *er*. Therefore, it is difficult to obtain an integrated view on the E2 response of these genes. To understand the differences of E2 responses of these genes, it is important to analyze the responses in a single species with consideration of all gene subtypes.

The transcriptional response of a gene to a hormone *in vivo* is affected by the complexity of the intact animal. Approaches by *ex vivo* experiment could greatly advance the understanding of tissue and/or cell basic properties and environmental adaptive responses without the complexity of the intact animals (Segner, 1998). In salmonids, the primary culture system of hepatocytes has been established, which was

used to analyze a hormonal effect on the genes or proteins (Klaunig et al., 1985; Schreer et al., 2005). The primary cultured hepatocytes give all the benefits of an intact cell (functional organelles, enzyme interactions, physiological cofactor and metabolite concentrations, etc.) without the complexity of the intact animal. In this *ex vivo* system, defined experimental conditions can be easily set and maintained, indicating the possibility for refined physiological studies in which the effects of exogenous factors are analyzed. In addition, multiple types of experimental conditions can easily be set with populations of a single type of cell obtained from a single animal.

It has generally been considered that a promoter region for a gene is responsible for its gene expression (Maniatis et al., 1987). In particular, the expressions of E2-responsive genes are strongly affected by the interaction between Er and the promoter region (O'Lone et al., 2004): the characteristic of the transcriptional response of a gene to E2 appears to reflect on the characteristics of its promoter. To clarify the characteristics of promoter region for a gene, it is effective to perform reporter gene assay *in vitro*. In this system, the direct interaction of a nuclear receptor and the promoter of a gene can be examined. Reporter gene assay have revealed the properties for transactivation of the E2-responsive genes by Er using mammalian cell lines (HEK 293, Hep G2, CHO-K1, HeLa etc.) in several studies (Le Drean et al., 1995; Menuet, 2004; Davis et al., 2010; Lee Pow et al., 2016).

Cutthroat trout is a close relative to rainbow trout, which is an important salmonid for fisheries and as a biological research model. Thus, the information from molecular biological databases [whole genome and expressed sequence tag (EST), etc], which have been established in rainbow trout, can be fairly adapted for the study using

cutthroat trout. Recently, cutthroat trout has been used as a model species for basic research on teleost ovarian follicle growth (Hiramatsu et al., 2015: Luo et al., 2013; Mizuta et al., 2013, 2017, Mushirobira et al., 2013, 2018; Ryu et al., 2013). It should be noticed that the mRNA expressions of dual *vtg* genes (*vtgAs*, *vtgC*) have been analyzed in cutthroat trout *in vivo* and *in vitro* (Mushirobira et al., 2013; Mushirobira, 2015; Mushirobira et al., 2018). The hepatic mRNAs of *vtgAs* and *vtgC* synchronously increased with the progress vitellogenesis during the reproductive cycle. Injections of E2 to male and juvenile fish synchronous increased the hepatic levels of both *vtg* mRNAs. The promoters of *vtgAs* (*vtgAs* promoters 1 and 2) and *vtgC* (*vtgC* promoter) genes were cloned from the genome. The reporter gene assay with co-expression of the trout Erα1 revealed that the E2-dependent transcriptional activity of *vtgAs* promoter 1 was highest among those of the other promoters. In addition, all three *vtg* promoters showed similar half-maximal effective concentrations (EC50) for E2.

As described above, E2 regulates the expressions of multiple genes in the liver. The elucidation of this mechanism leads to the further understandings of oocyte development, as well as the regulation mechanism of multiple genes by E2. The characteristic of the transcriptional response of a gene to E2 appears to reflect on the characteristic of its promoter. Based on these backgrounds, this study was performed in order to reveal the relationship between the expression profile of E2-responsive genes (*chg*, *vtg*, *erα1*) and the characteristics of the promoter region, using cutthroat trout as an experimental model fish. This dissertation includes following contents: 1) expression analysis of *chg*, *vtg* and *erα1* mRNAs in the liver of female trout during a reproductive cycle (Chapter II), 2) expression analysis of *chg*, *vtg* and *erα1* mRNAs in the liver of

male fish following E2 injections *in vivo* (Chapter III), 3) expression analysis of *chg*, *vtg* and *er* mRNAs in the male liver *ex vivo* (Chapter IV), 4) molecular characterization on the structures and the transcriptional functions of *chg*, *vtg* and *erα1* gene promoters *in vitro* (Chapter V). Finally, a model for the molecular mechanisms underlying regulation of hepatic *chg*, *vtg* and *er* genes by E2 was proposed (Chapter VI).

Chapter II. Changes in choriogenins, vitellogenins and estrogen receptor α1 mRNAs in the liver during a reproductive cycle of female cutthroat trout

Introduction

It has been shown that vitellogenesis is correlated well with elevated E2, Chg and Vtg levels in the blood of salmonid species (Hara et al., 2016). Thus, it is assumed that the elevated E2 levels lead to active expression of *chg*, *vtg* and *erα1* mRNAs in the liver. Therefore, analyzing the expression pattern of *chg*, *vtg* and *erα1* genes during reproductive cycle of female fish is important to understand how the genes response to E2 *in vivo*. To date, many studies have analyzed the Chg/*chg*, Vtg/*vtg* and Erα1/*erα1* levels during reproductive cycles of salmonid species (Hiramatsu et al., 1997; Shimizu et al., 2000; Fujita et al., 2005; Amano et al., 2010; Nagler et al., 2012). However, these estrogen-responsive genes or proteins have never been quantified together in single species nor considered their multiplicity. Thus, it is difficult to obtain the integrated view on the E2 response of these genes. Accumulating information on expression profiles of these genes in a single species would contribute to elucidate the mechanism underlying E2-dependent regulation of multiple gene expressions in the liver of salmonids.

The profiles of serum E2 and Vtgs (VtgAs/VtgC) protein levels and hepatic *vtg* (*vtgAs/vtgC*) mRNA levels during a reproductive cycle of cutthroat trout have been revealed (Mushirobira et al., 2013; Mushirobira, 2015). The both subtypes of *vtg*/Vtg showed similar patterns: *vtg*/Vtg levels increased with the progress of vitellogenesis, as

well as serum E2 levels. In the ovulated fish, serum Vtg levels remained high but serum E2 and hepatic *vtg* mRNA levels decreased.

The objective of this Chapter II was set to reveal relationship among serum E2 levels and hepatic *chg*, *vtg* and *erα1* mRNAs expressions during a reproductive cycle of female cutthroat trout, for further extension of our understanding on the regulation of hepatic estrogen-responsible, oogenesis-related, gene expressions. At first, three *chg* subtypes were cloned from the liver of cutthroat trout. Then, the expression patterns of hepatic *chg* and *erα1* mRNAs were quantified by real-time quantitative PCR (qPCR), using the same samples which were analyzed in the previous studies (Mushirobira et al., 2013; Mushirobira, 2015).

Materials and methods

Experimental fish and tissue samples

Cutthroat trout were obtained from a breeding stock held in flow-through fresh water under a natural ambient condition at Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University.

For cDNA cloning of *chg*s, a liver from a two-year-old female (body weight 355.3 g; fork length 315 mm) was obtained on September 10, 2013. The liver was cut into small pieces (2 mm × 2 mm) by scissors, incubated overnight in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C, and stored at -30°C until extraction of total RNAs.

For quantification of mRNA levels of *chg* and *erα1* mRNAs during reproductive cycle, same liver samples used for quantification of *vtg* mRNAs in previous study by Mushirobira et al. (2013) and Mushirobira (2015) were reused. Briefly, the samplings were conducted from January 2009 of two-year-old females to February 2010 of ovulated three-year-old fish; those were done monthly until June 2009 and every other month from August 2009 to February 2010. Five females were used in each sampling point. Fish were anesthetized with 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan) and weighed. Blood was sampled from caudal vein with syringes. Blood was allowed to clot, and serum was obtained by centrifugation and stored at -30°C for E2 assay (Mushirobira, 2015). Liver and ovary were dissected out. Liver was processed as described above. Ovary was weighed for calculation of gonad-somatic index (GSI: gonad weight/body weight × 100). Ovarian follicles were isolated from a portion of

ovary with forceps, and the diameters of follicles were measured using a stereoscopic microscope. Oocyte developmental stages were categorized based on the mean diameter of ovarian follicle into perinucleolus stage (< 0.5 mm diameter), lipid droplet stage (0.5 ~ 1.0 mm diameter) and vitellogeninc stage (≥ 1.0 mm diameter).

Molecular cloning of *chgHα*, *chgHβ* and *chgL*

Total RNA was extracted from the liver samples with Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. Concentration of total RNA was measured with NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). 1 μg total RNA was reverse-transcribed by PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan), according to the manufacturer's instruction. The resulting cDNAs template were stored at -30°C.

Primer sets (Table 1) were designed from rainbow trout *chgHα* (NM_001124273) *chgHβ* (NM_001124600), and *chgL* (NM_001124274) sequences in order to amplify each entire open reading frame in cutthroat trout. Polymerase chain reaction (PCR) was performed using PrimeSTAR® Max DNA Polymerase (Takara Bio) as follows: 1 μl 1st stranded cDNA, 5 μl PrimeSTAR® Max premix, 1 μl each forward and reverse primer (10 μM) and nuclease-free water up to final volume of 10 μl. The PCR amplification was carried out as following thermal parameters: 30 cycles at 98°C for 10 s, 55 °C for 5 s and 72 °C for 1 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 manufacturer's instruction. The PCR products were subjected to an A-tailing reaction (Knoche and Kephart, 1999)

followed by a ligation into pGEM-T Easy Vector (Promega, Madison, WI, USA) according to the manufacturer's instruction. The ligated products were transformed into XL-1 competent cells (Strategene, La Jolla, CA, USA). Recombinant clones were cultured overnight at 37°C on an Luria-Bertani (LB) plate containing 50 μg/ml ampicillin and 25 μg/ml tetracycline, followed by selection of colonies by blue-white screening. Selected clones were grown in LB medium, and then used to extract and purify plasmid DNA by Wizard Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer's protocol. Purified plasmid DNAs were sequenced using BigDye terminator v3.1 Cycle Sequencing Kit (Thermo fisher Scientific) and 3130xl Genetic Analyzer (Thermo fisher Scientific) according to the manufacturer's protocol.

Homology and domain analyses of Chgs

Homology and domain analyses of Chgs were performed using Genetyx ver. 11 (GENETYX, Tokyo, Japan) and CD-Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), respectively. The cleavage site of peptide sequence was predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The repetitive structure of Chgs were estimated using RADAR (https://www.ebi.ac.uk/Tools/pfa/radar/).

Quantitative real-time reverse transcription PCR (qPCR)

Total RNAs were extracted from the liver samples, and measured their concentrations as described above. 200 ng total RNA was reverse-transcribed by

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

Primer name	Primer sequence	Primer direction	Use
chgHα-F	CTTGTTCTGAATCCATGGCC	Forward	Cloning of *chgHα*
chgHα-R	AGATGTCTCATCTTTTATTGTC	Reverse	Cloning of *chgHα*
chgHβ-F	GTAGCGAAGCCATTGCGA	Forward	Cloning of *chgHβ*
chgHβ-R	CGAGGACAGTCAGATTTATTTG	Reverse	Cloning of *chgHβ*
chgL-F	ATCAATTCTTATTGTGAAGCCATG	Forward	Cloning of *chgL*
chgL-R	AACCATCAACTTGTTTATTTCAATG	Reverse	Cloning of *chgL*
erα1 -F	AAAACTCTCACCACAGCAGCCG	Forward	Cloning of *erα1*
erα1 -R	TCACGGAATGGGCATCTGGTC	Reverse	Cloning of *erα1*
chgHα-F qPCR	GACAGCCACTATGACCTGCTC	Forward	qPCR for *chgHα*
chgHα-R qPCR	TCCACGTGGAGAACTGCATC	Reverse	qPCR for *chgHα*
chgHβ-F qPCR	GCTACAGAAAGAGGAGAGACATTCC	Forward	qPCR for *chgHβ*
chgHβ-R qPCR	TTCTAGTTGGTGAGCTCCCTTGG	Reverse	qPCR for *chgHβ*
chgL-F qPCR	AGCCAGCTAAGGATGACTACCAAC	Forward	qPCR for *chgL*
chgL-R qPCR	TCGCGTCATTTGTCCTGATGAG	Reverse	qPCR for *chgL*
erα1 -F qPCR	ACTGGTGTTTGTGTCCTCCAG	Forward	qPCR for *erα1*
erα1 -R qPCR	ACTGTACGACTGCTGCCTATCG	Reverse	qPCR for *erα1*

SuperScript® VILO cDNA Synthesis kit (Thermo Fisher Scientific), according to the manufacturer's instruction. Aliquots of total RNA of all samples were pooled, reverse-transcribed and used as an inter-assay control (IAC) to normalize values among plates. In addition, reactions without reverse trancriptase enzyme were used as no reverse transcriptase control (NRT).

Gene specific primers (Table 1) for qPCR amplification of *chg* subtypes (*chg*: *chgHα*, *chgHβ* and *chgL*) and *erα1* were designed using Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) to cover intron/exon boundaries which were predicted from rainbow trout genome database (Accession No. GCA_900005705.1). The primers for elongation factor 1-α (*ef1-α*) were designed as previous described (Luo et al., 2013).

All qPCR reactions were carried out in a volume of 10 µl. Primers were added to the reaction at final concentration of 150 nM except for *chgHα* (50 nM). Each reaction mix contained 5 µl of SYBR® Green PCR Master Mix (Thermo Fisher Scientific), 0.5 µl of template (either reverse-transcription: RT, NRT product, or standard plasmid cDNA) and 0.5 µl of each forward and reverse primer. Standard curves were generated using a serial dilution of plasmid DNA containing the target gene (10^2 to 10^7 copies per reaction). The PCR amplifications and fluorescence detection were performed using the Step One Plus (Thermo Fisher Scientific) as following cycling parameters: one cycle of 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Standard curves were generated using a serial dilution of plasmid DNA containing the target gene (10^2 to 10^7 copies per reaction). The expression levels of the target gene were normalized to the expression levels of the *ef1-α*. Results were reported as copy number

of target gene / copy number of *ef1-α*. No PCR amplification was observed from no-reverse-transcription control templates. Primer specificity was confirmed by dissociation curve analysis of PCR products.

Statistics

Data were analyzed by two-way ANOVA followed by Tukey-Kramer honestly significant difference (HSD) test, using a JMP 7 Software program (SAS Institute Inc., Cary, NC, USA). The difference between groups was considered significant at $P < 0.05$. All the results are expressed as means ± SE. Correlation analyses between serum E2 levels (Mushirobira, 2015) and hepatic mRNA levels were performed using the Excel software package (Office 2010, Microsoft, Redmond, WA, USA).

Results

Primary structure analyses of *chg*s

The cDNAs of *chgHα*, *chgHβ* and *chgL* were isolated and sequenced (Accession Nos. *chgHα*, LC331063; *chgHβ*, LC331064; *chgL*, LC331065). The sequences of *chgHα, chgHβ and chgL* contained open reading frames encoded 532, 517 and 441 amino acids with predicted mass of 59.4 kDa, 57.1 kDa and 49.9 kDa, respectively. The encoded cutthroat trout ChgHα, ChgHβ and ChgL had ZP domains and consensus furin cleavage sites (CFCS) (Fig, 2). The trefoil domains were found in the ChgHα and ChgHβ but not in the ChgL. The similarities of ChgHα, ChgHβ, and ChgL between cutthroat trout and rainbow trout were 93.3%, 97.7% and 99.3%, respectively. The peptides of ChgHα and ChgHβ of cutthroat trout lacked the 32 and 7 amino acids between the signal peptides and the ZP domain compared to those of rainbow trout, respectively (Fig. 3). In the lacked regions, there were repetitive structures of proline (P) and glutamine (Q). The basic units of the repetitive structure were PLPQR(W)PAQ for ChgHα and PPQRPAQ for ChgHβ. In cutthroat trout, ChgHα and ChgHβ lacked four and one repetitive units compared with those in rainbow trout, respectively.

Annual changes in hepatic *chg* and *erα1* mRNA levels

Hepatic *chgHα* and *chgL* mRNA levels showed similar expression patterns. Both mRNAs kept low levels from January to June and started to show an increasing tendency in August (Fig. 4). These mRNA levels reached plateau in October, showing significant increases compared to those from January to June, and then remained high

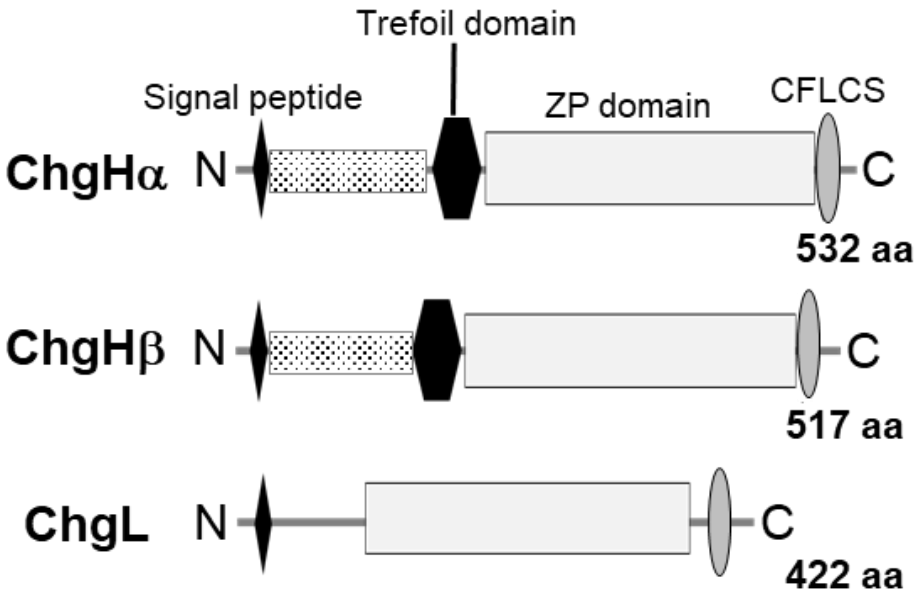

Fig. 2 Schematic representation of cutthroat trout choriogenin proteins (Chg).
Depicted are several elements that comprise the polypeptide chains of cutthroat trout Chgs (ChgHα, ChgHβ, ChgL). Elements aligned from N-terminus (N) to C-terminus (C) include an Signal peptide sequence (diamond shape), repetitive region (dotted quadrangle), ZP domain (white quadrangle) and consensus furin-like cleavage-site (CFLCS; discoid shape). ChgHα and ChgHβ also contain a trefoil domain (hexagonal shape).

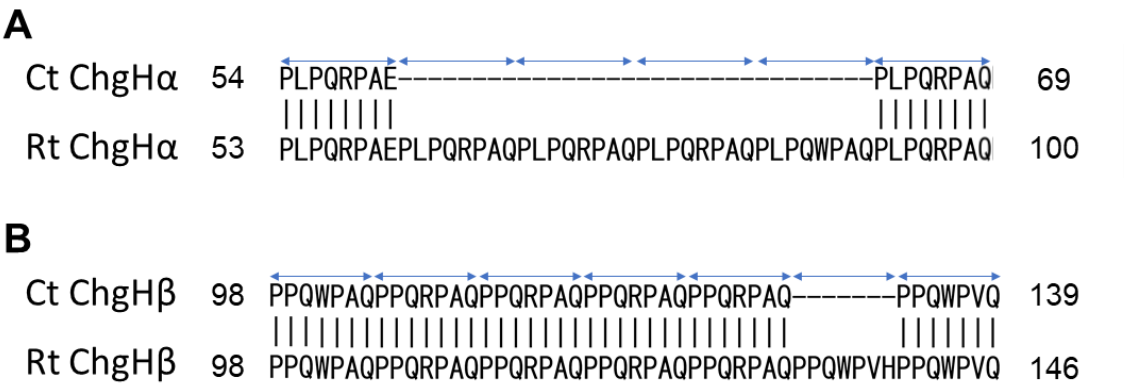

Fig. 3 Alignment of partial repetitive regions in cutthroat trout and rainbow trout choriogenin H proteins (ChgH).
Depicted are sequence that showed difference between cutthroat trout and rainbow trout. Repetitive unit are indicated by arrows. A: cutthoroat trout ChgHα (Ct ChgHα) and rainbow trout ChgHα (Rt ChgHα), B: cutthoroat trout ChgHβ (Ct ChgHβ) and rainbow trout ChgHβ (Rt ChgHβ). The numbers on the right and left refer to the position of the amino acids. Amino acids lacked in the Ct ChgHs are shown by dashes (-). Identical amino acids are indicated by vertical lines between sequences.

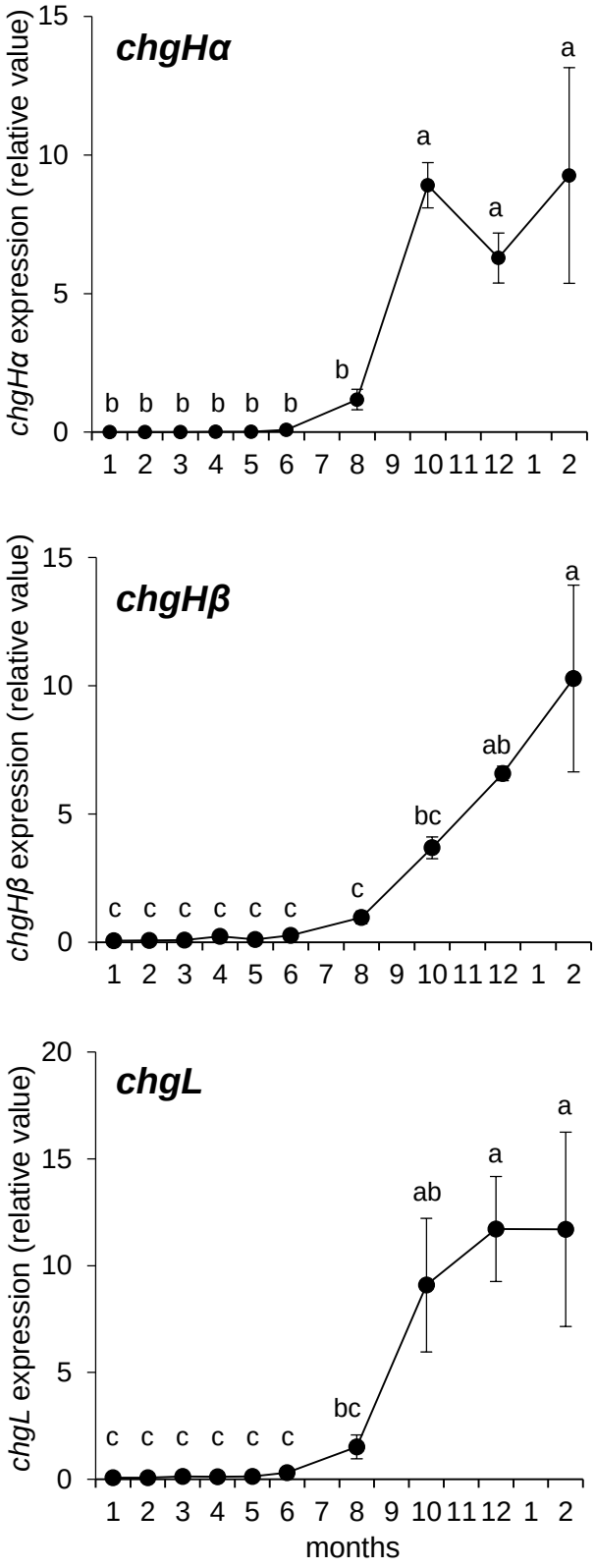

Fig. 4 Reproductive changes in expression of choriogenin transcripts (top panel: *chgHα*; middle panel: *chgHβ*; bottom panel: *chgL*) in the cutthroat trout. Different letters denote that values are significantly different ($P < 0.05$).

values until February. Hepatic *chgHβ* mRNA expression kept low levels from January to June, and then gradually increased until February, exhibiting significant increase between August and December, as well as between October and February (Fig. 4).

Hepatic *erα1* mRNA expression kept low levels from January to June, then significantly increased between June and August, and gradually decreased until February with significant decrease between August and December (Fig. 5).

Relationship between serum E2 levels and *chg* or *erα1* mRNA levels

Correlation between serum E2 levels and *chg* or *erα1* mRNA levels were analyzed using data from January 2009 to December 2010. All *chg* mRNA levels showed highly positive correlations with serum E2 levels (Fig. 6; *chgHα*: R^2= 0.78, $P < 0.001$; *chgHβ*: $R^2 = 0.92$, $P < 0.001$; *chgL*: $R^2 = 0.75$, $P < 0.001$). Heparic *erα1* mRNA levels did not show any significant correlation with serum E2 levels (Fig. 7).

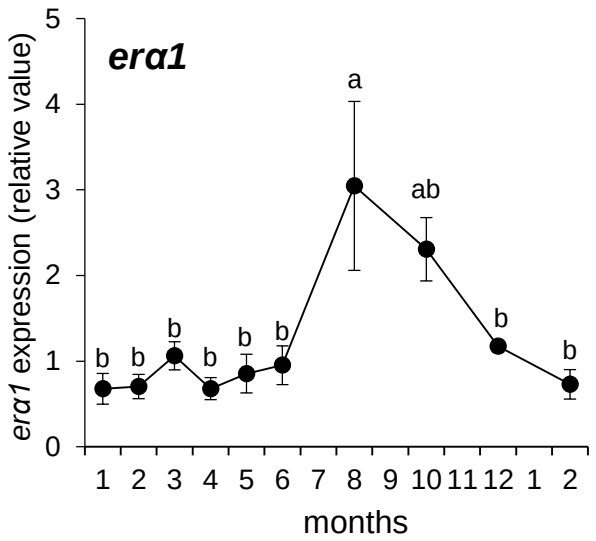

Fig. 5 Reproductive changes in expression of estrogen receptor *α1* (*erα1*) transcripts in the cutthroat trout.
Different letters denote that values are significantly different ($P < 0.05$).

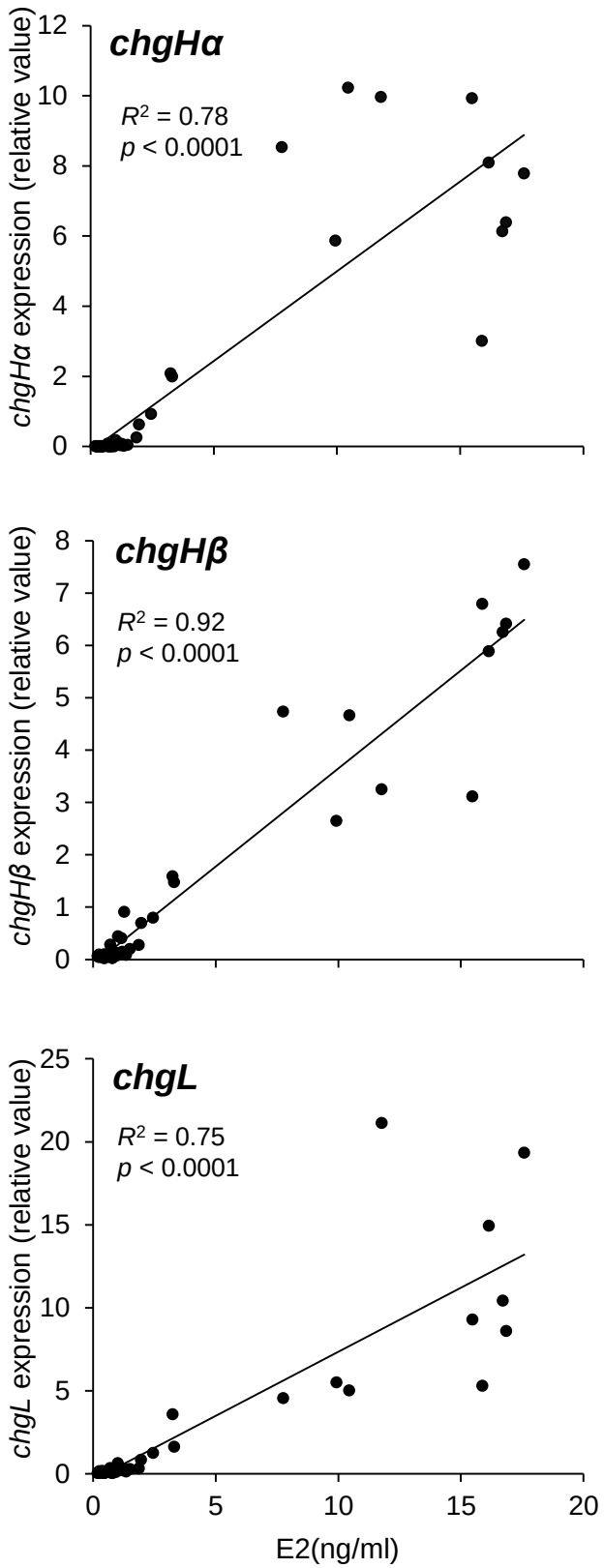

Fig. 6 Correlation of estradiol-17β (E2) with hepatic choriogenin mRNA levels (top panel: *chgHα*; middle panel: *chgHβ*; bottom panel: *chgL*) in the cutthroat trout. Post-ovulated samples (February 2010) were excluded from the analysis.

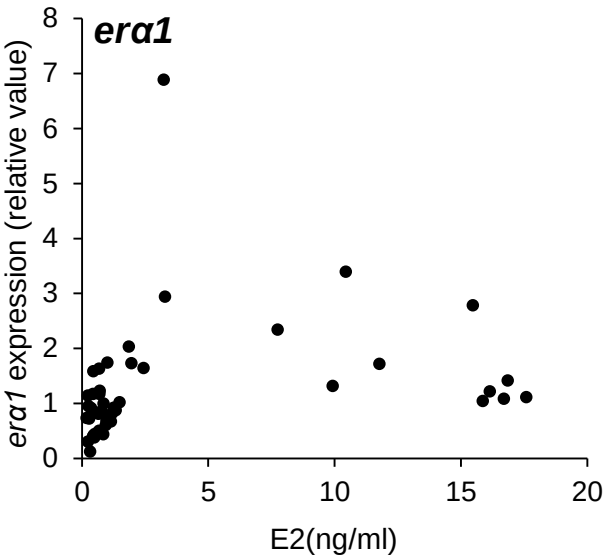

Fig. 7 Correlation of estradiol-17β (E2) with hepatic estrogen receptor α1 (*erα1*) mRNA levels in the cutthroat trout.
Post-ovulated samples (February 2010) were excluded from the analysis.

Discussion

The polypeptide sequences of cutthroat trout Chgs (ChgHα, ChgHβ and ChgL) deduced from cloned cDNAs have ZP domains and CFLS, which are typical characteristics of ZP protein family. ZP proteins are thought to polymerize into filaments using ZP domains when the protein are cleaved at CFCS (Jovine et al., 2005). Thus, cutthroat trout Chgs were assumed to exhibit the basic function as ZP proteins. In ChgHα and ChgHβ, but not in ChgL, trefoil domains are also found. Difference of Chg function depending on the presence or absence of trefoil domain remains unclear. However, it has been shown that trefoil domain prevents the degradation of the ZP proteins (Bork, 1993; Gajhede et al., 1993). Therefore, the presence or absence of trefoil domains in ChgHs and ChgL may affect their stabilities.

The amino acid sequences of cutthroat trout Chgs showed high similarities with those of rainbow trout Chgs (ChgHα: 93.3%, ChgHβ: 97.7%, ChgL: 99.3%), but cutthroat trout ChgHα and ChgHβ lacked some sequences in repetitive structures relative to those of rainbow trout ChgHs. The presence of repetitive structures in ChgHs are found in many teleosts (Kanamori et al., 2003), but the functions of repetitive structures on ChgHs are not known. The repetitive structures of ChgHs are different even in closely related species, such as cutthroat trout and rainbow trout. Therefore, the repetitive structures with abundant PQ are likely to vary among species.

In October when serum E2 levels increased sharply (Mushirobira, 2015), *chgHα* and *chgL* mRNA levels showed significant increase and reached plateau. On the other hand, *chgHβ* mRNA levels significantly increased in December when serum E2 levels

showed highest value (Mushirobira, 2015)n and kept increasing thereafter. These results revealed that reproductive change in *chgHβ* mRNA was unique among *chgs* in cutthroat trout. In the *in vivo* E2 exposure experiment of rainbow trout, *chgHα* and *chgHβ* mRNA levels were up-regulated by lower concentration of E2 than that were effective for *chgL* mRNA levels (Thomas-Jones et al., 2003). These results suggested that distinct *chg* subtypes exhibit different sensitivity to E2 in each trout. Since the effect of Chg subtypes on the properties of the chorion have not been clarified yet, it is difficult to explain the physiological significance of differential E2 sensitivity in Chg subtypes. In gilthead seabream (*Sparus aurata*), it has been shown that differential localization of the Chg/Zp subtypes in chorion were evident, although the chorion precursor proteins are not distinguished from ovary- and liver-derived types (Modig et al., 2008). The difference in expression patterns of hepatic *chgs* mRNAs possibly reflect such differential localization in the chorion of trouts. Further studies are needed to investigate the effect of Chg subtypes on the properties of the chorion, and the localizations of Chg subtypes, to analyze the relationship between those differences and their E2 responsiveness.

In teleosts, chorion formation (choriogenesis) generally precedes yolk formation (vitellogenesis) in ovarian follicle (Selman and Wallace, 1989). However, mRNA levels of all three *chg* subtypes in the liver of female cutthroat trout started to increase in August: like in two *vtg* subtypes (Mushirobira et al., 2013). Thus, none of increase in *chg* mRNA levels preceded the increase in *vtg* mRNA levels. The tendency of synchronous increase in Chg and Vtg protein levels were reported in Sakhalin taimen (*Hucho perryi*) (Shimizu et al., 2000) and masu salmon (Fujita et al., 2005). These

results suggest that increases in *chg*/Chg levels do not precede those in *vtg*/Vtg levels, although choriogenesis precedes vitellogenesis. This discrepancy may be due to the involvement of ovary-derived Zp in the chorion formation. In medaka, *zp* genes are expressed in the ovary, as well as *chg* in the liver (Kanamori, 2000). In masu salmon, ovarian *zp* mRNA and hepatic *chg* mRNA are expressed in the ovary and the liver, respectively (Fujita et al., 2003). Mechanisms for the regulation of *zp* genes in salmonid ovary are completely unknown. The study using zebrafish revealed that *zp3* (*zpC*) gene does not show the estrogen response, and its promoter does not contain ERE (Liu, 2006). In addition, *zp3* mRNA expresses at stage I and II oocytes (primary growth stage), but the expression levels gradually decline and become barely detectable in early stage III oocytes (vitellogenesis stage). Therefore, the expression of ovary-derived Zp proteins, which are not responsive to E2, may contribute to the earlier formation of chorion than yolk deposition. Further research directed toward the analysis of ovarian *zp* genes and Zp proteins are required to clarify the roles of ovarian *zp*/ZP in salmonid choriogenesis.

In cutthroat trout, while the levels of serum E2 and hepatic *vtg* mRNAs showed low values in the ovulated fish of February 2013 (Mushirobira et al., 2013; Mushirobira, 2015), the *chgs* mRNA levels kept high values. The serum levels of ChgH and ChgL protein decrease in the ovulated masu salmon, as well as serum E2 and Vtg levels do (Fujita et al., 2005). Also in Sakhalin taimen, serum levels of two Chg proteins decreased in ovulated fish (Shimizu et al., 2000). Thus, the profiles of hepatic *chg* mRNAs are possibly inconsistent with serum Chg proteins in salmonids, unlike *vtg*/Vtg. However, the profiles of Chgs protein in cutthroat trout and *chgs* mRNA in masu salmon and Sakhalin taimen remain to be investigated. Further studies are needed to

confirm the relationship between hepatic *chg* mRNAs and serum Chg proteins in these species.

Unlike *chg* and *vtg* mRNAs, *erα1* mRNA levels significantly increased only in August, at the beginning of vitellogenesis, followed by the gradual decrease with progress of vitellogenesis. Of the four *er* subtypes in the liver of female rainbow trout, *erα1* mRNA levels increase during vitellogenesis (Nagler et al., 2012). Also in large mouse bass (*Micropterus salmoides*), only *erα* mRNA shows high levels among *er* subtypes (*erα*, *erβ* : *erβ1*, *erγ*: *erβ2*) during vitellogenesis (Sabo-Attwood et al., 2004). The differential expression pattern of *erα1* between cutthroat trout and the other fish may be due to involvement of the other Er subtypes in the regulation of *chg* and *vtg* expression. In goldfish, it has been shown that hepatic *vtg* expression is not only regulated by Erα, but also Erβ1 and Erβ2 (Nelson and Habibi, 2010) . Rainbow trout has an additional *erα* subtypes, *erα2*, whose role remains unclear. Thus, in cutthroat trout, the Er subtypes other than Erα1 may be also involved in the expressions of *chg* and *vtg* mRNAs, leading to differential expression patterns of *erα1* from the other fish. Further study is needed to analyze the functions of all Er subtypes to clarify their roles in oocyte development.

Levels of *chg* mRNAs for cutthroat trout were highly correlated with those of serum E2 levels (*chgHα* : R^2 = 0.78 ; *chgHβ* : R^2 = 0.92 ; *chgL* : R^2 = 0.75) from January (perinuclear stage) to December (vitellogenic stage) like *vtg* mRNAs as previously described (*vtgAs*: R^2 = 0.91, *vtgC*: R^2 = 0.83; Mushirobira, 2015). These results suggest that *chg* and *vtg* mRNAs are expressed mainly under E2 regulation during the period from perinuclear stage to vitellogenic stage. However, in ovulated fish, although both

vtg mRNA and serum E2 levels decrease (Mushirobira et al., 2013), *chg* mRNAs kept the high levels. Thus, *chg* and *vtg* expressions appear to be differentially regulated in the ovulated fish. Unlike *chg* and *vtg* mRNAs, *erα1* mRNA did not show any correlation with serum E2 levels, suggesting that *erα1* is under different mechanism from those of *chg* and *vtg*. The mechanism that causes above mentioned difference between expressions of *erα1* and *chg*s/*vtg*s remains unclear. It is expected that the structure of promoter region responsible for gene expression are different among *chg*s, *vtg*s and *erα1*, resulting in the different expression patterns of these genes.

In summary, this study revealed the hepatic mRNAs expressions of three *chg* subtypes and *erα1* during reproductive cycle of female cutthroat trout. The previous report of serum E2 levels and hepatic *vtg* mRNA levels (Mushirobira et al., 2013; Mushirobira, 2015) in addition to the results of the present study enable us to compare the profile of the serum E2 and hepatic *chg, vtg* and *erα1* mRNAs in female cutthroat trout during reproductive cycle. Simply, the both *chg* and *vtg* mRNAs increased along with the progress of vitellogenesis and with the serum E2 levels. In the ovulated fish however, *chg* mRNAs kept high levels, unlike serum E2 and *vtg* mRNAs. Meanwhile, hepatic *erα1* mRNA levels exhibited a peak in August (at the beginning of vitellogenesis) before the levels of serum E2 and *chg* and *vtg* mRNAs started to increase significantly. These results suggest that expression levels of *chg*, *vtg* and *erα1* genes are potentially regulated through E2 stimulation by different mechanisms.

Chapter III. Transcriptional responses of choriogenins, vitellogenins and estrogen receptor α1 to estradiol-17β administration in the liver of cutthroat trout

Introduction

The results of Chapter II showed that hepatic mRNA levels of three *chg*s (*chgHα*, *chgHβ* and *chgL*) and two *vtg*s (*vtgAs* and *vtgC*) increased along with serum E2 levels during the reproductive cycle of cutthroat trout. The levels of *chg* and *vtg* mRNAs were significantly and positively correlated with the serum E2 levels during vitellogenesis. On the other hand, hepatic *erα1* mRNA levels exhibited a peak in August (at the beginning of vitellogenesis) before increase in levels of serum E2, as well as hepatic *chg* and *vtg* mRNAs. Expression patterns of these mRNAs appeared to be different, suggesting that their expression differ in regulation by E2. However, the mechanisms underlying such difference are unknown.

Effect of E2 on the expression of hepatic *chg*, *vtg* and *erα* (*erα1* in salmonids) genes have been analyzed by E2 treatment *in vivo* in various teleost species including salmonids (Hiramatsu et al., 1997; Westerlund et al., 2001; Kang et al., 2002; Fujita et al., 2004; Sabo-Attwood et al., 2004; Boyce-Derricott et al., 2009; Amano et al., 2010). These studies have revealed the up-regulation of *chg*s, *vtg*s and *erα*/*erα1* genes by E2. However, these studies have been conducted in different species and/or different treatment condition. In addition, most studies did not consider the multiplicity of *chg*, *vtg* and *er* genes. Therefore, it is difficult to obtain the integrated view on the E2 responses of these genes from previous studies. Thus, it is important to analyze the E2 responses of these genes in a single species under the same E2 treatment conditions

with consideration of all subtypes of E2-responsive genes.

Recently, Mushirobira et al. (2018) have examined *in vivo* effects of single E2 injection on hepatic *vtgAs* and *vtgC* mRNA expressions in precocious male and immature cutthroat trout. As the results, both *vtg* mRNAs synchronously increased at the same timing. In this chapter, the responses of *chg* and *erα1* mRNAs to E2 were analyzed to compare their responses each other, as well as with those of *vtg*s, in cutthroat trout *in vivo*, using the same samples used for the previous *vtg* mRNAs analysis.

Materials and methods

Experimental fish and tissue samples

Fish and tissue samples were obtained as described in the previous study for quantification of *vtg* mRNAs (Mushirobira et al. 2018). One-year-old cutthroat trout consisting of a mixture of immature individuals and precociously mature males (body weight 93.9 ± 40.1 g; fork length 208 ± 28 mm; mean ± standard deviation) were obtained from Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University. They were reared in outdoor aquaria at the Faculty of Fisheries Sciences, Hokkaido University, receiving a continuous flow of well water under natural photothermal conditions. The fish were administered a single intraperitoneal injection of E2 dissolved in propylene glycol at one of three doses (low: 50 μ g/kg body weight; middle: 500 μ g/kg body weight; and high: 5000 μ g/kg body weight) or a solvent (propylene glycol) only for control. Blood and liver samples were collected from each group (N = 5 fish) at 2 and 5 days post-injections. Blood samples were incubated overnight at 4°C, and then, sera were separated by centrifugation and stored at -30°C for quantification of E2 (Mushirobira et al., 2018). Liver samples were immersed in RNAlater solution (Thermo fisher Scientific, Waltham, MA, USA) overnight at 4°C, and then stored at -30°C until RNA extraction. Blood and tissue samples were also collected as described above from fish prior to hormone treatment (initial control group).

Quantitative real-time reverse transcription PCR (qPCR)

Extraction of total RNAs from liver tissues, reverse transcription of RNA samples and qPCR assays for *chgHα*, *chgHβ*, *chgL* and *erα1* were performed as described in Chapter II with following modification. Aliquots of total RNA of samples in all E2-treated groups were pooled, reverse-transcribed and used as an inter-assay control (IAC) to normalize the values among plates. FastStart Universal Master Mix (Roche, Basel, Switzerland) was used as the master mix for PCR amplification. Each primer was added to the reaction at final concentrations of 150 nM.

Statistics

Data at the same days following treatments were analyzed by two-way ANOVA followed by Tukey-Kramer HSD test. Data at the different days following treatments were analyzed by Student's t-test. These statistical analyses were performed using a JMP 7 Software program (SAS Institute, Cary, NC, USA). The difference between groups was considered significant at $P < 0.05$. All the results are expressed as means ± SE. For statistical analyses, samples that showed levels below the detection limit (100 copy/reaction mix) in qPCR were treated as indicating the detection limit.

Results

Effect of E2 on hepatic *chg* mRNA expression

At both 2 days and 5 days post-injections (dpi), mRNA levels of *chg* subtypes in the E2 treatment groups tended to increase in dose-dependent manners (Fig. 8). At 2 and 5 dpi, the significant increase in *chgHα* mRNAs occurred at high-dose (5000 μg/kg) groups compared to control groups. Meanwhile, low E2 dose (50 μg/kg) and middle E2 dose (500 μg/kg) groups were not significantly different from the control groups in *chgHα* levels at 2 and 5 dpi. The *chgHβ* and *chgL* mRNAs in middle and high E2 dose groups at 2 dpi and in high dose groups at 5 dpi showed significant increase compared to those of their corresponding control groups. Meanwhile, low E2 dose groups at 2 and 5 dpi, as well as middle E2 dose groups at 5 dpi were not significantly different from their corresponding control groups in the *chgHβ* and *chgL* mRNA expressions.

From 2 dpi to 5 dpi, a slight but significant increase in *chgHα* mRNA levels occurred in control groups. In *chgHα*, the mRNA levels significantly increased from 2 dpi to 5 dpi in middle- and high-dose groups, while no such significant difference was found in the low-dose group. In *chgHβ* and *chgL*, significant increase were evident only in the mRNA levels of high-dose groups between 2 dpi and 5 dpi, while no increase were found in in low- and middle-dose groups.

Effect of E2 on hepatic *erα1* mRNA expression

Levels of *erα1* mRNA in the middle-dose group at 2 dpi were significantly higher than those in the control group (Fig. 9); at 2 dpi, high, but not significant, levels of

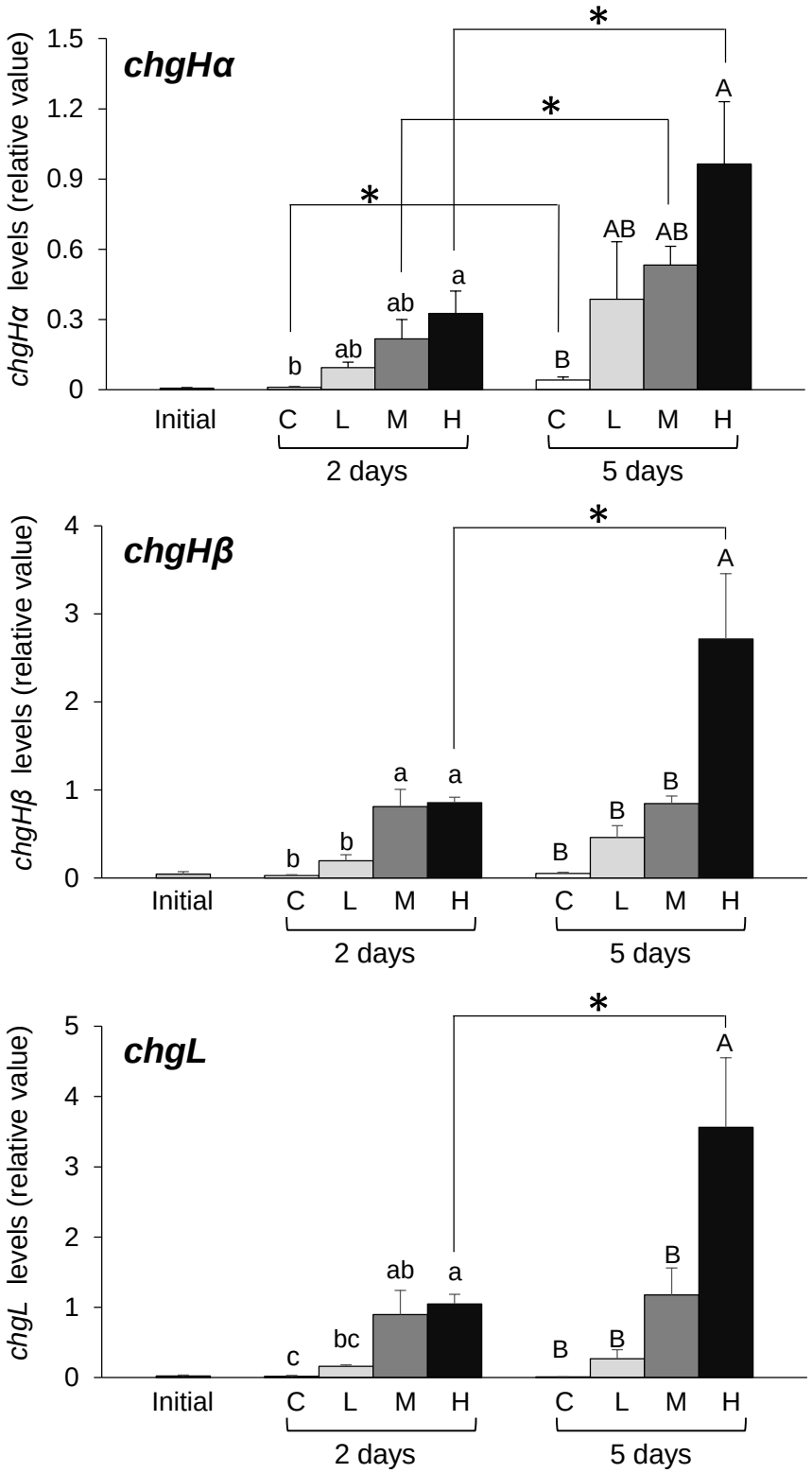

Fig. 8 Hepatic levels of choriogenin transcripts (top panel: *chgHα*; middle panel: *chgHβ*; bottom panel: *chgL*) before (Initial) or after (2 days or 5 days) injection of estradiol-17β (E2) at three distinct doses (L: 50 μg/kg body weight, M: 500 μg/kg body weight, and H: 5000 μg/kg body weight) and a solvent control (C: propylene glycol).

Columns indicate mean values and vertical brackets indicate standard errors. Mean values bearing different letter superscripts or asterisks are significantly different ($P < 0.05$)

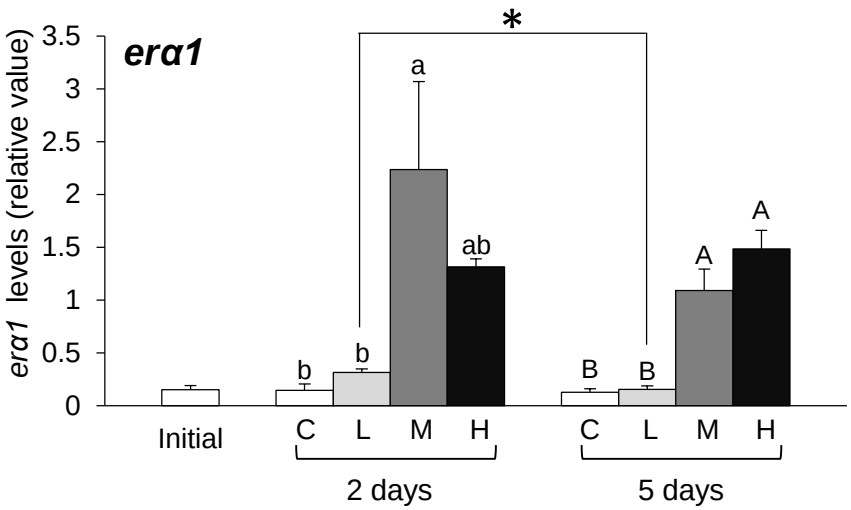

Fig. 9 Hepatic levels of estrogen receptor α1 transcripts (*erα1*) before (Initial) or after (2 days or 5 days) injection of estradiol-17β (E2) at three distinct doses (L: 50 μg/kg body weight, M: 500 μg/kg body weight, and H: 5000 μg/kg body weight) and a solvent control (C: propylene glycol).
Columns indicate mean values and vertical brackets indicate standard errors. Mean values bearing different letter superscripts or asterisks are significantly different ($P < 0.05$)

erα1 mRNA was found in high-dose groups. At 5 dpi, *erα1* mRNA levels in both of middle- and high- dose groups were significantly higher than those in the control group. Meanwhile, *erα1* mRNA levels in low-dose groups did not show significant differences compared to those in the corresponding control groups at 2 and 5 dpi. A significant decrease in *erα1* mRNA levels of low-does groups occurred from 2 dpi to 5 dpi, while no significant change was found between 2 dpi and 5 dpi in middle- and high-dose groups.

Discussion

The expression of three *chg* mRNAs showed positive responses to *in vivo* E2 injection in cutthroat trout, indicating that the *chg* gene expressions are under the control of E2 in this species, like in other salmonids (Shimizu et al., 2000; Hyllner et al., 2001; Westerlund et al., 2001; Fujita et al., 2004). In cutthroat trout, the *chgHβ* and *chgL* mRNAs showed higher sensitivity to E2 than *chgHα* mRNA at 2 dpi following injections while all *chg* mRNAs showed the same sensitivity at 5 dpi. In rainbow trout receiving 4.8 ng/L E2 exposure for 14 days *in vivo*, *chgHα* mRNA is more sensitive to E2 than other *chg*s, while *chgHα* and *chgHβ* are more sensitive than *chgL* in the rainbow trout receiving 9.7 ng/L E2 exposure for 48 hrs (Thomas-Jones et al., 2003). In Arctic char (*Salvelinus alpinus*), mRNA levels of three *chg* subtypes significantly increase at the same timing (6 hrs) following E2 injection (10 mg/kg body weight) (Westerlund et al., 2001). Thus, the responses of *chg*s to E2 appear to depend on characteristics of experimental fish (age, sex, species, etc.), and/or treatment conditions.

During the reproductive cycle of cutthroat trout, *chg*s mRNA levels significantly increased at different timing: *chgHβ* mRNA levels significantly increased in December (vitellogenic stage), while *chgHα* and *chgL* mRNA levels increased in October (vitellogenic stage) (Chapter II). It seems that the sensitivity of three *chg* subtypes to E2 in cutthroat trout following E2 injection does not reflect the timing at which corresponding *chg* subtype mRNA levels significantly increase during the reproductive cycle. This discrepancy may be caused by the differences in the elevation patterns of serum E2 levels between the two analyses. In reproductive cycle, it takes several

months for serum E2 levels to reach the maximal values from the beginning of vitellogenesis (Mushirobira, 2015). Meanwhile, in the experiment of E2 administration *in vivo*, serum E2 levels reach the highest values within a few days following administration. (Mushirobira et al., 2018). These differential kinetics of serum E2 levels may result in the difference in sensitivities of *chg* subtypes between the reproductive cycle and E2 administration *in vivo*.

As described in Chapter II, the *chg*s mRNA levels did not increase at earlier timing than *vtg*s mRNAs during the reproductive cycle of cutthroat trout, although chorion formation starts prior to yolk accumulation in teleosts including salmonids. In the present study, the sensitivity of *chg*s mRNAs to E2 was not high compared to those of *vtg* mRNAs (Mushirobira et al., 2018). These results suggest that hepatic *chg* expressions may not contribute to the early stage of chorion formation that occurs before vitellogenesis. Instead, chorion precursor proteins derived from ovarian *zp* genes are possibly involved as discussed in Chapter II. This hypothesis is supported by a result that *zpB* and *zpC* mRNAs are expressed in the previtellogenic oocytes but not in the lipid droplet oocytes of cutthroat trout (Kawakita, 2013). In zebrafish, expression of ovarian *zpB* (*zp2*) and *zpC* (*zp3*) mRNAs were not E2-dependent (Liu, 2006). However, *erβ2* (*er2a*) knockout (KO) zebrafish showed lower ovarian *zpB* and *zpC* mRNAs levels than the levels in wild types in early vitellogenic follicles (Lu et al., 2017). These results suggest that expression of ovarian *zp* genes are under the regulation of *erβ2*, although these genes do not have E2-responsiveness. Further studies on the regulation of ovarian *zp* gene expressions are required to confirm the involvement of *zp* genes in chorion formation of cutthroat trout.

The *erα1* mRNA levels in middle- and high-does groups reached plateau at 2 dpi, as well as *vtgC* mRNA, while the levels *chg* mRNAs in high-does groups and *vtgAs* mRNA increased from 2 pdi to 5 dpi. This particular expression pattern of *erα1* is in accordance with the previous results obtained from rainbow trout (Pakdel et al., 1991). *er* (considered as *erα1*) mRNA levels showed highest values between 2 and 6 dpi and decreased after 8 dpi, while *vtg* (considered as *vtgAs*) mRNA levels gradually increase and reach highest levels at 15 dpi in male rainbow trout injected with 1.5 mg/kg E2. These results suggest that *erα1* mRNA levels reach maximum values before *chg*s and *vtgAs* mRNA levels reach maximum in response to E2. The maximal levels of *erα1* were seen at earlier timing than those of *chgs* mRNA and *vtgs* mRNA during reproductive cycle of cutthroat trout, as described in Chapter II. The increase of *erα1* mRNA at earlier timing than the increases of *chg* and *vtg* mRNAs in reproductive cycle may be due to the particular response of *erα1* gene to E2 *in vivo.* The increase of *erα1* mRNA expression should result in the increase of Erα1 protein synthesis. Thus, the increased Erα1 protein probably leads to the up-regulation of *chg*s and *vtg*s mRNAs.

As stated before, teleosts have at least three Er subtypes (Erα, Erβ1 and Erβ2), and Erα is thought to be the major regulator of *chg*s and *vtg*s expressions. Recently, Tohyama et al., (2017) established *erα* (*esr1*) KO male and female medaka using a Transcription Activator-Like Effector Nuclease (TALEN) approach. The *erα* KO male medaka shows lower *vtg* levels than wild type medaka when they are exposed to estrogen (17α-ethinylestradiol), indicating *erα* is involved in *vtg* expression. However, *erα* KO female medaka do not show any significant defects in their gonadal development. In female zebrafish with each *er* subtype KO (*esr1*: *erα*, *esr2a*: *erβ2*,

esr2b: *erβ1*) using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system , normal reproductive development and function were confirmed (Lu et al., 2017). These results suggested that Erα function could be compensated by the other Erβ subtypes at least in part, indicating Erβs are likely to affect the function of Erα. The Erβs possibly play some roles in the expressions of *chg*s and *vtg*s genes. Further investigation is required to confirm the role of Erβs in the expression of *chg* and *vtg* genes.

In summary, the differential responses in mRNA expression of *chg*s, *vtg*s and *erα1* to E2 were confirmed in male or immature cutthroat trout following *in vivo* E2 injections. In E2-treatment groups, hepatic *erα1* and *vtgC* mRNA levels reached plateau at 2 dpi, while the other mRNA levels kept increasing from 2 dpi to 5 dpi. These results suggest that their expressions are under different E2 regulation.

Chapter IV. Transcriptional responses of choriogenins, vitellogenins and estrogen receptor α1 to estradiol-17β in primary cultured hepatocytes of cutthroat trout

Introduction

Hepatic *chg*, *vtg* and *erα1* expressions have been analyzed in cutthroat trout in previous Chapters; Chapter II: changes in their mRNA levels during the reproductive cycle of female fish, Chapter III: changes in their mRNA levels following E2 treatment *in vivo* in mature male and immature fish. The results from these Chapters have suggested that the E2 responses of these genes are different.

The physiological response *in vivo* is affected by the complexity of the intact animal. Thus, it is difficult to exclude the effects of several factors, such as individual differences and other endocrine hormones, on the expressions of E2-responsive genes. Further investigations are required to analyze the E2 responses of *chg*, *vtg* and *erα1* genes without the complexity of the intact animal. Primary cultured hepatocytes and fish cell lines are suitable experimental models for such analysis, because the analyses using these cultured cells can be performed without the complexity of the intact animals. However, the fish cell lines derived from rainbow trour hepatoma (RTH-149) or zebrafish normal liver (ZF-L) seem to lose the ability of Vtg production (Christianson-Heiska and Isomaa, 2008). Meanwhile, the primary cultured hepatocytes have the ability to produce Chg, Vtg and Er in response to estrogens (Flouriot et al., 1997; Celius and Walther, 1998). Therefore, in this Chapter, the primary cultured hepatocytes were used for further investigation to analyze the E2 response in mRNA expression of *chg*s, *vtg*s and *er*s.

As described in Chapter I, there are at least three *er* subtypes (*erα*, *erβ1, erβ2*) in

teleosts. The possible involvement of Er subtypes other than Erα1 in *vtg* and *erα* expression has been shown in several studies (Nelson and Habibi, 2010; Griffin et al., 2013). However, the information for the roles of Er subtypes other than Erα1 on the expressions of *chg*s, *vtg*s and *erα1* genes are limited. To understand the role of Er subtypes other than Erα1 in the expression of these genes, it is important to clarify their transcriptional responses to E2. The Erα1, which is thought to be the major regulator for the expression of hepatic genes responsible for fish oogenesis, is up-regulated by E2, thus possibly contributing to active expressions of *chg* and *vtg* genes. If Er subtypes other than Erα1 are up-regulated by E2, these Er subtype proteins possibly contribute the active production of Chgs and Vtgsα. The E2 response of *erα2*, an additional Erα subtype in salmonids, is controversial: some studies (Osachoff et al., 2013; Casanova-Nakayama et al., 2018) report that the *erα2* mRNA levels are up-regulated by E2, while the another study (Boyce-Derricott et al., 2009) does not confirm such E2 response of *erα2* mRNAs. The E2 responses of Erβs appear to depend on species and/or treatment conditions (Menuet, 2004; Sabo-Attwood et al., 2004; Nelson et al., 2007; Boyce-Derricott et al., 2009). In rainbow trout, Erβ subtypes are not up-regulated by E2 (Boyce-Derricott et al., 2009; Osachoff et al., 2013; Casanova-Nakayama et al., 2018). The E2 responses of *er* subtypes other than *erα1* remain unclear in cutthroat trout.

In this Chapter, in order to clarify the effect of E2 on transcriptional responses of all *chg*, *vtg* and *er* subtypes in the liver, E2 responses of these genes in primary cultured hepatocyte of cutthroat trout were analyzed.

Materials and methods

Experimental fish and tissue sample

Cutthroat trout used in this study were obtained from a breeding stock held in flow-through fresh water under the natural ambient condition at Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University. These fish were transferred and reared in outdoor aquaria at the Faculty of Fisheries Sciences, Hokkaido University (Hakodate, Japan), receiving a continuous flow of well water under natural photothermal conditions before use in the following experiments.

For cloning of *er* cDNAs, livers from females with following biological characteristics were collected; for *erα*2, body weight (BW): 920.5 g, total length (TL): 345 mm, gonadosomatic index (GSI: gonad weight/body weight × 100): 5.8; for *erβ1*, BW: 355.3 g, TL: 320 mm, GSI: 1.4; for *erβ2*, BW: 129.3 g, TL: 222 mm, GSI: 0.30. 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 the use for RNA extraction.

For the experiments of primary cultured hepatocytes, male cutthroat trout (BW: 420 ~ 480 g, TL: 31.4 ~ 41.0 mm) were used.

Molecular cloning of *erα2*, *erβ1* and *erβ2*

Molecular cloning of *erα2*, *erβ1* and *erβ2* were performed as described in Chapter II with following modifications. The extracted total RNAs (1250 μg) were reverse-transcribed. Primer sets were designed from rainbow trout *erα2*

Table 2 Name, nucleotide sequence, direction, and use of primers

Name	Sequence	Direction	Use
er α2 -F	GCCATCTCACCCCAGAAACT	Forward	Cloning for *er α2*
er α2 -R	ACTCTCACCTCCACAAATGTCA	Reverse	Cloning for *er α2*
er β1 -F	CACGGATGGATTGCTACTCC	Forward	Cloning for *er β1*
er β1 -R	GAACAGTGCTAATGCCTGAAAGG	Reverse	Cloning for *er β1*
er β2 -F	CACTGATGACAGGCTTGGCAG	Forward	Cloning for *er β2*
er β2 -R	AATGGTCACAGACACTGATAAAG	Reverse	Cloning for *er β2*
er α2 -F qPCR	AGCCTCCCCAGCCAGTCTATC	Forward	qPCR for *er α2*
er α2 -R qPCR	TGAGCCTGACCCTGACTCCAC	Reverse	qPCR for *er α2*
er β1 -F qPCR	TCCATTGTCTCTGCACCATCG	Forward	qPCR for *er β1*
er β1 -R qPCR	TTCCTCAGAGGCTTACTGCTCTC	Reverse	qPCR for *er β1*
er β2 -F qPCR	TCCAAACGAGGCCTGTCATTC	Forward	qPCR for *er β2*
er β2 -R qPCR	TCTTCATGCTAGAGAGGTGCTG	Reverse	qPCR for *er β2*

(NM_001124558), *erβ1* (NM_001124753) and *erβ2* (NM_001124570.1) to amplify each entire open reading frame (Table 2). PCR for each *er* subtype was performed using PrimeSTAR® Max DNA Polymerase (Takara Bio, Shiga, Japan) as follows: 1 µl 1st stranded cDNA, 5 µl PrimeSTAR® Max premix, 1 µl each forward and reverse primers (10^{-6} M) and nuclease-free water up to final volume of 10 µl. The PCR amplification were carried out with following thermal parameters: 40 (*erα2* and *erβ1*) or 35 (*erβ2*) cycles at 98°C for 10 s, 55 °C for 5 s and 72 °C for 3 min.

Primary culture of hepatocytes

Hepatocytes were isolated from male cutthroat trout using a two-step collagenase perfusion technique as described previously (Klaunig et al., 1985) with following modifications. Briefly, trout was anesthetized in 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan). The liver was perfused with 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 blood from the liver. The liver was then perfused with the same solution without EGTA and glucose but with $CaCl_2$ (5 mM), 0.05% collagenase (Wako, Tokyo, Japan), and 0.005% trypsin inhibitor (Sigma-Aldrich, St. Louis, MO). After the perfusion, the liver was dispersed in ice-cold L-15 medium (Thermo Fisher Scientific) supplemented with 10 mM HEPES (pH 7.4), 1% Antibiotic Antimycotic Solution (final concentration: 100 units/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B; Wako) and 5% fetal bovine serum (FBS, Thermo Fisher Scientific). The cell suspension was filtered through cell strainer (Ikemoto Rika, Tokyo, Japan), and the filtrate was centrifugated at 100 g for 90 s at 4°C.

The pellet was resuspended in fresh medium and centrifugated at 100 g for 2 min at 4°C. The cell pellet was washed three times. Cell viability was about 95% when 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, Corning, 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). The cultured cells were settled for 24 hrs in order to adhere to the culture plate.

Hormone Treatment

Experient 1: effects of continuous treatment of E2

Cells in each well were washed once by 0.5 ml of serum- and insulin-free L-15. Then, cells were treated with 10^{-6} M E2. Control cultures were treated with solvent (ethanol) only. The amount of ethanol in the medium did not exceed 0.1%. The half volume (0.25 ml) of the culture medium was changed every 24 hrs. After 24 hrs and 72 hrs following the treatment, the medium was removed and 200 μl Isogen (Nippon Gene, Tokyo, Japan) was added to each well. Following 5 min incubation, the samples were stored at -80°C until use. The samples in each group were obtained from three wells (n = 3).

Experiment 2: effects of various dose of E2

Cells were treated with various concentrations of E2 (10^{-11} ~ 10^{-6} M). Treatments

and samplings were performed as described in Experiment 1 with following exceptions; culture medium was not changed after treatment, and the samples in each group were obtained from four wells (n = 4).

Quantitative real-time reverse transcription PCR (qPCR)

qPCR for all subtypes of *chg*, *vtg* and *er* were performed as described in Chapter II with following modifications. Total RNA (200 ng) was reverse-transcribed by SuperScript® IV VILO cDNA Synthesis kit (Thermo Fisher Scientific). Aliquots of total RNA in all E2-treated groups was pooled, reverse-transcribed and used as an inter-assay control (IAC) to normalize the values among plates. Primer sets for *erα2*, *erβ1* and *erβ2* (Table 2) were designed in Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). Primer pairs were designed to cover intron/exon boundaries which were predicted from rainbow trout genome database (Accession No. GCA_900005705.1).

Primers were added to the reaction at final concentration of 150 nM except for *erβ2* (50 nM). No PCR amplification was observed from no-reverse-transcription control templates. Primer specificty was confirmed by dissociation curve analysis of PCR products.

Statistics

Data were analyzed by two-way ANOVA followed by Tukey-Kramer HSD test using a JMP 7 Software program (SAS Institute, Cary, NC, USA). The difference between groups was considered significant at $P < 0.05$. All the results are expressed as

means ± SE. For statistical analyses, samples that showed levels below the detection limit (100 copy/reaction mix) in qPCR were treated as indicating the detection limit.

Results

Primary structure analyses of *erα2*, *erβ1* and *erβ2*

The cutthroat trout *erα2*, *erβ1*, and *erβ2* (*erα2*: 1782 bp, *erβ1*: 2324 bp, *erβ2*: 2354 bp, respectively) were isolated and sequenced. The sequences of *erα2*, *erβ1* and *erβ2* contained open reading frames encoded 556, 594 and 606 amino acids, respectively. The encoded cutthroat trout Erα2, Erβ1 and Erβ2 exhibited domain features typical of estrogen receptor (A/B, C, D, E, F domain). The Erα2, Erβ1 and Erβ2 sequences shared high similarity (*erα2*: 99.3%, *erβ1*: 99.3% *erβ1*: 98.8%, respectively) with the homologous sequences of the rainbow trout (GenBank accession no. *erα2*: NM_001124558, *erβ1*: NM_001124753, *erβ2*: NM_001124570.1).

Experiment 1: effects of continuous treatment of E2

Levels of *chgHα*, *chgHβ* and *chgL* mRNAs in E2 treatment group were significantly higher than those in the corresponding control groups at 24 hrs and 72 hrs post initiation (hpi) of the treatment (Fig. 10). In E2-treatment group, *chgHβ* and *chgL* mRNA levels significantly increased from 24 hpi to 72 hpi, while *chgHα* mRNA levels did not. Levels of *vtgAs* and *vtgC* mRNAs in E2 treatment group were significantly higher than those in the corresponding control groups at 24 hpi and 72 hpi (Fig. 11). In E2-treatment group, levels of both *vtg* mRNAs significantly increased from 24 hpi to 72 hpi. Levels of *erα1* mRNA levels in E2 treatment group significantly increased relative to those in corresponding control groups at 24 hpi and 72 hpi (Fig. 12). In E2-treatment group, levels of *erα1* mRNA significantly increased from 24 hpi to 72 hpi. The *erα2* mRNA

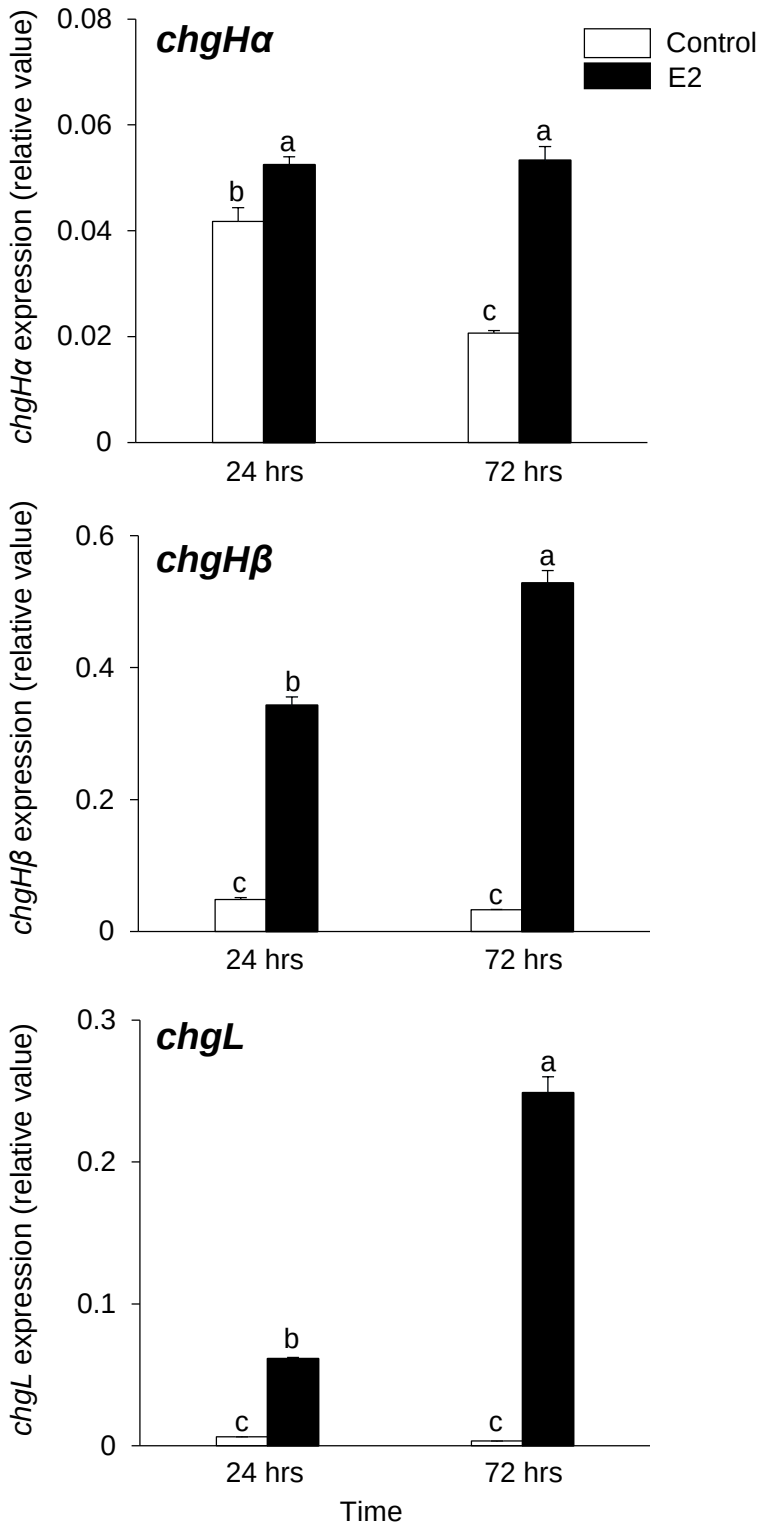

Fig. 10 Effects of continuous treatment of estradiol-17β (E2) or a control solvent (Control) on the expression of choriogenin (*chg*) subtype mRNAs transcripts (top panel: *chgHα*; middle panel: *chgHβ*; bottom panel: *chgL*) in primary cultured hepatocytes of male cutthroat trout.

Hepatocytes were treated with 10^{-6} M of E2. Half of the culture medium was replaced by the fresh medium every 24 hrs. At 24 hrs and 72 hrs after the initiation of E2 treatment, cells were harvested and *chg* mRNAs were 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 ($P < 0.05$).

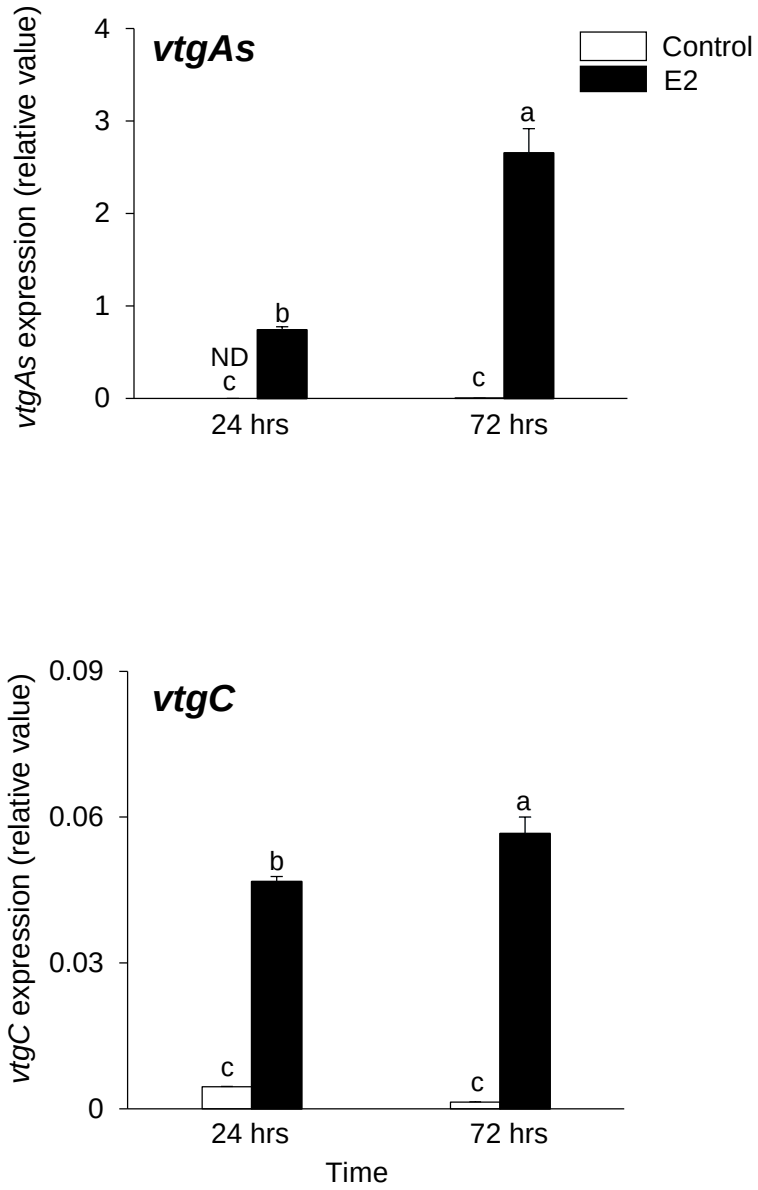

Fig. 11 Effects of continuous treatment of estradiol-17β (E2) or a control solvent (Control) on the expression of vitellogenin (*vtg*) subtype mRNAs transcripts (top panel: *vtgAs*; bottom panel: *vtgC*) in primary cultured hepatocytes of male cutthroat trout.
Hepatocytes were treated with 10^{-6} M of E2. Half of the culture medium was replaced by the fresh medium every 24 hrs. At 24 hrs and 72 hrs after the initiation of E2 treatment, cells were harvested and *vtg* mRNAs were quantified by quantitative real-time PCR. ND values denote all analyzed samples undetectable by quantitative reverse transcription PCR (below the threshold detection limit of the instrument) Columns indicate mean values and vertical lines indicate standard errors. Different letters denote that values are significantly different ($P < 0.05$).

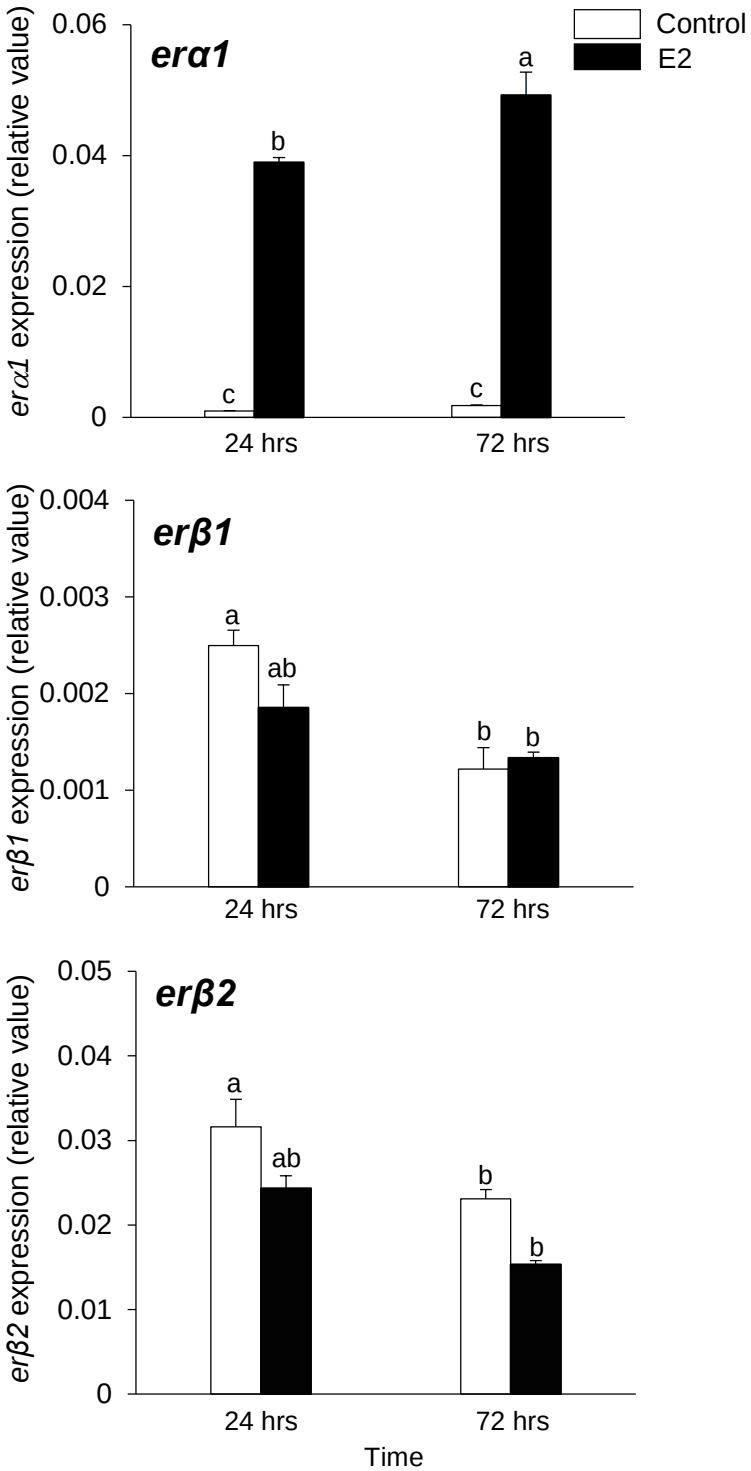

Fig. 12 Effects of continuous treatment of estradiol-17β (E2) or a control solvent (Control) on the expression of estrogen receptor (*er*) subtype mRNAs (top panel: *erα1*; middle panel: *erβ1*; bottom panel: *erβ2*) in primary cultured hepatocytes of male cutthroat trout. Hepatocytes were treated with 10^{-6} M of E2.

Half of the culture medium was replaced by the fresh medium every 24 hrs. At 24 hrs and 72 hrs after the initiation of E2 treatment, cells were harvested and *er* mRNAs were 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 ($P < 0.05$).

levels were undetectable in all groups (data not shown). Treatment of E2 did not affect mRNA levels of *erβ1* nor *erβ2* at 24 dpi and 72 dpi when compared them with the levels in the corresponding control groups (Fig. 12).

Experiment 2: effects of various dose of E2

In cultured hepatocytes at 24 hpi and 72 hpi, levels of *chgHα*, *chgHβ* and *chgL* mRNAs increased by E2 treatments in dose-dependent manners (Fig. 13). The lowest doses of E2 inducing a significant increase in the expression level (LOEC) were 10^{-10} M for *chgHβ* and 10^{-9} M for *chgHα* and *chgL* in both sampling points. Levels of *chgHα*, *chgHβ* and *chgL* mRNAs in the high concentration groups (10^{-7} and 10^{-6} M E2) significantly increased from 24 hpi to 72 hpi after the initiation of E2 treatments. Levels of *vtgAs* and *vtgC* mRNA in E2 treatment groups also showed dose-dependent increase (Fig. 14). The LOEC of E2 were 10^{-9} M for *vtgAs* and 10^{-8} M for *vtgC* in both sampling points. The *vtgAs* and *vtgC* mRNAs levels in the high concentration groups (*vtgAs*: 10^{-7} and 10^{-6} M; *vtgC*: 10^{-6} M) showed significant increase from 24 hpi to 72 hpi. Levels of *erα1* mRNA at the 24 hpi increased by E2 treatments in a dose-dependent manner (Fig. 15). The LOEC of E2 for *erα1* mRNA expression was 10^{-9} M in 24 hpi. Unlike other genes, levels of *erα1* mRNA significantly decreased from 24 hpi to 72 hpi in $10^{-9} \sim 10^{-6}$ M E2-treatment groups; the levels of *erα1* of all E2-treatment groups at 72 hpi did not show any significant difference compared to those of the control group.

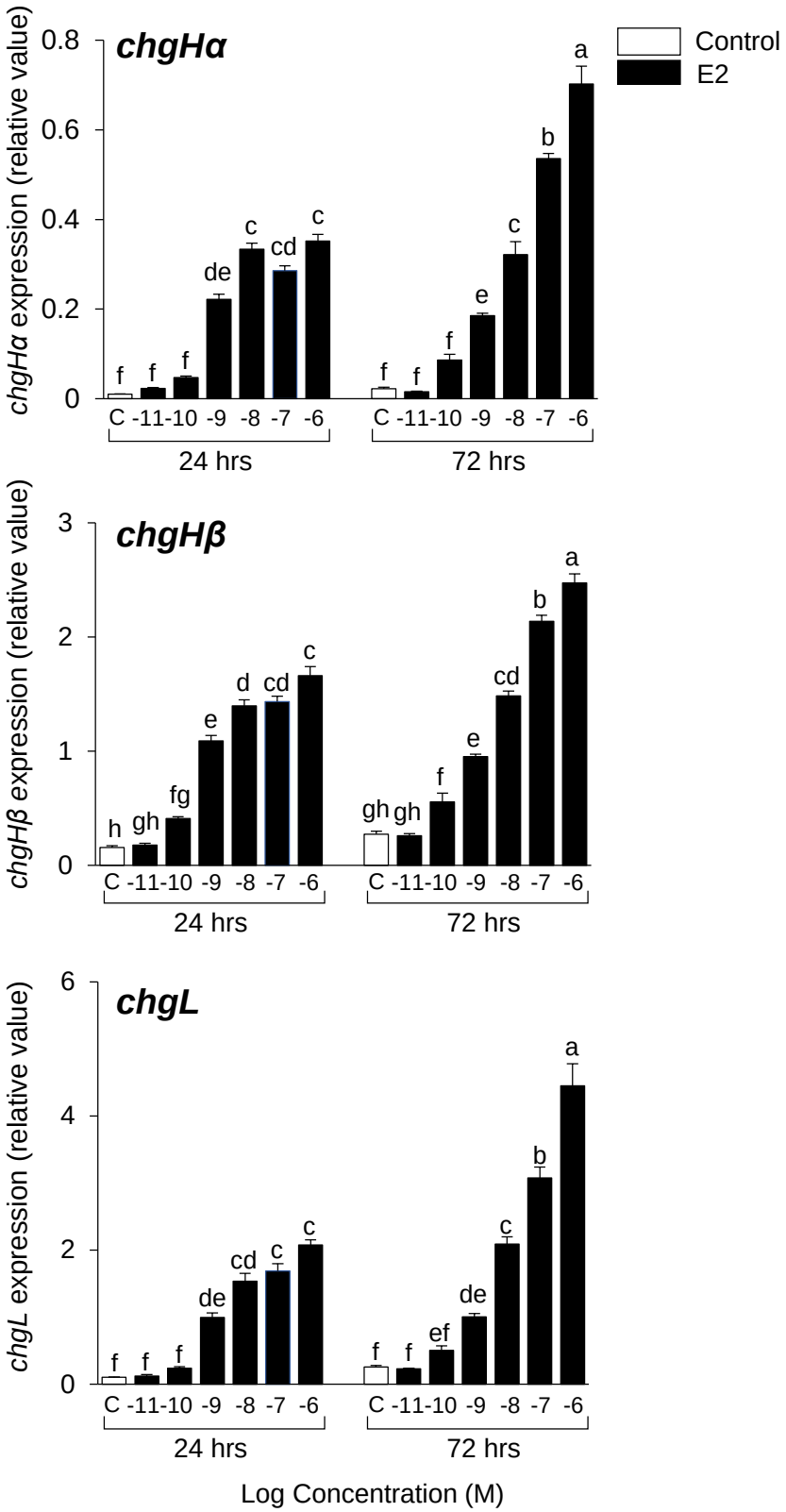

Fig. 13 Responses to different dose of estradiol-17β (E2) or a control solvent (C) of choriogenin (*chg*) subtype mRNA expression (top panel: *chgHα*; middle panel: *chgHβ*; bottom panel: *chgL*) in primary cultured hepatocytes of male cutthroat trout.
Hepatocytes were treated with 10^{-11} ~ 10^{-6} M (-11 ~ -6 in horizontal axis) of E2 for 72 hrs without replacement of the medium. At 24 hrs and 72 hrs after the initiation of E2 treatment, cells were harvested and *chg* mRNAs were 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 ($P < 0.05$).

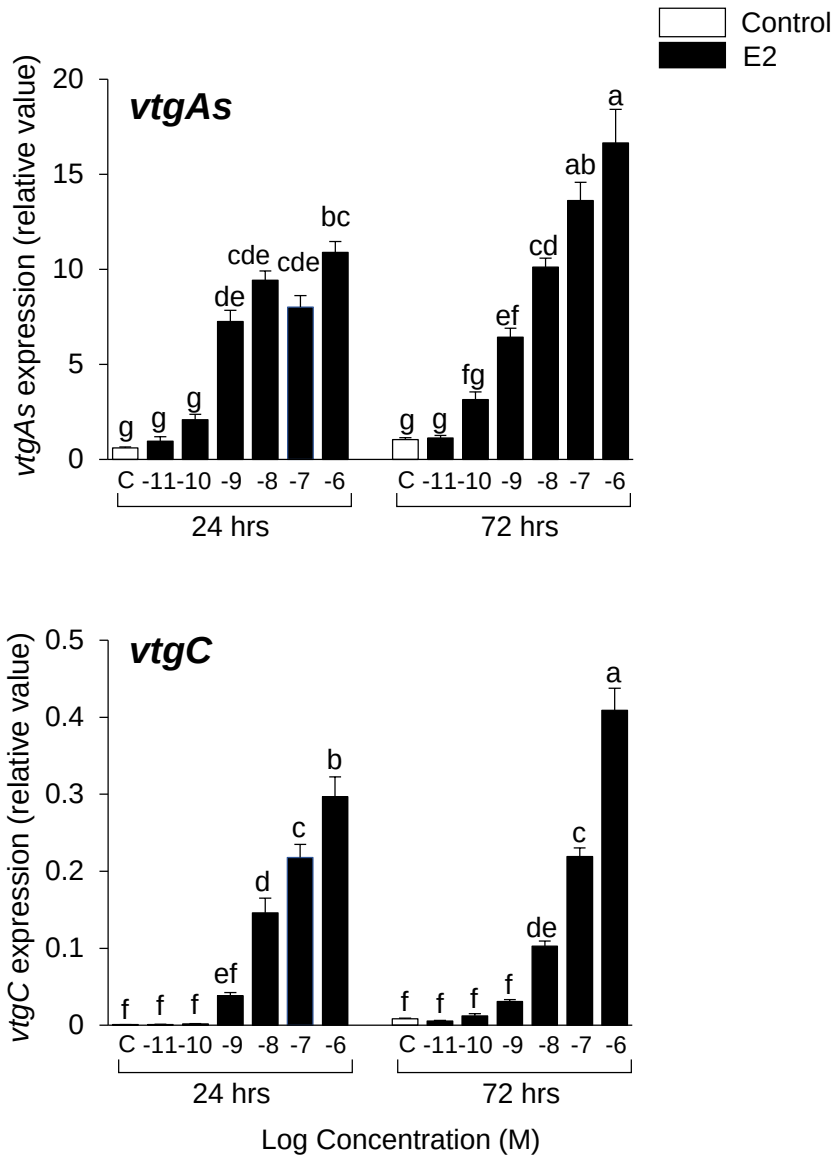

Fig. 14 Responses to different dose of estradiol-17β (E2) or a control solvent (C) of vitellogenin subtype mRNA expressions (top panel: *vtgAs*; bottom panel: *vtgC*) in primary cultured hepatocytes of male cutthroat trout.
Hepatocytes were treated with 10^{-11} ~ 10^{-6} M (-11 ~ -6 in horizontal axis) of E2 for 72 hrs without replacement of the medium. At 24 hrs and 72 hrs after the initiation of E2 treatment, cells were harvested and *vtg* mRNAs were quantified by quantitative real-time reverse transcription reverse transcription PCR. Columns indicate mean values and vertical lines indicate standard errors. Different letters denote that values are significantly different ($P < 0.05$).

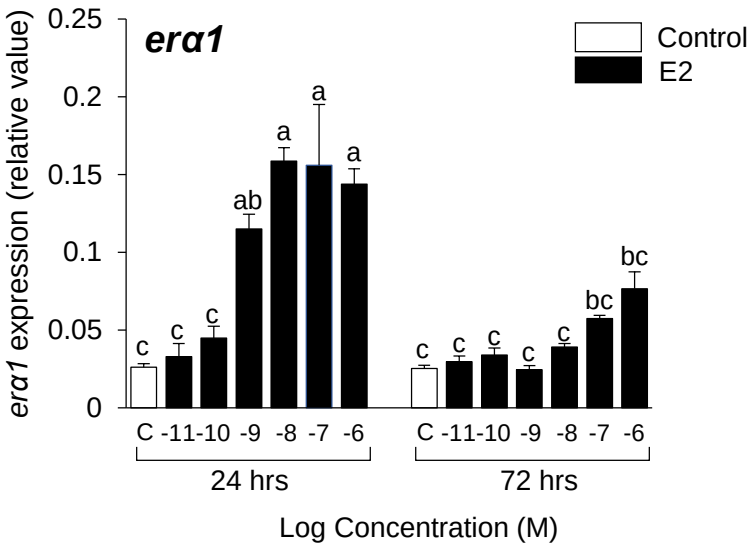

Fig. 15 Responses to different dose of estradiol-17β (E2) or a control solvent (C) of estrogen receptor α1 (*erα1*) mRNA expression in primary cultured hepatocytes of male cutthroat trout.

Hepatocytes were treated with 10^{-11} ~ 10^{-6} M (-11 ~ -6 in horizontal axis) of E2 for 72 hrs without replacement of the medium. At 24 hrs and 72 hrs after the initiation of E2 treatment, cells were harvested and *erα1* mRNAs were 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 ($P < 0.05$).

Discussion

The predicted cutthroat trout Erα2, Erβ1 and Erβ2 polypeptide sequences showed high similarities with homologous sequences of the rainbow trout (Erα2: 99.3%, Erβ1: 99.3%, Erβ2: 98.8%). Primary structures of cutthroat trout Erα2, Erβ1 and Erβ2 exhibited typical functional domains reported for the vertebrate ERs/Ers, and the amino acids required for DNA and ligand bindings were well conserved (data not shown). These structural similarities suggest that cutthroat trout Erα2, Erβ1 and Erβ2 have the basic functions of vertebrate ERs/Ers, such as ligand (estrogens) binding and transactivation of target genes (Menuet et al., 2002; Davis et al., 2010).

To check the responsiveness of *chg*, *vtg* and *er* gene expression to E2, effects of continuous treatments of high E2 dose (10^{-6} M) on each gene expression were examined in the Experiment 1. With replenishment of E2 every 24 hpi of the treatments, levels of *chg*, *vtg* and *erα1* mRNAs increased by E2 treatments at both 24 hpi and 72 hpi. Levels of these mRNA were also up-regulated by E2 treatment *in vivo* in the liver of male and immature cutthroat trout (Chapter III), as well as those in the liver of other salmonids (Amano et al., 2010; Boyce-Derricott et al., 2009; Hiramatsu et al., 1997; Mushirobira et al ., 2018; 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 *erα1* is in common in salmonid species.

In Experiment 1, *erα2* mRNA was undetectable and both *erβ1* and *erβ2* mRNAs did not show estrogen responses, while *erα1* mRNA levels were up-regulated by the E2 treatment. The levels of *erα2* mRNA were lower than those of the other *er* subtypes in

the liver of immature female rainbow trout (Casanova-Nakayama et al., 2018). The lower expression levels of *erα2* compared to those of the other *er*s were also confirmed in male cutthroat trout following E2 treatments *in vivo* (Nagata et al., unpublished). Thus, non-detection *erα2* mRNA in primary cultured hepatocytes was perhaps due to the low expressions of *erα2* mRNA *in vivo*. Using rainbow trout fed with E2-containing pellets for five days, Casanova-Nakayama et al. (2018) have confirmed the E2 response of *erα2* mRNA in the liver . Osachoff et al. (2013) showed upregulation of hepatic *erα2* mRNA levels in rainbow trout exposed to E2 for 7 days, while *erα2* did not show E2 response for 2 days exposure. Boyce-Derricott et al. (2009) did not confirm the E2 response of hepatic *erα2* mRNA from rainbow trout after 24 hrs following E2 injections. These results suggested that it takes relatively long time for E2 to induce *erα2* mRNA expression. Meanwhile, the mRNA levels of *erβ*s in rainbow trout (Boyce-Derricott et al., 2009; Osachoff et al., 2013; Casanova-Nakayama et al., 2018) were not up-regulated by E2 treatments in any exposure periods, as was confirmed in the primary cultured hepatocyte of cutthroat trout in this study. Thus, in salmonids, only *erα1* appears to show strong and acute upregulation by E2 among the *er* subtypes. Strong upregulation of *erα1* by E2 in the liver possibly contributes to induce the active (i.e., strong and acute) synthesis of hepatic *chg* and *vtg* mRNAs.

Upregulations of *chgHα*, *chgHβ*, *chgL, vtgAs*, *vtgC*, and *erα1* mRNA levels by E2 treatments were confirmed in the Experiment 1. In Experiment 2, the effects of various doses (10^{-11} ~ 10^{-6} M) of E2 on expressions of these estrogen-responsive genes were examined. Transcriptional levels of *chg*s and *vtg*s at 24 hpi and 72 hpi of E2 treatments increased in a dose-dependent manner. In addition, *erα1* mRNA levels in E2-treatment

groups at 24 hpi also showed dose-dependent increase. These increase patterns suggested these genes are under strict E2 regulation.

At 24 hpi and 72 hpi, the LOECs of E2 in mRNA expression of *chg*s and *vtg*s were *chgHβ* (10^{-10} M) < *chgHα*, *chgL*, *vtgAs* (10^{-9} M) < *vtgC* (10^{-8} M). These results suggest that *chgHβ* is most sensitive gene to E2, and *vtgC* is the least. However, these sensitivities shown in primary cultured hepatocytes were different from the results obtained from E2 administration *in vivo* in Chapter III. In the results of Chapter III, expression of *chgHβ*, *chgL*, *vtgAs* and *vtgC* mRNAs showed higher sensitivity to E2 than it of *chgHα* mRNA at 2 dpi beside expression of *vtgAs* and *vtgC* mRNAs showed higher sensitivity to E2 than those of three *chg* subtype mRNAs. The physiological responses *in vivo* are affected by several factors such as individual differences and endocrine hormones. Therefore, the E2 responses of genes *in vivo* in Chapter III can be inconsistent with those in primary cultured hepatocytes. For example, some studies reported that the endocrine factors other than E2 affect *chg* and *vtg* expressions. In Arctic char (*Salvelinus alpinus*), cortisol administration up-regulates plasma Chg levels but downregulates plasma Vtg protein levels in conjunction with E2 treatment (Berg et al., 2004). Hepatic *vtg* mRNA expression is affected by triiodothyronine (T3) treatment through upregulation of *erα* mRNA levels in goldfish (*Carassius auratus*). Involvement of growth hormone (GH) on hepatic Vtg synthesis has been shown in Japanese and European (*A. anguilla*) eels (Kwon and Mugiya, 1994; Peyon et al., 1996). Furthermore, androgens are involved in hepatic Vtg synthesis in eels (Peyon et al., 1997; Kwon et al., 1994), rainbow trout (Mori et al., 1998) and goldfish (Hori et al., 1979). However, to date, the effect of these hormones on the expression of *chg*s, *vtg*s and *erα1* genes are

unknown in cutthroat trout. Further investigations are required to clarify the role of these supplemental endocrine factors on the expression of hepatic estrogen-responsive genes in cutthroat trout.

From 24 hpi to 72 hpi, *chgHα*, *chgHβ*, *chgL*, *vtgAs*, and *vtgC* mRNA levels increased or kept high values in high-E2-dose groups of both Experiment 1 (culture with replacement of medium) and Experiment 2 (culture without replacement of medium). Meanwhile, *erα1* mRNA levels in high dose E2 treatment groups increased in Experiment 1 but decreased in Experiment 2 from 24 hpi to 72 hpi. The differences in E2-responsiveness of *erα1* expression between Experiment 1 and 2 were possibly caused by the difference in the way of E2 treatment. Similar results have been shown in the study using primary cultured hepatocytes of rainbow trout (Flouriot et al., 1996): *erα1* mRNA levels kept high values from 24 hpi to 72 hpi in 10^{-6} M E2 treatment groups when E2-contained culture medium was replaced every 24 hpi, but *erα1* mRNA levels decreased from 24 hpi to 72 hpi following 10^{-6} M E2 treatments when the medium was not replaced. Thus, the data obtained in rainbow trout is in agreement with the data of cutthroat trout. It has been shown that primary cultured hepatocytes of *Xenopus laevis* rapidly metabolize E2 in culture medium (Tenniswood et al., 1983), suggesting that primary cultured hepatocytes of trout also metabolize E2 during treatments. Thus, in experiment 2, the *erα1* mRNA levels appeared to decrease from 24 hpi to 72 hpi after treatments with decrease of the E2 concentrations in the medium.

In *Xenopus laevis* and rainbow trout, it has been shown that E2 regulates not only transcriptional process (mRNA synthesis) but also post-transcriptional process (mRNA stabilization) of *vtg* and *er* gene expressions, although the mechanisms of

post-transcriptional regulation by E2 are unknown (Brock and Shapiro, 1983; Flouriot et al., 1996). In addition, it has been shown that the transcriptional rates are different between *vtg* and *er* genes in the analysis using primary cultured hepatocytes of rainbow trout (Flouriot et al., 1996). The transcriptional rate of *er* gene is faster than that of *vtg* gene. These transcriptional rate and post-transcriptional regulation should be reflected in the mRNA levels of *chg*s, *vtg*s, and *erα1*. Thus, further investigation are encouraged to focus on the effects of E2 on the rates of synthesis and clearance for *chg*s, *vtg*s and *erα1* mRNAs.

The expressions of *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC*, and *erα1* mRNAs showed differential patterns in Experiment 1 and Experiment 2. In addition, the LOECs of E2 on the expression of *chg*, *vtg* and *erα1* mRNAs were different among these genes in Experiment 2. These results obtained from primary cultured hepatocytes suggest that these estrogen responsive genes are differentially regulated by E2 in the liver. The differential expressions among these mRNAs may be due to involvement of Er subtypes other than Erα1 in the expressions of *chg*, *vtg* and *erα1* genes. Limited information on functional differences of Er subtypes are available. However, as described in Chapter I, recent studies have revealed that ERβ is involved in the expression of *vtg* and *erα1*. (Nelson and Habibi, 2010; Griffin et al., 2013). In medaka, Lee Pow et al. (2016) have shown that Erα, Erβ1, and Erβ2 have differential characteristics in transactivation of *vtg* genes *in vitro*. The higher binding affinities of teleost ERβs to estradiol than those of Erα has now been described in other fish species, namely, zebrafish and channel catfish (Xia et al., 1999, 2000; Menuet et al., 2002). Therefore, functional differences of Er subtypes in the expression of *chg*, *vtg* and *erα1* mRNAs may contribute to the

differential expressions of the E2-responsive genes.

In conclusion, this study revealed the expression profiles of all subtypes of *chg*, *vtg* and *er* mRNA in primary cultured hepatocytes of a single species. In primary cultured hepatocytes of cutthoroat trout, levels of *chg*, *vtg* and *erα1* mRNAs were up-regulated by E2. The sensitivity of *chg*s and *vtg*s to E2 were determined as *chgHβ* < *chgHα*, *chgL*, *vtgAs* < *vtgC*. Transcriptional levels of *erα1* decreased without replenishment of E2 from 24 hpi to 72 hpi of the treatment, unlike those of *chg*s and *vtg*s. These results indicated that *chg*, *vtg*, and *erα1* mRNAs showed the differential responses to E2, suggesting that expression of *chg*, *vtg*, and *erα1* genes are potentially regulated through E2 stimulation by different mechanisms.

Chapter V. Molecular cloning and characterization of choriogenins, vitellogenins and estrogen receptor α1 gene promoters in cutthroat trout

Introduction

To date, promoter regions of *chg*, *vtg* and *erα1* genes, which contain consensus ERE or ERE-like sequences, have been cloned in several teleosts (Le Roux et al., 1993; Teo et al., 1998; Menuet et al., 2004; Ueno et al., 2004; Zeng et al., 2005; Mushirobira et al., 2018). Reporter gene assays have revealed the involvement of 1/2 ERE and ERE-like sites, as well as ERE sites, in the transactivation of target genes by Er (Le Drean et al., 1995; Menuet et al., 2004; Ueno et al., 2004; Mushirobira et al., 2018). In addition to these sites, binding sites for transcription factors such as AP-1 and Sp1, which can interact with Er to transactivate estrogen-responsive genes in mammalian cells (Björnström and Sjöberg, 2005), are confirmed in rainbow trout (AP-1; Le Roux et al., 1993) and cutthroat trout (Sp1 and AP-1; Mushirobira et al., 2018).

As described in the previous chapters, differential E2 responses in cutthroat trout *in vivo* and *ex vivo* were observed among *chg*, *vtg*, and *erα1* mRNAs. It can be generally explained that differences in expression patterns of genes possibly depend on the characteristics of the promoter region, suggesting that *chg*, *vtg*, and *erα1* promoters have differential characteristics, leading to their distinct expression profiles in response to estrogenic stimulation. Recently, the two promoters of *vtgAs* (1 and 2) and one promoter of *vtgC* for cutthroat trout have been cloned and analyzed (Mushirobira et al., 2018). Reporter gene assays *in vitro* using Erα1 have revealed that the three *vtg* promoters are transactivated by Erα1 in the presence of E2. The *vtgAs* promoter 1 alone, contains consensus ERE and exhibits the highest maximal transcriptional activity. The

concentrations of E2 that induces 50% of gene reporter activity (half-maximal effective concentrations, EC50) are similar among all *vtg* promoters.

In this chapter, a series of experiments were designed to directly investigate the effects of Er subtypes on promoter regions of *chg*, *vtg* and *erα1* genes. At first, *chg* and *erα1* promoters were cloned from cutthroat trout genome. The additional promoter regions of both *vtgAs* and *vtgC* were also cloned and analyzed, because the lengths of these promoters previously reported were relatively short. Finally, the roles of ERα1, Erα2, Erβ1 and Erβ2 in driving gene expression were investigated in reporter gene assays using promoter regions of *chg*, *vtg* and *erα1* genes. It should be noted that *vtgAs* promoter 1, which exhibited the higher transcriptional activity of the two *vtgAs* subtypes (Mushirobira et al., 2018), was used as a representative *vtgAs* promoters. Thus, *vtgAs* promoter 1 is described as *vtgAs* promoter in the following sentences unless otherwise noted.

Materials and methods

Experimental fish and tissue samples

Two-year-old female cutthroat trout were obtained from Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University. They were transferred and reared in outdoor aquaria at the Faculty of Fisheries Sciences, Hokkaido University, receiving a continuous flow of well water under natural photothermal conditions. Blood was collected from one female fish 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 of genomic DNA extraction.

Genomic DNA extraction

Extraction of genomic DNA from blood sample was performed as described previously (Mushirobira et al., 2018). One μl of blood was mixed with 300 μl of cell lysis solution (10 mM Tris-HCl, pH 8.0, containing 100 mM EDTA and 2% sodium dodecyl sulfate and 2 μl of 530 Unit/ml proteinase K (Wako, Osaka, Japan). The solution was incubated at 55°C for 1 hr. After the incubation, the cell lysate was mixed with 100 μl of 7.5 M ammonium acetate and centrifuged at 13,000 g for 3 min at 4°C. After the centrifugation, the cell lysate was placed on ice for 5 min and centrifuged again for 5 min. The resulting supernatant was mixed with 600 μl of isopropanol following a centrifugation at 13,000 g for 8 min. The pellet (genomic DNA) was washed with 600 μl of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 0.1

mM EDTA, pH 8.0). The genomic DNA was stored at -80°C.

Molecular cloning of *chg*, *vtg* and *erα1* gene promoters

A genome walking library was used for amplification of *chgHβ*, *chgL* and *erα1* gene promoter. Four genome walking libraries were made from the trout genomic DNA using GenomeWalker Universal Kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. Gene specific primers (GSP) were designed from the coding region of each gene (Table 3). Primary PCR was carried out as follows: 5 µl of PrimeSTAR Max Premix (Takara Bio), 0.2 µl of adaptor primer (AP) 1, 0.2 µl of 10 µM GSP (for *chgHβ*: *chgHβ* G.W. R1, for *chgL*: *chgL* G.W. R1, for *erα1*: *erα1* G.W. R1), 0.2 µl of library template (*chgHβ*, genomic DNA digested with EcoRV; *chgL* and *erα1*, genomic DNA digested with StuI); 7 cycle of 98 °C for 10 s and 72°C for 3 min and 32 cycles of 98 °C for 10 s and 68 °C for 3 min. Secondary PCR was carried out as follows: 5 µl of PrimeSTAR Max Premix, 0.2 µl of AP1, 0.2 µl of 10 µM GSP 2 (for *chgHβ*: *chgHβ* G.W. R2, for *chgL*: *chgL* G.W.-R2, for *erα1*: *erα1* G.W.-R2), 0.2 µl of each template; 5 cycle of 98 °C for 10 s and 72°C for 3 min and 20 cycles of 98 °C for 10 s and 68 °C for 3 min.

Genomic DNA template without the above treatments of genome walking procedures were used for amplification of *chgHα*, *vtgAs* and *vtgC* gene promoters. Primers (Table 3) for amplification of *chgHα*, *vtgAs* and *vtgC* 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 2 µM forward

primer,.1 µl of 2 µM reverse primer, 0.2 µl of genomic DNA template; 35 cycles of 98 °C for 10 s, 55°C for 10 s and 72°C for 3 min.

Table 3 Name, nucleotide sequence, direction, and use of primers

Name	Sequence	Direction	Use
chgH α promoter F	ACTCCCCCAACCTTCCTCTT	Reverse	Cloning of *chgH α* promtoer
chgH α promoter R	GTTCTGAGGGGGTTGGTAAGG	Reverse	Cloning of *chgH α* promoter
chgH β G.W. R1	CCCTGGTTTTTCCAAGTAAATCTGAG	Reverse	Genome walking for *chgH β*
chgH β G.W. R2	GAACAGTGCTAATGCCTGAAAGG	Reverse	Genome walking for *chgH β*
chgL G.W. R1	CTGACGATTGGGTCTGAAGGGTTGCTG	Reverse	Genome walking for *chgL*
chgL G.W. R2	TTTACTGAAGGGTGGCCAATTCTGAG	Reverse	Genome walking for *chgL*
er α1 G.W. R1	CTGACGATTGGGTCTGAAGGGTTGCTG	Reverse	Genome walking for *er α1*
er α1 G.W. R2	TTTACTGAAGGGTGGCCAATTCTGAG	Reverse	Genome walking for *er α1*
vtgAs promoter 1 F	AAGATGCAATTCGTCAGACTTCG	Forward	Cloning of *vtgAs* promtoer 1
vtgAs promoter 1 R	AAAGTTAACAGATTGACTCGCTACA	Reverse	Cloning of *vtgAs* promtoer 1
vtgC promoter F	ATCAAACCATGCAATAATCTGAGTC	Forward	Cloning of *vtgC*
vtgC promoter R	GGCCAAGGCCACAAGGT	Reverse	Cloning of *vtgC*
pcDNA3.1 Linealize F	GTTTAAACCCGCTGATCA	Forward	Subconing of *er α2*, *er β1* and *er β2*
pcDNA3.1 Linealize R	GCTAGCCAGCTTGGGTCT	Reverse	Subconing of *er α2*, *er β1* and *er β2*
er α2 pGL3.1 infusion F	CCCAAGCTGGCTAGCACCATGTACCCTG AGGAGACC	Forward	Subconing of *er α2*
er α2 pGL3.1 infusion R	TCAGCGGGTTTAAACTCATGGAATGGG GCTCTG	Reverse	Subconing of *er α2*
er β1 pGL3.1 infusion F	CCCAAGCTGGCTAGCACCATGTCACAAT ATAGAAGACTC	Forward	Subconing of *er β1*
er β1 pGL3.1 infusion R	TCAGCGGGTTTAAACTCACCACTGTCTT TCTACC	Reverse	Subconing of *er β1*
er β2 pGL3.1 infusion F	CCCAAGCTGGCTAGCACCATGGCATGTT CTCCTGAAAG	Forward	Subconing of *er β2*
er β2 pGL3.1 infusion R	TCAGCGGGTTTAAACTTACTGAGGTACA CATCTCC	Reverse	Subconing of *er β2*
pGL4.10 linearize F	CTGTTGGTAAAGCCACCATGGAAG	Forward	Subconing of *chgH α*, *chgH β*, *chgL* and *er α1* promoter
pGL4.10 linearize R	GCGAGCTCAGGTACCGGC	Reverse	Subconing of *chgH α*, *chgH β*, *chgL* and *er α1* promoter
chgH α pGL4.10 infusion F	GGTACCTGAGCTCGCACTCCCCCAACCTT CCTC	Forward	Subconing of *chgH α* promoter
chgH α pGL4.10 infusion R	TGGCTTTACCAACAGAGACAATCCGAGG AACCC	Reverse	Subconing of *chgH α* promoter
chgH β pGL4.10 infusion F	GGTACCTGAGCTCGCATCACGATCGTGA AATACGT	Forward	Subconing of *chgH β* promoter
chgH β pGL4.10 infusion R	TGGCTTTACCAACAGAAGAACTAATCCG CAATGTTC	Reverse	Subconing of *chgH β* promoter
chgL pGL4.10 infusion F	GGTACCTGAGCTCGCACTATAGGGCACG CGTGG	Forward	Subconing of *chgL* promoter
chgL pGL4.10 infusion R	TGGCTTTACCAACAGCAGCAATGTTCAC TGGATG	Reverse	Subconing of *chgL* promoter
er α1 pGL4.10 infusion F	GGTACCTGAGCTCGCCCTCGGCAAACTT AATGATG	Forward	Subconing of *er α1* promoter
er α1 pGL4.10 infusion R	TGGCTTTACCAACAGAGATTTAAAAAAG GTAGAAACAA	Reverse	Subconing of *er α1* promoter
chgH α pGL4.10 2000 bp infusion F	GGTACCTGAGCTCGCGATATTTCTGTGTC TTATTTTC	Forward	Subconing of *chgH α* promoter for 2000 bp
chgH β pGL4.10 2000 bp infusion F	GGTACCTGAGCTCGCTCATAATGCATCAA TTTGACAT	Forward	Subconing of *chgH β* promoter for 2000 bp
chgL pGL4.10 2000 bp infusion F	GGTACCTGAGCTCGCATCCGGGTTTGCAG AATATG	Forward	Subconing of *chgL* promoter for 2000 bp
er α1 pGL4.10 2000 bp infusion F	GGTACCTGAGCTCGCCTTCTCCCATTTAA C	Forward	Subconing of *er α1* promoter for 2000 bp
vtgAs promoter 1 2000 bp pGL4.10 infusion F	GGTACCTGAGCTCGCGGGCAGTCAGAAT G	Forward	Subconing of *vtgAs* promoter 1 for 2000 bp
vtgAs promoter 1 2000 bp pGL4.10 infusion R	TGGCTTTACCAACAGGGCCAGTGTGATGT GAGG	Reverse	Subconing of *vtgAs* promoter 1 for 2000 bp
vtgC promoter 2000 bp pGL4.10 infusion F	GGTACCTGAGCTCGCCCGCACCATAGCA C	Forward	Subconing of *vtgC* promoter for 2000 bp
vtgC promoter 2000 bp pGL4.10 infusion R	TGGCTTTACCAACAGGGTGAAATCCAG G AGTGGGA	Reverse	Subconing of *vtgC* promoter for 2000 bp

The PCR products were ligated into cloning vectors, transformed into XL-1 competent cells (Strategene, La Jolla, CA, USA), and sequenced, as described in Chapter II.

Computational search for putative transcription factor binding sites

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

Reporter gene assays

Construction of plasmid vectors for reporter gene assays were performed using In-Fusion HD Cloning Kit (Takara Bio) according to the manufacturer's instructions. Primer sets (Table 3) for In-fusion cloning were designed using 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 *erα1* were subcloned into pGL4.10[*luc2*] Vector (Promega). The open reading frames of *erα2*, *erβ1* and *erβ2* were subcloned into pcDNA3.1(+) Vector (Thermo Fisher Scientific). Erα1 expression plasmid, pcDNA3.1-Erα1, was the same preparation that 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 (Corning, Corning, NY, USA) at 3×10^4 cells/well in phenol-red free Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% charcoal/dextran treated fetal bovine serum (Hyclone, Logan, UT, USA). The cells were pre-incubated for 24 hrs 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-inserted pGL4.10 (pGL4.10-*chgHα*, pGL4.10-*chgHβ*, pGL4.10-*chgL*, pGL4.10-*vtgA,* pGL4.10-*vtgC* and pGL4.10-*erα1*) or the empty pGL4.10 (negative promoter-construct control), 200 ng of the *er*s-inserted pcDNA3.1 (pcDNA3.1-*erα1*, pcDNA3.1-*erα2*, pcDNA3.1-*erβ1* and pcDNA3.1-*erβ2*), and 100 ng of pRL-TK Vector (served as an internal control to normalize possible variations in each transfection efficiency, Promega) using X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. After 4 hrs of incubation at 37°C, the cells were treated with various concentrations of E2 (final concentration: 10^{-14} – 10^{-5} M) or hormone vehicle (ethanol). The amount of ethanol in the medium did not exceed 0.1%. Following 40 hrs 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 assays were conducted in quadruplicate.

Statistics

All data were expressed as means ± SE. Data were analyzed by two-way ANOVA

followed by Tukey-Kramer HSD test, using a JMP Pro 14 Software program (SAS Institute Inc., Cary, NC, USA). The difference between groups was considered significant at $P < 0.05$. Hill's curve and half-maximal effective concentrations (EC50) for E2 were calculated using JMP Pro 14 Software program.

Results

Molecular cloning and sequence analysis of *chg*, *vtg*, and *erα1* promoters

The *chgHα* DNA promoter sequence consisted of 2851 bp located upstream of the translation initiation site and additional regions of Exon/Intron 113 bp (Fig. 16). No complete palindrome ERE (GGTCAnnnTGACC) was 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. AP-1 and Sp1 were predicted at following positions: AP-1: -2077 to -2070 (AGACTCAC), -1198 to -1191 (ATGATTCA), -1197 to -1190 (TGATTCAT), -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, -103 to -88 (GTCAGCCAAGGAGGTG) and -55 to -41 (GTATAAAAGCAGCAA), respectively.

The *chgHβ* DNA promoter sequence consisted of 2270 bp located upstream of the translation initiation site and additional regions of Exon/Intron 101 bp (Fig. 17). No complete palindrome ERE was identified 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. AP-1 were predicted at

-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. 16 A 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 to 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; 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

-1201 TGAATGATTCATTTGGTCGTTGGTAAACATTATAGGAAGAACCTACTACT

-1151 GAAAGAGACACGAGAAACCACATTGAACTTTGCAAAAGCCCACCTAAACA

-1101 AGTCTAAATCCTGTGAAAATGTTCTGTGGACCACTGAAACAAAATTAGAG

Fig. 16 (Continued)

Fig. 16 (Continued)

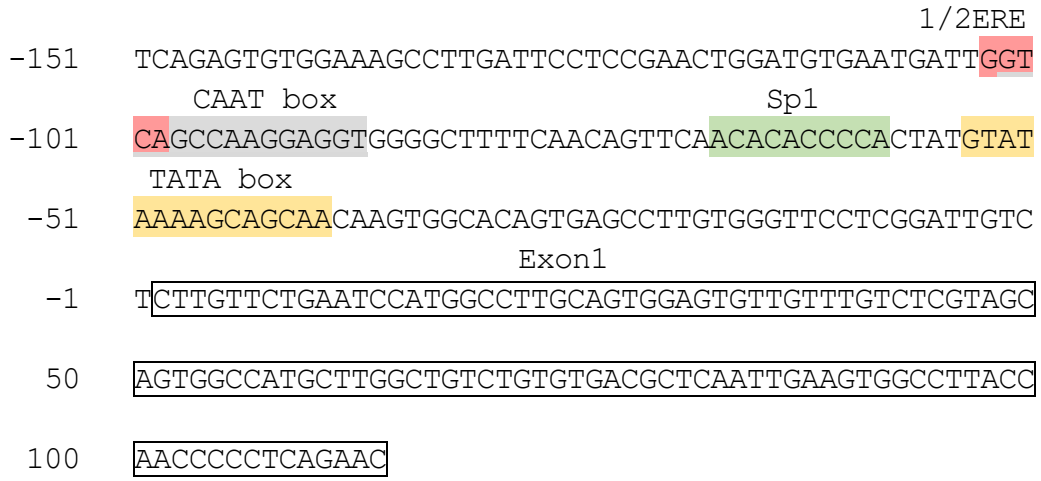

Fig. 16 (Continued)

```
-2270   ATCACGATCGTGAAATACGTTGCGCACATTGGCACTCAAACAAAATAATA
                                    ERE-like
-2220   TCCCACATCGCAGGTGGGCAAAAGGACACACTAAGTCTGATCCCCAATTA

-2170   GAGGTAACGATCATCAGCTGCCTCCAATTGGGAACCATGCACACACACAC
                                                    1/2ERE
-2120   ACCAACATAGAAATATAATACCTAGAAACCCCCCTAGTCACGCCCTGACC

-2070   TAAAACACAGGGCGTGACATGGTGTGAATACTGTCCTAACTTATTCTGCA

-2020   AGTGCTGGATTTGCTAGTTAACGTATATGGTTTGATAACTAATGAACTCT

-1970   GTAAATGTCTATCTTTTACCTCATAATGCATCAATTTGACATCAATTTAT

-1920   GTTGCAACCAAATAATATATGGATGTGGCTGTAAATACTGCATCATCATT

-1870   CTCCAATGGAAAGAGTCATTGGAAAAAAGAGTCATCTGTGCTATATTTTG

-1820   GAAATGTAGTTTAGTGTACAATGCACTACTTACCTGGGTTGTATATAACA

-1770   ATATAATCCACTCATTATTTAAATTACAGTAATATATAAATAATTTCAAT

-1720   TAGAGAGCCAGGCAAAACTGTATGATTTGACAAGTCACATAGAAAAGGTG

-1670   ATCTTGTACAATGTTGCATCGGGCAGAAATGGCATACAACAACATGCTAT

-1620   GTTTTCACAGTAGCCACCTGCTTTACAATACACCTATTTCTGGTTCTGTC

-1570   CTATATATGTAGTGTATACGGCAGGGGTTCCCAAACTGTTTTTTTGGCCG

-1520   CAACCCCATTTTGAAATTAAAATGTTTTTGTAATCCCGCCATGTGAAAAA

-1470   TGTTATGTTATAAACGGCCAATGTTTACTTTTTTTATTTGGGCTATGACA

-1420   GTTTATTACAAATCACTTTGACAGTACCTTTGAAAGTATTTCAATCTGAT
```

Fig. 17 A 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 to 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; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

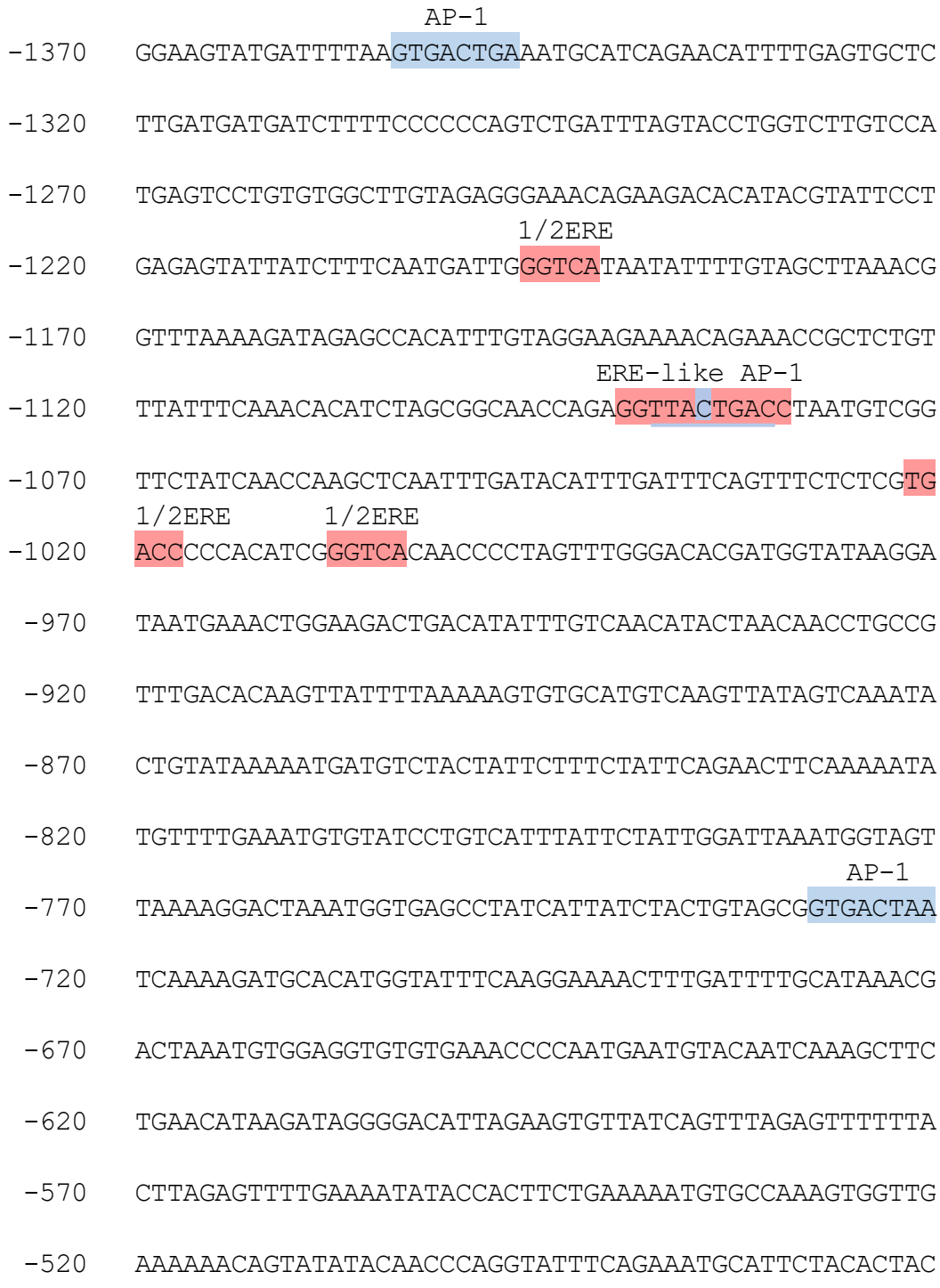

Fig. 17 (Continued)

Fig. 17 (Continued)

following positions: AP-1: -1354 to -1347 (GTGACTGA), -1088 to -1081 (TTACTGAC) and -728 to -721 (GTGACTAA) The conserved TATA box and CAAT box were found at positions, -17 to -3 (TTATAAAGGTGGCCG) and -234 to -219 (AGCCTCCAATGACATG), respectively.

The *chgL* DNA promoter sequence consisted of 3636 bp located upstream of the translation initiation site and additional regions of Exon/Intron 111 bp (Fig. 18). 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. AP-1 and Sp1 were predicted at 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 conserved TATA box and CAAT box were found at positions, -830 to -815 (TGTGCCCAATGGGCAG) and -57 to -43 (TTATAAAACTGGCCA), respectively.

The *vtgAs* DNA promoter sequence consisted of 2872 bp located upstream of the translation initiation site and additional regions of Exon/Intron 225 bp (Fig. 19). The consensus palindrome canonical ERE was identified in *vtgAs* promoter at position -690

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-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. 18 A 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 to 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; 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

-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. 18 (Continued)

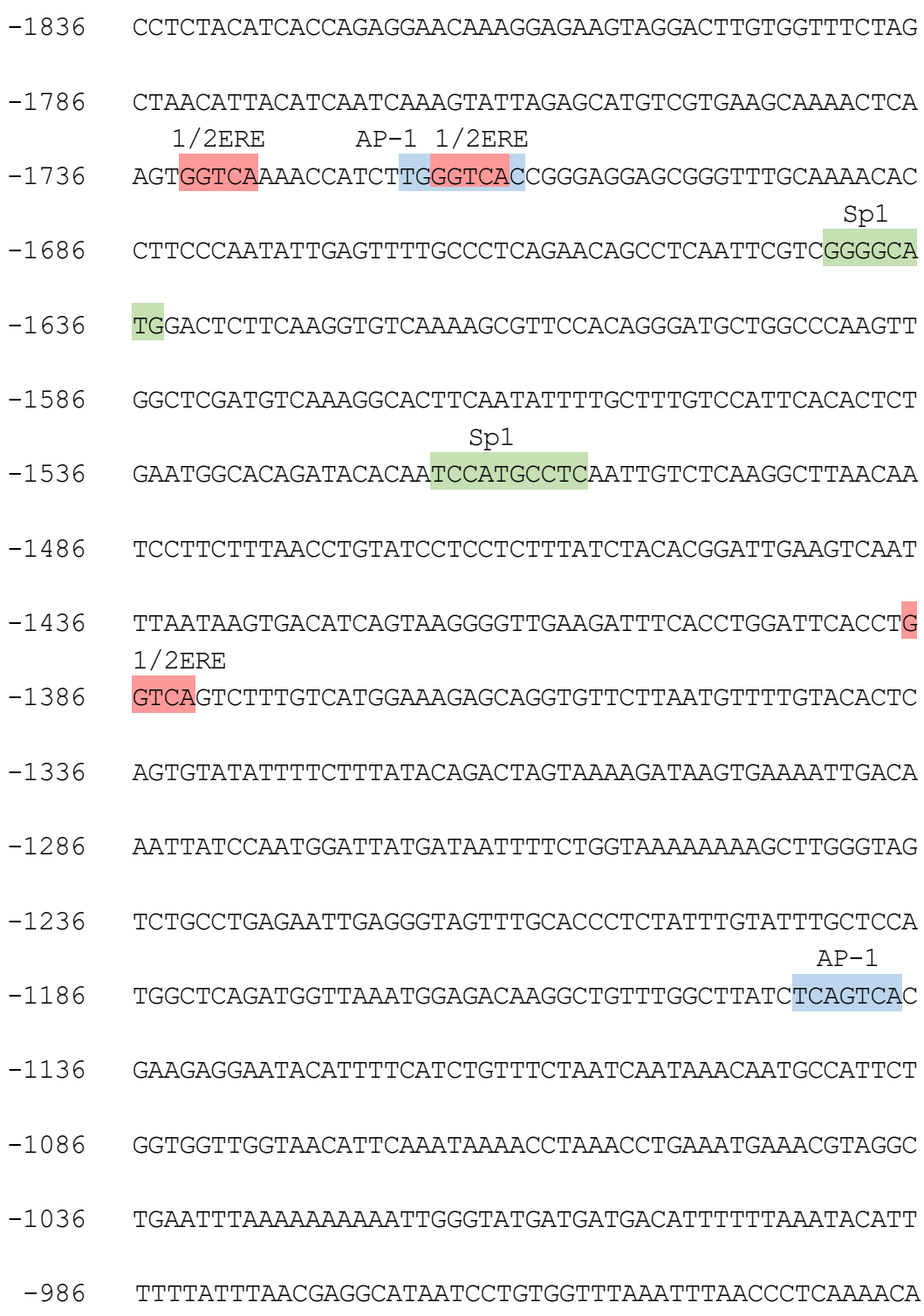

Fig. 18 (Continued)

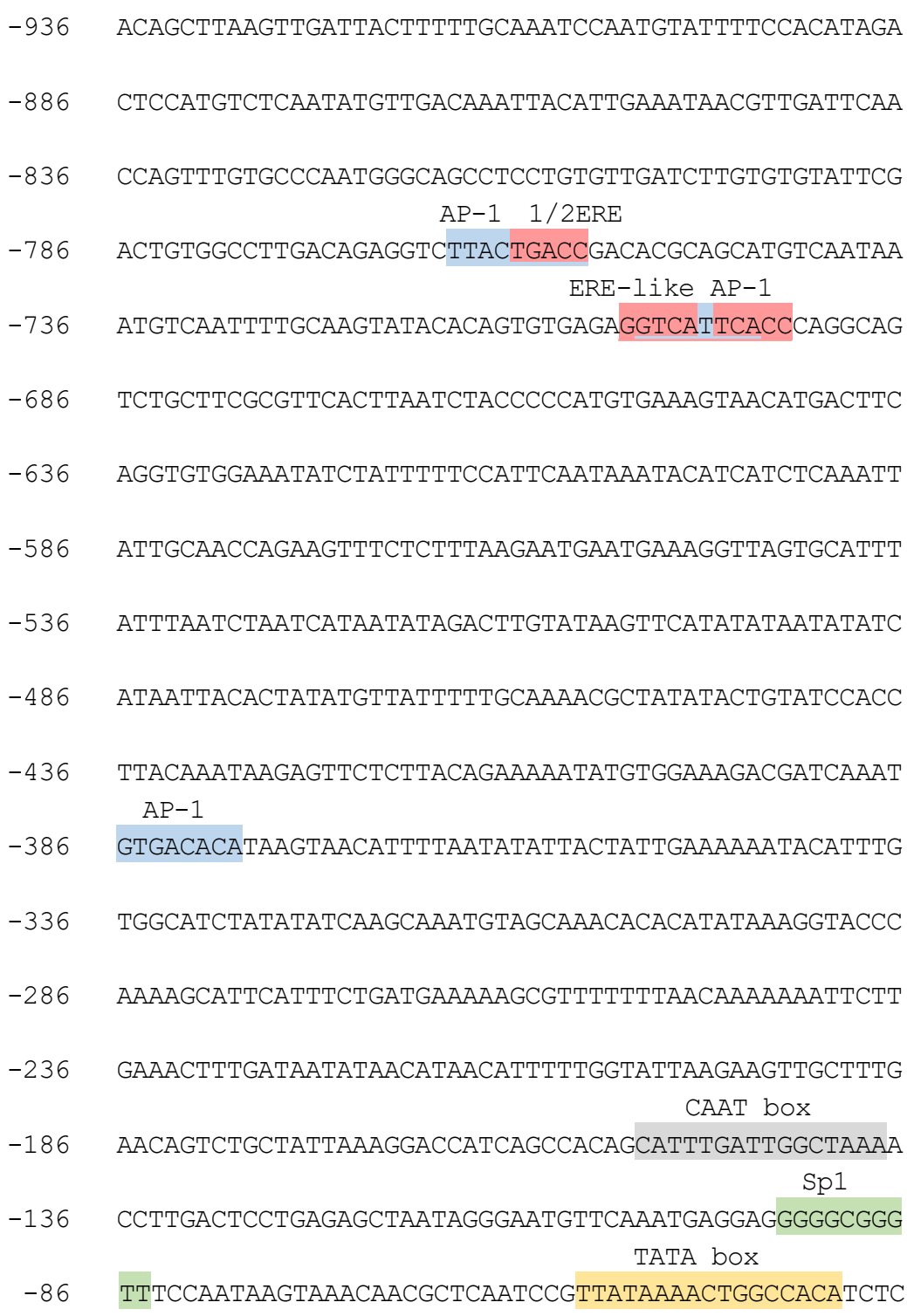

Fig. 18 (Continued)

Fig. 18 (Continued)

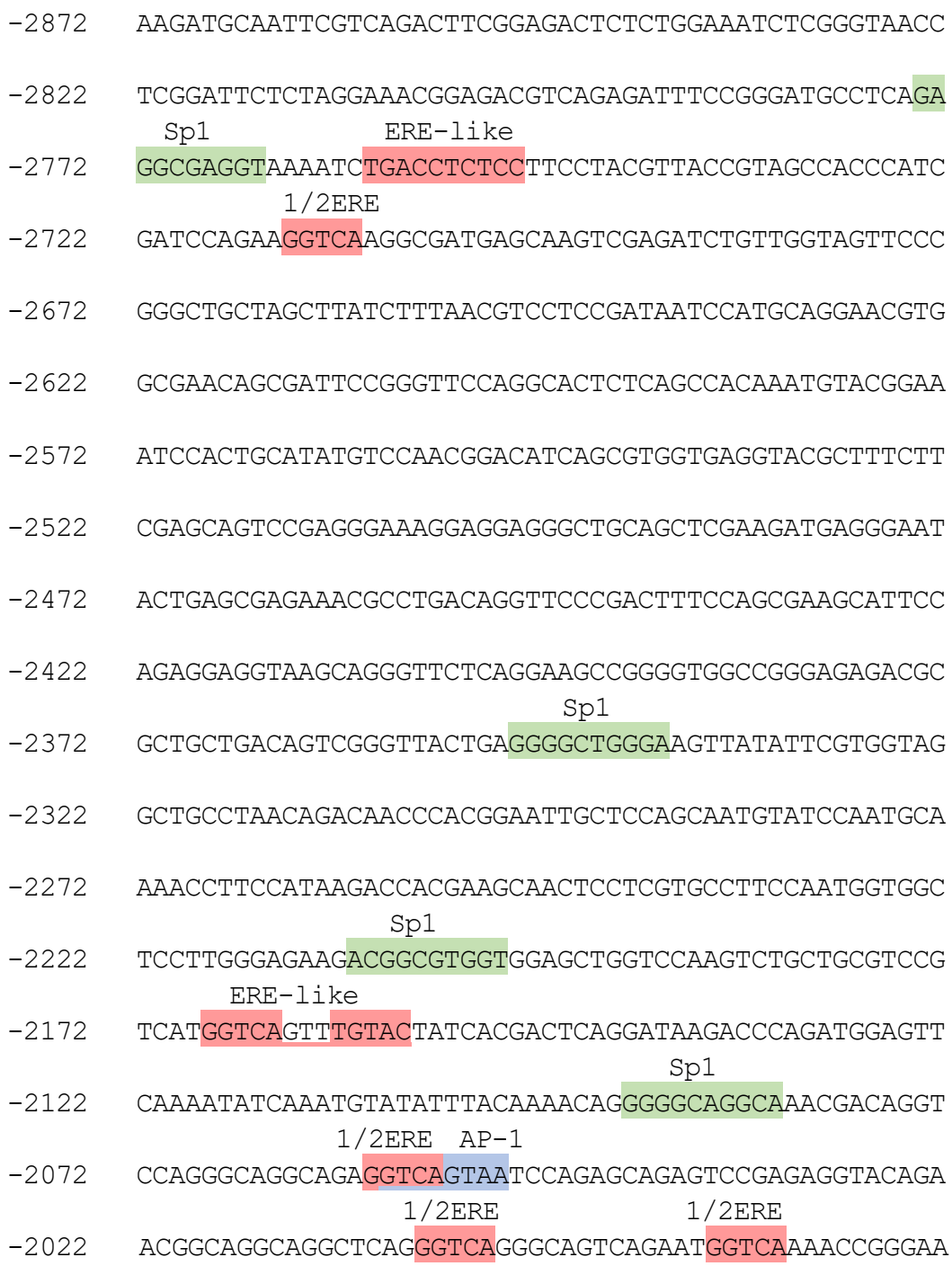

Fig. 19 A putative promoter sequence of the cutthroat trout vitellogenin As gene including 5'flanking region, exon 1, intron 1 and exon 2. The exons are indicated by open boxes. Nucleotides are numbered to 5' end of exon 1, intron 1 and exon 2, with negative numbers representing the 5'flanking region. Estrogen responsive element (ERE), ERE-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

Fig. 19 (Continued)

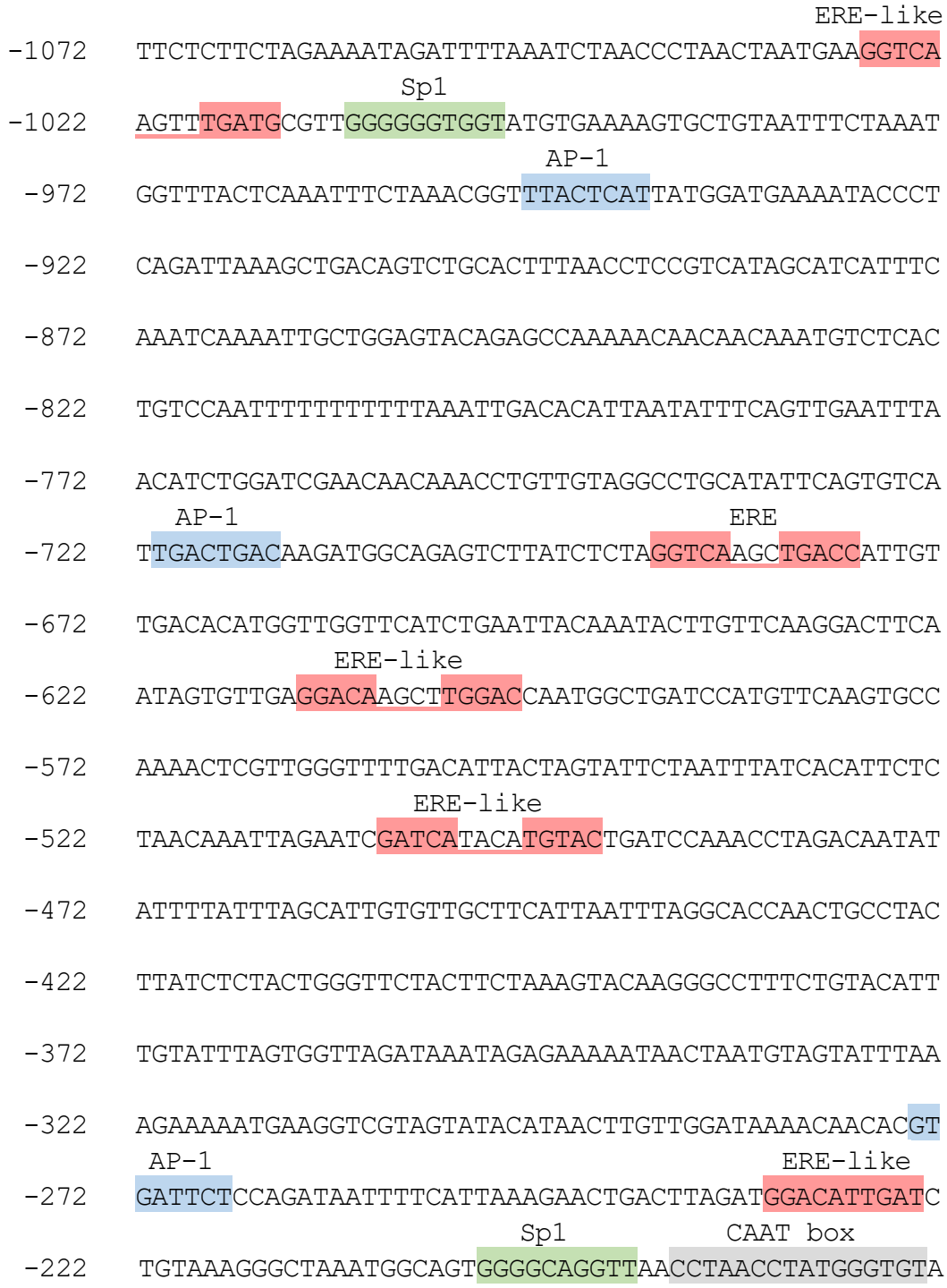

Fig. 19 (Continued)

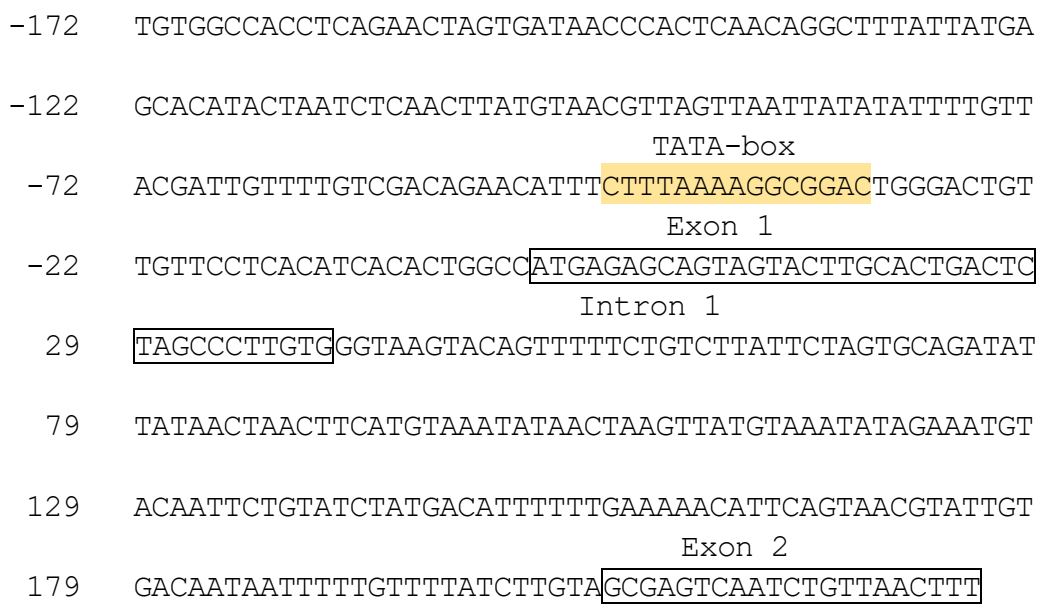

Fig. 19 (Continued)

to -678 (GGTCAagcTGACC). Eight ERE-like sequences and six 1/2 ERE were predicted at the following positions: ERE-like: -2767 to -2754 (GGTGAaatcTGACCT), -2758 to -2749 (TGACCTCTCC), -2168 to -2156 (GGTCAgttTGTAC), -1027 to -1014 (GGTCAagttTGATG), -690 to -678 (GGTCAagcTGACC), -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. AP-1 and Sp1 were predicted at 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 conserved TATA box and CAAT box were found at positions, -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 additional regions of Exon/Intron 39 bp (Fig. 20). No complete palindrome ERE was identified in the analyzed promoter sequences. Six ERE-like sequences and eight 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

-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. 20 A 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 to 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; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

```
                                                          1/2ERE
-2125   ACTGGCATGCGTATTCACTGAGATTTTTAACCTCTCGCTGACCGAGTCTG

-2075   CAATACGTACAGGTTTAAGCAGACCACCATAGTCCCTGTGGCTAAGGAAG

-2025   CGAAGGTAACCTACCAAAATAATTACCGCACCATAGCACTCACGTATGTA

-1975   GCCATGAAGTGCTTTGAAAAGCTGATCATGGATCATATCAATACCATTAT

-1925   CCCAGAAACCCCAGACCCAGCGTTCAACACCATAGGGCCCACAAAGCTAG

-1875   TCACTAAGCTAAGTACCATGGAACTAAACACCTCCCTCTGCAACTGGATC

-1825   CTGGATTTCCTGACGGGACACCCCCAGGTGGTAAGGGTAGGCAACAACAC

-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
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Fig. 20 (Continued)

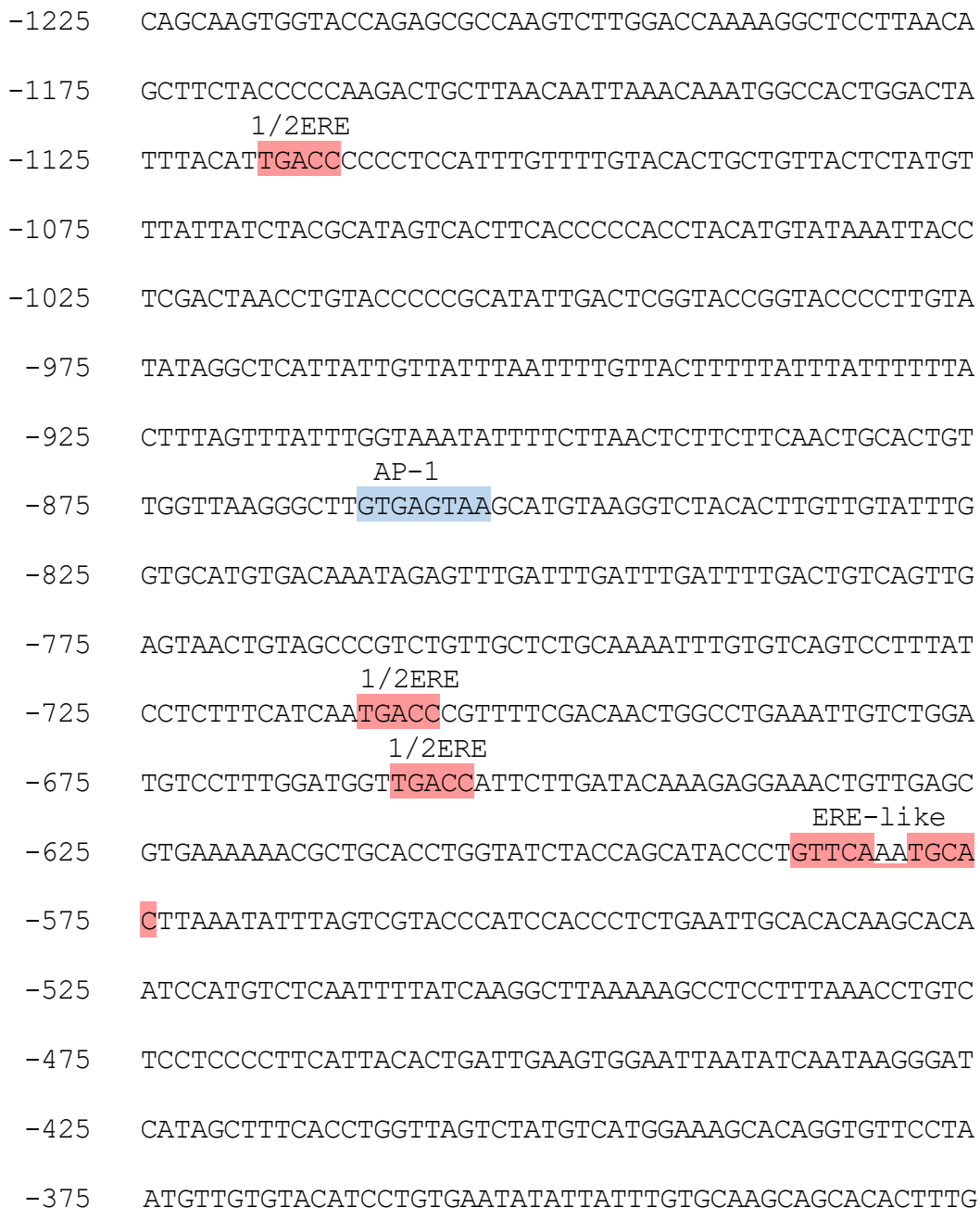

Fig. 20 (Continued)

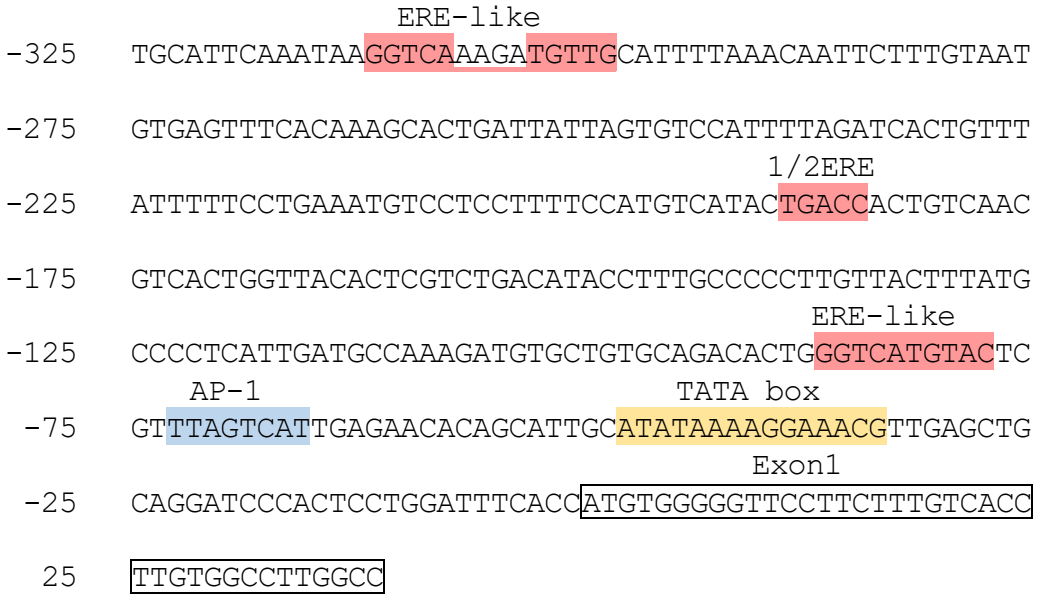

Fig. 20 (Continued)

(GGTCAaagaTGTTG) and -87 to -78 (GGTCATGTAC); 1/2ERE: -2193 to -2189, -2087 to -2083, -1316 to -1312, -1273 to -1269, -1118 to -1114, -712 to -708, -660 to -656 and -189 to -185. AP-1 and Sp1 were predicted at 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 found at the positions, -48 to -34 (TTATAAAACTGGCCA).

The *erα1* DNA promoter sequence consisted of 6155 bp located upstream of the translation initiation site and additional regions of Exon/Intron 1041 bp (Fig. 21). No complete palindrome ERE was identified in the analyzed promoter sequences. Two ERE-like sequences and eight 1/2 ERE were predicted at the following positions: ERE-like: -1107 to -1090 (GAGCAaccgaggcTTGAC) and -525 to -511 (GGTCAagagtTGTCC); 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. AP-1 and Sp1 were predicted at 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), -267 to -260 (TTAGTCAG); Sp1: -5855 to -5846 (AGGGCAGTGT), -5808 to -5799 (GGGGCGGTAT), -4409 to -4400 (ACCCAGCCAG), -3604 to -3595

```
-6155   CCTCGGCAAACTTAATGATGGTGTTGGAGTTAAGCTTAGCCATTCAGGTA

-6105   TGGGTGAGCAGGGAGTTCAGGAGGGGACTGAGCATGCACCCCTGAGGGGC

-6055   CCCTGTGTTGAGGATCAGCGAGGCAGATGTGTTGTTACCTACCCTTACCA

-6005   CCTGGGGGCGGCCCATCAGGAAGTCCACGATCCAGTTGCAGTGGGAGGTG

-5955   TTTAGTCCCAGGGTTCTTAGCTTGATGATGAGCTTTGAGGGTACTATGGT

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

-5705   TCTACGGGTTGGTAGTCATTTAAGCGTTGTTACCTTGGTGTTCTTGGGCA

-5655   CAGTTCTATGGTGGTCTACTTGAAACATGTTGGTGTTACAGACTCGGTCA

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

-5455   GTTCGGAACAGCTGATGCTCTCATGCATGCTACAGTGTTGCTTGCCTCAA
                                                  AP-1
-5405   AGCAAGCATGGAAGTTATTTAGCTTGTCTGGTAGGCTTGTGTCACTGGGC

-5355   AGCTCGTGGCTGTGCTTCCCTTTTGTAGTCCGTAATAGTTTGCAGGCCCT

-5305   GCCACATCCAATGAGCGTCGGAGCCGGTGTAGTACAATTCAATCTCAGTC
```

Fig. 21 A putative promoter sequence of the cutthroat trout estrogen receptor α1 gene including 5'flanking region and exon 1, intron 1 and exon 2 . The exons are indicated by open boxes. Nucleotides are numbered to 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; 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

-5105 TGTATGTACGGTCACTGTGGGGACGACGTCATCAATGCACTTATTGGCGA

AP-1

-5055 AGCCAGTGACTGATGTGGTGTACTCCTTAATGCATTCGGAAGAATCCCGG

-5005 AACATATTCCAGTCTGTGCTAGCAAAACAGTCCTGTAGCTTAACATCTGT

1/2ERE

-4955 GTCAACTGACCACTTCCTTATTGAGCAAGTCACTGGTACTTCCTGCTTTA

-4905 GTTTTTGCTTGTAAGCAGGAATCAGGAGGATAGCGTTATGGTCAGATTTG

-4855 TCAAATGGAGGGCGATGGAGAGCTTTGTACACGTCTCTGTGTGTGGAGTA

-4805 AAGGTCGTCTAGAGTTTTTTTTCCCCCTCTGGCTGCACATTTAACAAGCT

-4755 GCTAGAAATGAGGTAAAACTGATTTAAGTTTCCCTGTATTAAAGTCCCCG

-4705 GCCACTAGGAGCGCCGCCTCTGGATTAGCATTTTCTTGTTTGCTTATGGC

-4655 AGAATACAGCTCATTGAGAGCCGTCTTAGTGCCAGCATCGGTTTGTAGTG

-4605 GTAAATAGACAGCTACGAAAAATATAGATGTAATTATCTTGGTAAATAGT

-4555 GTGGTCTACAGCTTATCATGAGATACTTTACCTCTGGACTTCCTTAATAT

-4505 TAGATTTCATGCATCAGCTGTTATTTTCAAATAGACACAGACCGCCACCC

-4455 CTTGTCTTACCGGAGGCAGCTGTTCTATCTTGCCGATGGACAGAAAACCC

Sp1

-4405 AGCCAGTTATCCATGTTGTTGTTCAGCCACGACTAAGTGAAACATAAGAT

Fig. 21 (Continued)

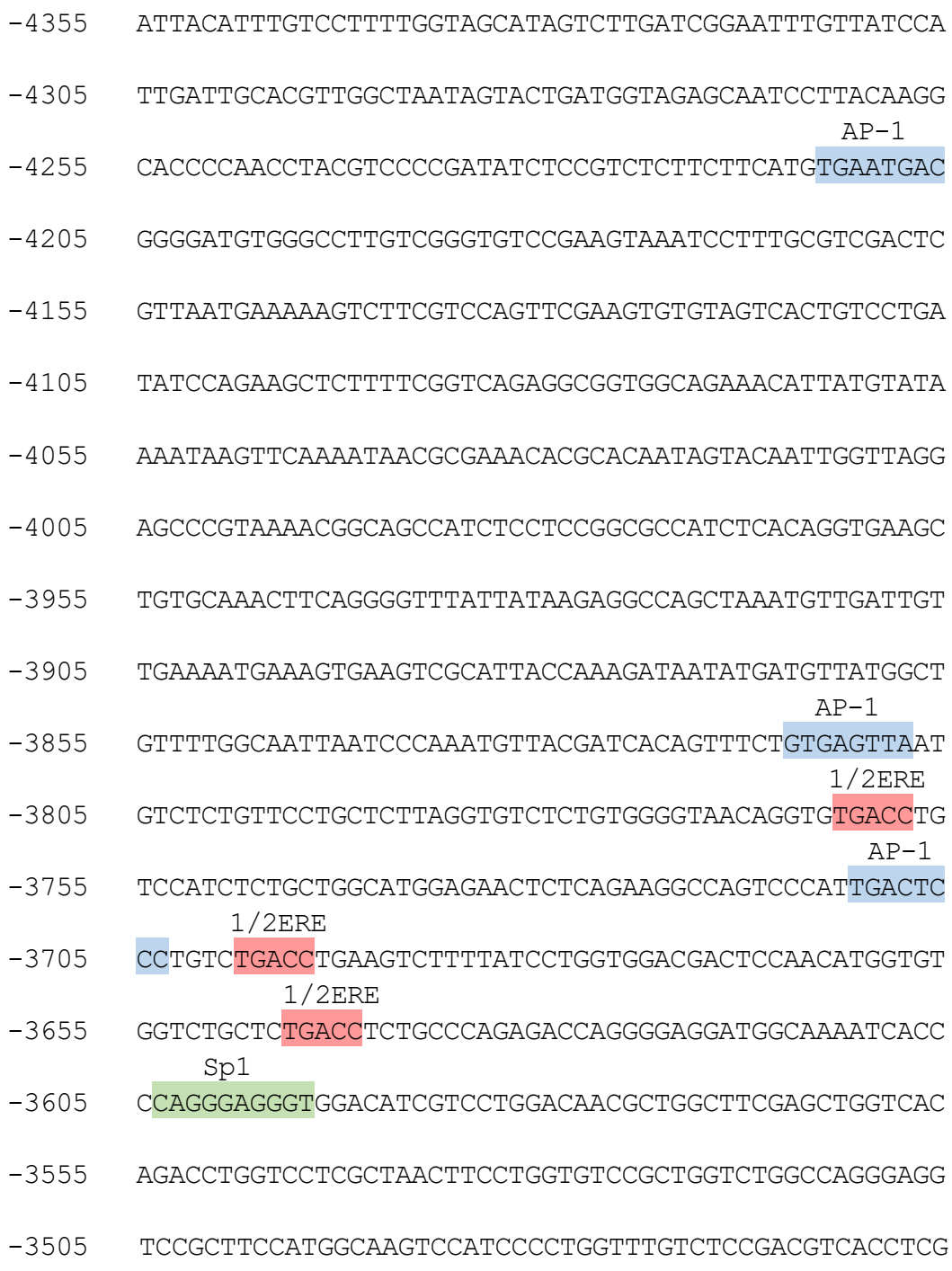

Fig. 21 (Continued)

-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. 21 (Continued)

-2555 AAATAGCATAGTATAACATTACTGTATGACAAAAAACACCTAATTTCTTA

-2505 TATTTTAGGATGATTGTGCTCATCGTCACATTTTTCTCTCATGCAGTCTA

-2455 AACTCAACAGTGCACGCCACTCAGCAAAGGTATGCTACAGTTTAATACCA

-2405 TCTGCAAAAAAATGTGCTAAGGTACATAACTAATTCAAAACACTGTAGCC

-2355 TACTTCAAAACAACACTGTAGCCTACACTCAATAATATTTAAATGCATAT

-2305 GCCCATGAAACTGATTGCAAATGGTTGGCGTGCAGATTCTGCATGTATGT

AP-1

-2255 TTCACCGGTAAAACTTGGAAGAATTATCATTCACGATTGACAGTGAGAAA

Sp1 1/2ERE

-2205 TGCGAAGGGAGGGTGACCAGAAGTTTGGGTGTGCGTAGAGTTAAAGAAAC

-2155 ACATGCACAGAACGTGAGTGGGATCTTCAGCTCTGGGGAAAGGGATCCAG

1/2ERE

-2105 TGGGATGGGGCTGTGACCTCCGGATCCCCAGCCTCTGCCATTTAAATAAT

-2055 GATCAACTGCTGGTTAGCAATCCATCCTGGCATAACTACAAAACAATGAC

-2005 GTTTACTTCTCCCATTTAACAAATGTGTTGACGGTTGTTTACCATCATTC

-1955 AGTACTAGGGTCGGGCTCTACCTCGATTCCAGTTCAAATCAGTTAATTTA

-1905 GAAACATTGAAATTCCAAGTCATGAATTGAAACATGCTTATCTGATCTTA

-1855 AGTGACTTTTTTCCAGACATATTATGTAATTTATGTAATGTTTTTGTCTC

-1805 CCCAACAGCGACTACACAAAATATAGATTTGAGACACATTTCATTCATTA

-1755 TCAGAGGCAATAAGATGCAAGGTGCAATTTGAACATTTACATTTTAAGTT

-1705 GGCCTGTCGTAAATAGTTTAGAATTGTTATTTTAAGTTGAGCCCTTATGT

Fig. 21 (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

-905 CAGCAGGTCAAACAGGAGGCCATTTTCTCCCAGTTTGCAGAGATAGAGGT

-855 TAATGAACCATCACTTTAATAAGCAAAAAGTTAACTTTCCCCCGTAAAAT

-805 CCAGCTGTCAATTTGTCAAGAAAGTTTGGAAAAATCAGAATCCCCAGTAT

Fig. 21 (Continued)

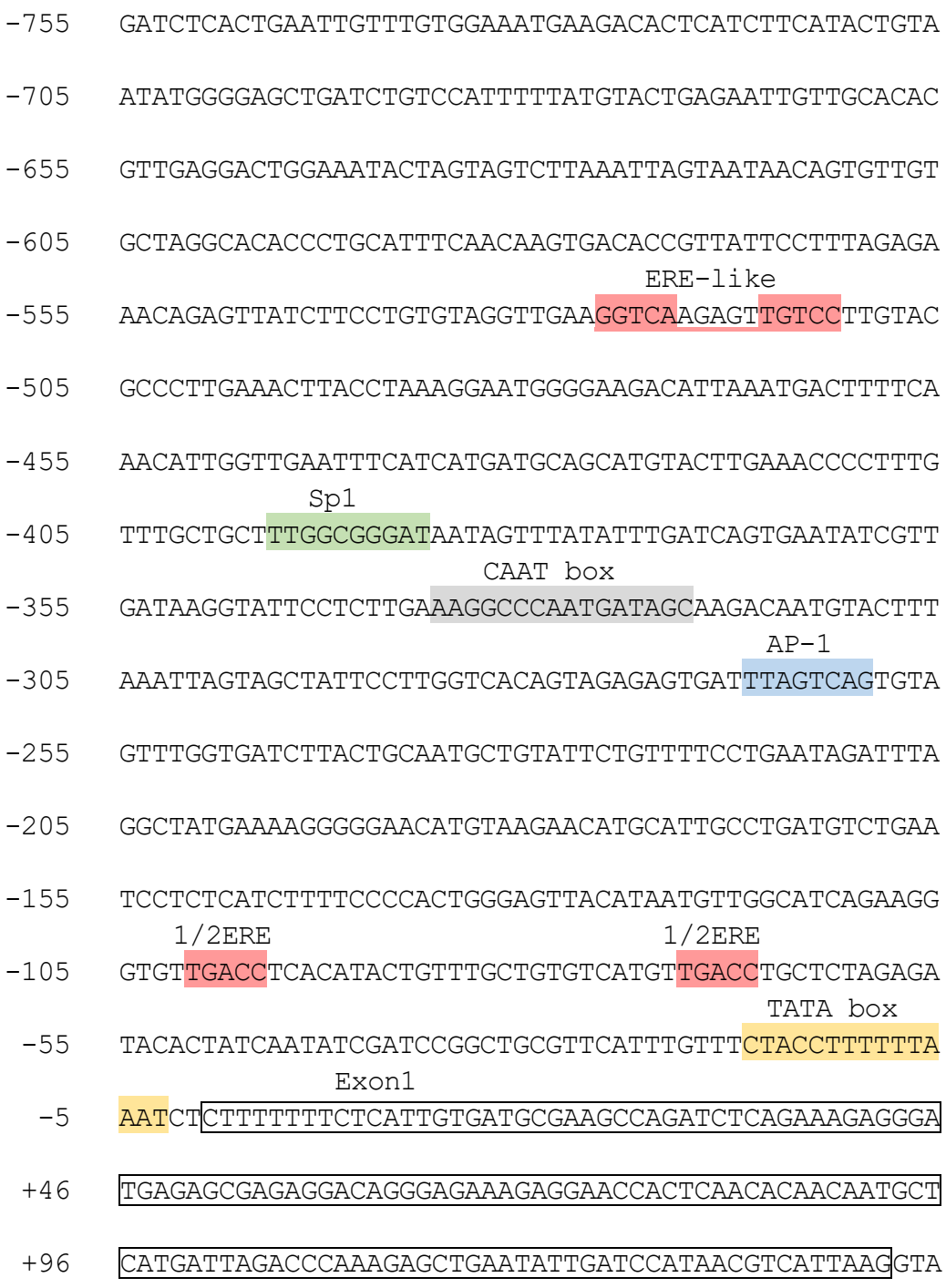

Fig. 21 (Continued)

Intron1

+146 AGTACTGTATATATGACGTCTTTGTGTGTATTTCATGTAATGGGTTCCTA

+196 TTTTATTGCTTCCTTCTCGTGGAATTGTTTTGTATAATTTTTCCATATTT

+246 GCACATAAATCCACTGATAGACACATTTAATAATGATTTAACTTCTTAGA

+296 AGAGTGAGTCAGAAAGGAGTGATATTCTGGGCCACGGCAGTACATGTAAC

+346 TAAATGACTGATAAGCATTCAGAGAGGAAGTCACACCAGGGTGTAACTCT

+396 CCTTGGAGCCTAGCAGCGTCCTCTAAAAGCTTTTCAGGGAAGAGAAAAAT

+446 AAGCTGCATTTTAGAAAAAAATGTAAGGGGACAGGGGAGTTGTTGGAGTG

+496 GTTGCCTCTTTCTTTCGTTCTCTCACTCTCCTTCTCTCTTGCTCTCTATC

+546 TCGAAATATCTGTCACTTGTTGTCCTTTCCTCCACCCCTCTCTCCCACAG

+596 CAGTGTCGATAGTTAAGGGAAAAGAGAAAAGCTCTGCCTCTCAAAAATAA

+646 GTGGGAGGGAGGAATGTTTTTGGGCTGTCTCCAGGCGGAACCTGGGAGGG

+696 AAAGAGAGAGCAAGGAGGGACGAGAAAAGAGAGAGAGAACCTAGTGAA

+746 TGCCTCTTCCCTTCCTCTTCCCAACAGCCAGTATTGAGTTGCTTAGCACG

+796 GGCTGTTAAGGAAGAAACAGAGCAAGAGAGGGACGAGAGAAAAGAGAGAG

+846 AGAAGACAGAACAGAGCCCTTCTCCCCTCCCACCCCTTAGTGAGCCAGTC

+896 TAAACCAAGCTGCTTGTCACTGCTGTTGTTCTGTGAATGTGATGCTGGTC

+946 AGACAGTCCCATACGCAGATTTCCAAACCTCTCGGAGCTCCTCTCAGATC

Exon2

+996 CCGAACGACCCTGGAGAGCCACGTCATCTCCACCCCAAAACTCTCACCAC

Fig. 21 (Continued)

+1046 AGCAGCCGACCACCCCCAACAGCAACATGTACCCTGAGGAGACACGCGGA

+1096 GGTGGTGGGGCGGCCGCCTTTAACTACCTGGACGGAGGGTATGACTACAC

+1146 AGCCCCTGCCCAA

Fig. 21 (Continued)

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

Numbers of ERE, ERE-like, 1/2 ERE, AP-1 and Sp1 sites in *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *erα1* promoters were shown in Table 4.

Transactivation of estrogen-responsive gene promoters by E2 via Erα1

The reporter plasmid containing either one of six promoters (*chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *erα1*) and the expression plasmid containing *erα1* were co-transfected into HeLa cells and the transactivation of the reporter gene was induced in the presence or absence of E2 (10^{-6} M), with reporter gene expression quantified by chemiluminescent intensities using the dual luciferase reporter assay system.

The luciferase reporter activity in all promoter-containing construct groups was higher in presence of E2 (10^{-6} M) than that of solvent control group (non-E2 controls) and non-promoter construct group, but the levels in the only *chgHα*, *chgHβ* and *vtgAs* promoters showed significant increase. (Fig. 22). The reporter activities in E2 treated groups of *chgHα* and *vtgAs* promoters were significantly higher than that of *chgHβ* promoter. The activity of *chgHβ* was higher than those of *chgL*, *vtgC*, and *erα1* in the E2 treated groups. Promoters of *chgL*, *vtgC* and *erα1* in the E2 treated groups showed similar reporter activities.

The dose-dependent responses (E2 doses: 10^{-14} M ~ 10^{-5} M) of the *chgHα*, *chgHβ,*

Table 4 The numbers of transcription factor binding sites in choriogenin, vitellogenin and estrogen receptor α1 promoters

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	8	6	7	10
vtgC	—	6	8	1	6
era1	—	2	19	9	11

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

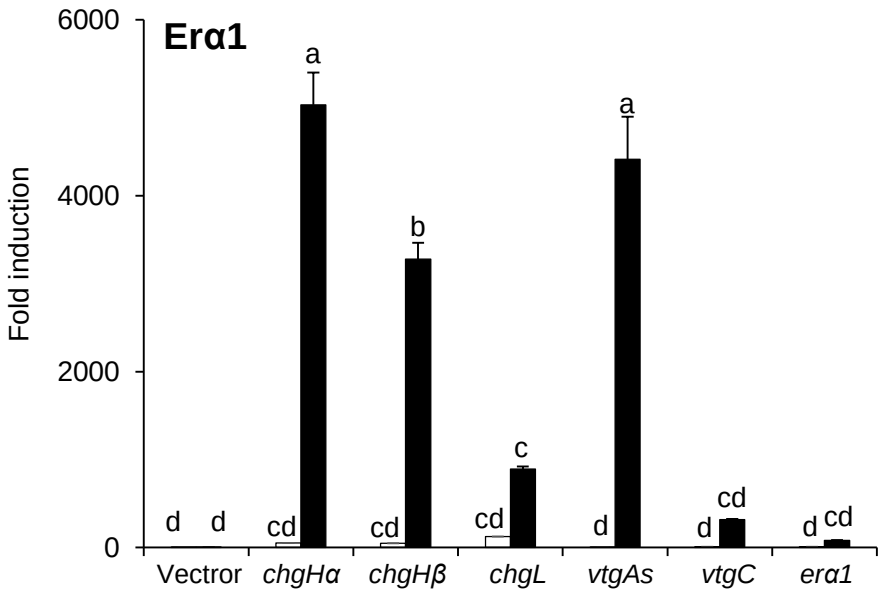

Fig. 22 Transcription activities of choriogenin (*chg*), vitellogenin (*vtg)* and estrogen receptor α1 (*erα1*) promoters mediated by Erα1 in the presence or absence of estradiol-17β (E2).

Hela cells were co-transfected with *erα1* in an expression vector and one of the following gene promoters in a reporter vector: *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *erα1*. Reporter vector without a gene promoter (vector) were used for the negative control. The transfected cells were treated with E2 (1 μM; black columns) or ethanol (vehicle control; open columns). The fold induction was initially normalized to the corresponding Renilla luciferase expression and averaged from 4 wells. The fold activation were represent with the that of empty vector (vehicle control) set to 1. Columns indicate mean values and vertical brackets standard errors. Differential letters denote a significant differences ($P < 0.05$).

and *vtgAs* promoters are shown in Fig. 23. All three promoters exhibited the same lowest-observed effect concentration (LOEC) at 10^{-8} M of E2, which was the minimum dose required to significantly increase reporter activity. The half-maximal effective concentrations (EC_{50}: the concentration of E2 that induces 50% reporter activity) for *vtgAs*, *chgHα*, and *chgHβ* promoters were 1.82 × 10^{-8} M, 2.75 × 10^{-8} M and 3.98 × 10^{-8} M respectively.

Transactivation of estrogen-responsive gene promoters by E2 via Erα2, Erβ1 and Erβ2

The reporter plasmid containing either one of six promoters (*chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC*, and *erα1*) and the expression plasmid containing either one of three *er* subtypes (Erα2, Erβ1, Erβ2**)** were co-transfected into HeLa cells and the transactivation of the reporter gene was induced in the presence or absence of 10^{-6} M E2 (Fig. 24).

In presence of E2, Erα2 transactivated all promoter-containing construct groups in the presence of E2 compared to the corresponding solvent control groups and non-promoter construct groups; the levels in all promoters except the *erα1* promoter showed significant increase. In presence of E2, *chgHβ* promoter showed the highest value. The reporter activities in E2 treatment groups were high in order of *chgHβ, chgHα, chgL, vtgAs*, *vtgC*, and *erα1*. The significant differences in E2 treatment groups were found among *chgHβ, chgHα, chgL, vtgAs*, and *vtgC* promoters. No significant differences was confirmed between *vtgC* and *erα1* promoters in E2 treatment groups.

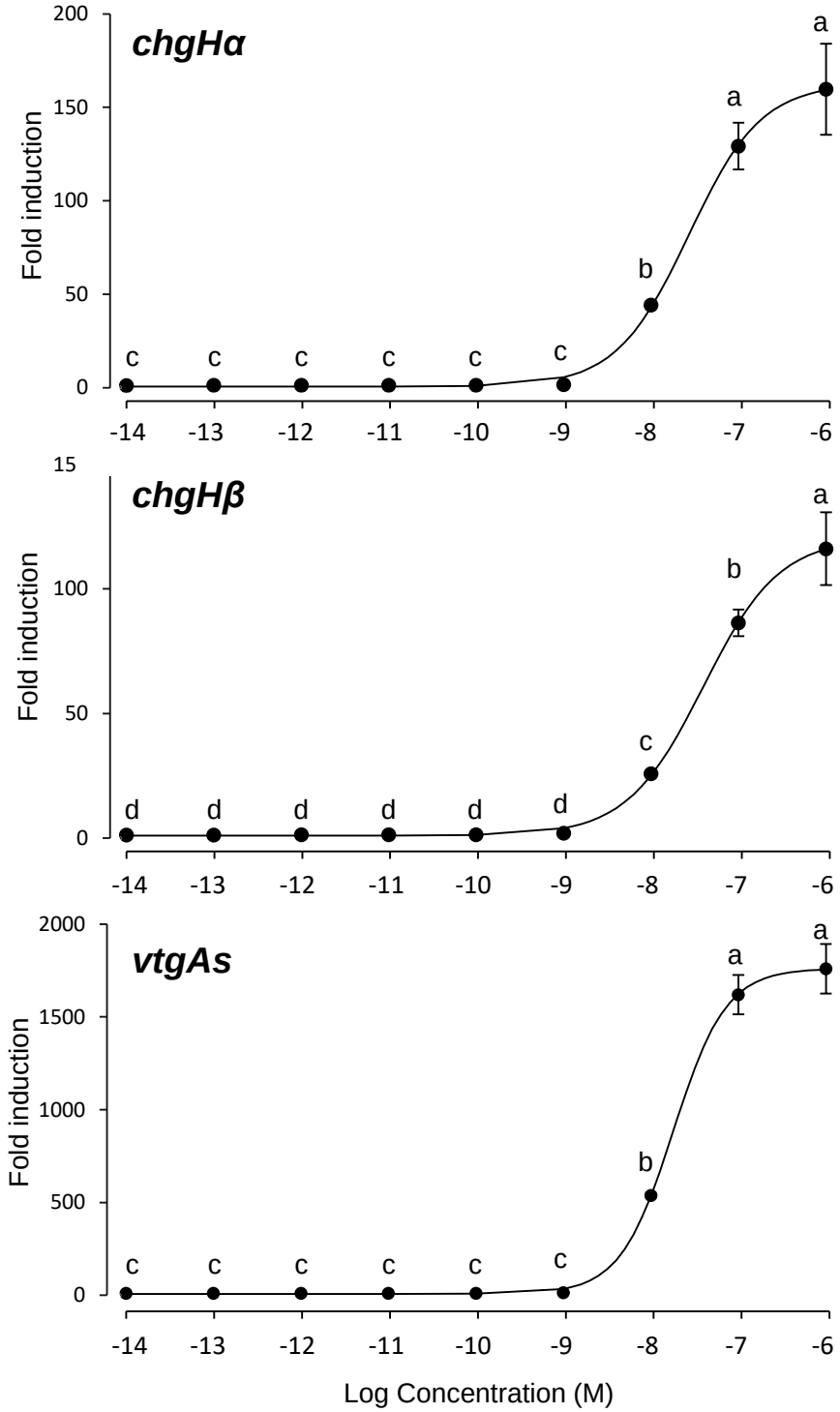

Fig. 23 Dose-response profiles in transcription activities of choriogenin Hα and Hβ (*chgHα*: top graph, *chgHβ*: middle graph) and vitellogenin As (*vtgAs*: bottom graph) promoters following estradiol-17β (E2) treatment.

Hela cells were co-transfected with two plamid costructs, estrogen receptor α1(*erα1*) in expression vector and one of the following promoters in reporter vector: choriogenin Hα and Hβ (*chgHα, chgHβ*) and vitellogenin As (*vtgAs*). Transiently transfected cells were either treated with ethanol (vehicle control) or various concentration of E2 (10^{-14}–10^{-6} M: shown as -14 ~ -6 in horizontal axis). The fold induction was initially normalized to the corresponding Renilla luciferase expression and averaged from 4 wells. The fold activation were represent with the that of vehicle control set to 1. The vertical brackets indicate standard errors. Different letters in each promoter group denote mean values that are significantly different between does ($P < 0.05$).

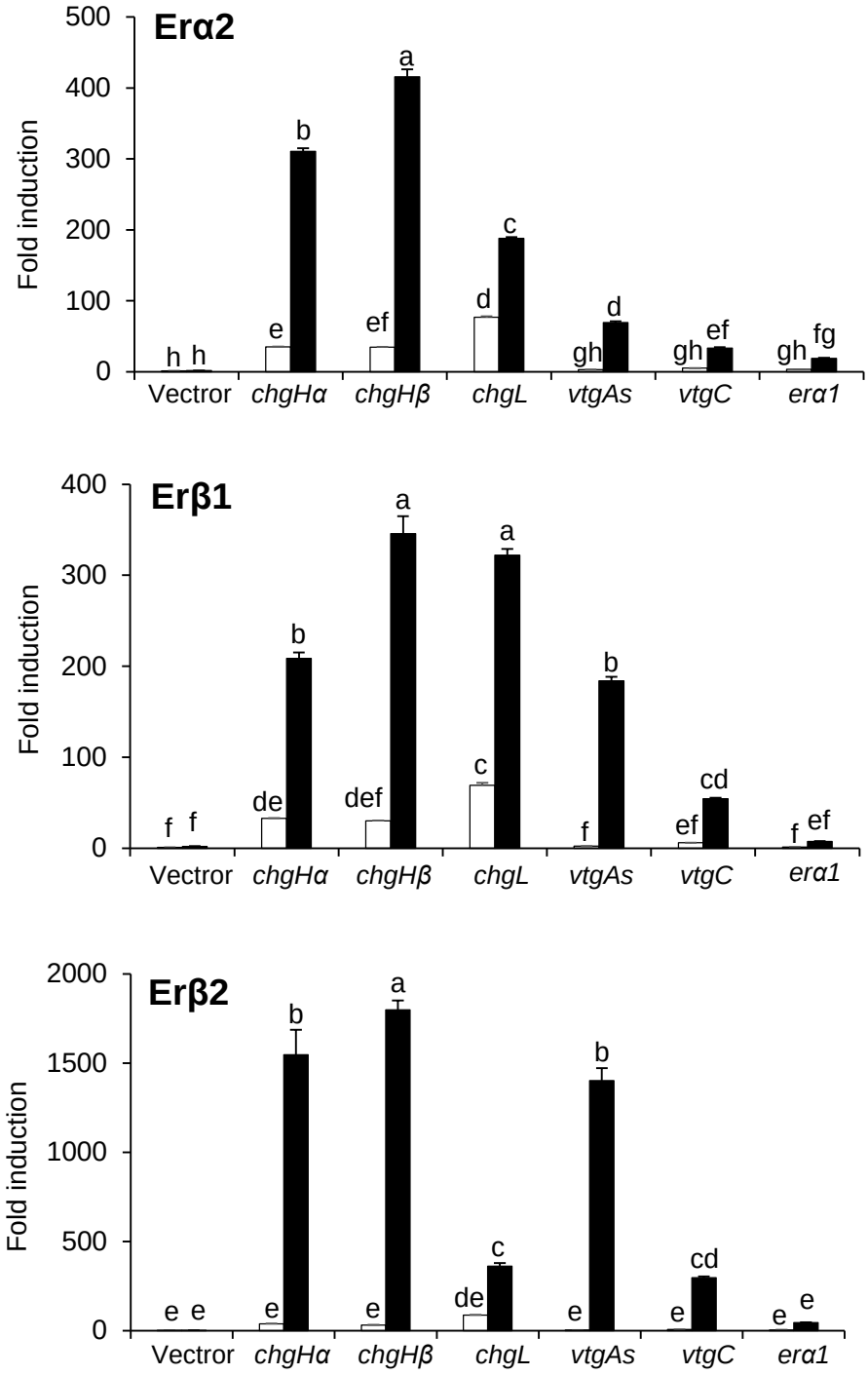

Fig. 24 Transcription activities of choriogenin (*chg*), vitellogenin (*vtg)* and estrogen receptor α1 (*erα1*) promoters mediated by Erα2 (top graph), Erβ1 (middle graph) and Erβ2 (bottom graph) in the presence or absence of estradiol-17β (E2).
Hela cells were co-transfected with two promoters, one of the following *er* in an expression vector: *erα2*, *erβ1 and erβ2* and one of the following gene promoters in a reporter vector: *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *erα1*. Reporter vector without a gene promoter (vector) were used for the negative control. The transfected cells were treated with E2 (1 μM; black columns) or ethanol (vehicle control; open columns). The fold induction was initially normalized to the corresponding Renilla luciferase expression and averaged from 4 wells. The fold activation were represent with the that of empty vector (vehicle control) set to 1. Columns indicate mean values and vertical brackets standard errors. Differential letters denote a significant differences ($P < 0.05$).

In presence of E2, Erβ1 transactivated all promoter-containing construct groups relative to the solvent control groups and non-promoter construct groups; the levels in all promoters except the *erα1* promoter showed significant increase. In presence of E2, *chgHβ* promoter showed the highest value. The reporter activities in E2 treatment groups were high in order of *chgHβ*, *chgL*, *chgHα*, *vtgAs*, *vtgC* and *erα1*. The levels in E2 treatment groups indicated significant differences between the following promoter pairs, *chgL* and *chgHα*, *vtgAs* and *vtgC*, and *vtgC* and *erα1*. The levels in E2 treatment groups did not show significant differences between the following promoter pairs, *chgHβ* and *chgL*, and *chgHα* and *vtgAs*.

In presence of E2, Erβ2 transactivated all promoter-containing construct groups compared to the solvent control groups and non-promoter construct groups, all promoters except the the *erα1* promoter showed significant increase. In presence of E2, *chgHβ* promoter showed the highest values. The reporter activities in E2 treatment groups were high in order of *chgHβ*, *chgHα*, *vtgAs*, *chgL*, *vtgC* and *erα1*. The levels in E2 treatment groups indicated significant differences between the following promoter pairs, *chgHβ* and *chgHα*, *vtgAs* and *chgL*, and *vtgC* and *erα1*. The levels in E2 treatment groups did not show significant differences between the following promoter pairs, *chgHα* and *vtgAs*, and *chgL* and *vtgC*.

Discussion

Previous report (Mushirobira et al., 2018) used the 1228 bp *vtgAs* promoter 1 and 1336 bp *vtgC* promoter. In the present study, the additional 5' promoter regions of both *vtgAs* and *vtgC* were further cloned and analyzed. The additional cloned regions of *vtgAs* promoter 1 and *vtgC* promoter do not have consensus ERE, but contain ERE-like (*vtgAs*: 4, *vtgC*: 2) and 1/2 ERE (*vtgAs*: 4, *vtgC*: 3) sequences.

Numbers of ERE, ERE-like, 1/2 ERE, AP-1 and Sp1 sites in *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *erα1* promoters were different, suggesting these promoters have differential transcriptional properties. A consensus sequence of ERE was confirmed only in *vtgAs* promoter while all promoters had ERE-like and 1/2 ERE sequences. In teleost, ERE-like and 1/2 ERE sites elicit a significant increase in estrogen-dependent synthesis of reporter protein, albeit with differences in reporter activities (Le Drean et al., 1995; Teo et al., 1998; Menuet et al., 2004). Thus, not only consensus ERE but also ERE-like and 1/2 ERE sites are likely to be responsible for the expression of *chg*, *vtg*, and *erα1* genes, which was supported by the results that *chg*, *vtg*, and *erα1* promoters of cutthroat trout were practically transactivated by all Er subtypes in the presence of E2. In addition, AP-1 and Sp1 binding sites, which interact with Er, were predicted in the promoters of *chg*, *vtg*, and *erα1*. These binding sites may also contribute to the transactivation of *chg*, *vtg*, and *erα1* genes. Thus, the functionality of each transcription factor binding site in the promoters remains unclear.

In reporter gene assays to examine the functions of teleost Er, several kinds of mammalian cell lines (CHO-K1, HepG2, HeLa, HEK-293 ,CHO, etc.) have been used

(Le Drean et al., 1995; Menuet et al., 2004; Davis et al., 2010; Lee Pow et al., 2016; Mushirobira et al., 2018). It has been shown that these cells have endogenous ERs and other estrogen-related proteins. For examples, CHO-K1 expresses functional endogenous ERβ (Thomas et al., 2003). On the other hand, HepG2 highly expresses (Transcripts Per Million; TPM: 15.3) G protein-coupled estrogen receptor 1 (gper1) mRNA, comparing to the other human cells (TPM; HeLa: 0.9, HEK-293: 1.9), according to the data in The Human Protein Atlas (https://www.proteinatlas.org/). The GPER has been shown to bind estrogens and initiate subsequent signaling cascades *in vitro*, while its function remains to be a controversial area in the field (Langer et al., 2010). Based on the levels of endogenous ERs and GPER, it is considered that CHO derivative cells (CHO and CHO-K1) and HepG2 are not suitable for the Er/ER-based reporter gene assay. HeLa and HEK-293 express low levels of ERα (TPM; HeLa: 0.1, HEK 293: 0.0), ERβ (TPM; HeLa: 0.4, HEK 293: 0.3) and GRER (TPM; HeLa: 0.9, HEK-293: 1.9), according to the data of The Human Protein Atlas. HeLa cell has been widely used for the reporter gene assay for Ers of teleosts (Menuet et al., 2004; Sumida and Saito, 2008; Lee Pow et al., 2016). Thus, HeLa cell 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 the 2000 bp promoter regions of all genes.

As described above, Erα (*erα1* in salmonids) is thought to be the major regulator of E2 signaling in the liver during vitellogenesis. Thus, the property of Erα1 for transactivation of estrogen-responsive genes is firstly analyzed using the reporter gene assay. In presence of E2, Erα1 transactivated all promoters, albeit with differences in

reporter activities. The Erα1-mediated reporter activities were different among *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *erα1* promoters, possibly due to their distinct promoter structures. In particular, *chgHα* promoter, which does not have consensus ERE, showed similar reporter activity to that of *vtgAs* which has consensus ERE. In previous report using *vtgAs* promoter of cutthroat trout, reporter activities of the consensus ERE-deleted type (deletion-type) in E2 treated groups were reduced to 77.5% of that of wild-type with the consensus ERE (Mushirobira et al., 2018). These results suggested that remaining activity (77.5%) is derived from binding sites other than consensus ERE. Therefore, the consensus ERE does not seem to be a critical factor responsible for most of the transactivation.

The reporter activities of v*tgAs* and *chgHα* promoters were similar; the reporter activity of *vtgAs* promoter was not highest among the tested promoters. Serum VtgAs were higher much than the VtgC levels during vitellogenesis and following E2 treatment in salmonids (Amano et al., 2010; Mushirobira et al., 2013). In the liver of cutthroat trout, *vtgAs* mRNA showed higher levels than *chgHα*, *chgHβ*, *chgL*, *vtgC* and *erα1* mRNAs during vitellogenesis and following E2 treatment, as described in the previous study (Mushirobira et al., 2013) and the previous Chapters. Thus, the reporter activity of *vtgAs* promoter 1 *in vitro* does not reflect the expression levels of its protein or mRNA *in vivo*. This discrepancy may be due to the multiplicity of *vtgAs* genes in salmonids. It has been reported that 20 genes and 10 pseudogenes encoding *vtgAs* are tandemly arranged in the rainbow trout genome (Trichet et al., 2000). The multiple copy number of *vtgAs* were also confirmed in other *Oncorhynchus* genus such as coho salmon (*O. kisutch*) and chinook salmon (*O. tshawytscha*) (Buisine et al., 2002). This

multiplicity rather than promoter activity of *vtgAs* is likely to contribute to the predominance of *vtgAs*/VtgAs levels *in vivo*.

Dose responses of E2 on the reporter activity were further examined for *chgHα*, *chgHβ*, and *vtgAs* promoters, because these selected promoters were significantly transactivated by Erα1 in the presence of E2 (10^{-6} M) compared to solvent control and non-promoter construct group. The LOEC and EC50 for E2 with expression of Erα1 appeared to be quite similar among *chgHα*, *chgHβ* and *vtgAs* promoters. The LOECs for E2 *ex vivo* as described in Chapter IV were different among *chg*, *vtg* and *erα1* after 24 hrs following E2 treatment (*chgHα*: 10^{-10} M, *chgHβ*: 10^{-10} M, *vtgAs*: 10^{-9}). The sensitivity of these genes in reporter gene assay with expressing Erα1 was not reflected in the sensitivity of mRNAs *ex vivo*, indicating several factors other than Erα1 is involved in the expression of these genes. Recently, possible involvement of Erβ subtypes in *vtg* expression were shown in several studies (Nelson and Habibi, 2010; Griffin et al., 2013; Lee Pow et al., 2016), indicating that all Er subtypes may regulate the estrogen responsive genes such as *chg* and *erα1*, as well as *vtg*. Therefore, the further experiments focused on the Er subtypes other than Erα1 were conducted.

To analyze the effect of Erα2, Erβ1, and Erβ2 on *chg*, *vtg,* and *erα1* promoters, reporter gene assay using Erα2, Erβ1, and Erβ2 were performed. The Erα2, Erβ1, and Erβ2 transactivated the reporter activities of all *chg* and *vtg* promoters in the presence of E2. To our knowledge, this is the first report to confirm the function of Erα2 in salmonids. The Er subtypes other than Erα1 are also involved in the expression of *chg* and *vtg*. In female rainbow trout, in addition to *erα1* with high expression levels, the other Er subtypes are expressed with low levels in the liver during vitellogenesis

(Nagler et al., 2012). In male cutthroat trout following E2 treatment in Chapter III, four Er subtypes were expressed in the liver (data not shown). In addition, *erβ1* and *erβ2* mRNAs were also expressed in primary cultured hepatocytes as described in Chapter IV. The characteristic of teleost Erβs being able to bind estradiol with higher affinity than Erα has been described in zebrafish, tilapia (*Oreochromis mossambicus*), medaka and channel catfish (*Ictalurus punctatus*) (Xia et al., 1999, 2000; Menuet et al., 2002; Davis et al., 2010; Lee Pow et al., 2016). In the present study, each Er subtype differentially transactivated *chg*s, *vtg*s and *erα1* promoters. For example, in presence of E2, the transactivation levels of the promoters mediated by Erα1 were *chgHα* ≳ *vtgAs* > *chgHβ* > *chgL* > *vtgC* >*erα1* while those mediated Erα2 were *chgHβ* > *chgHα* > *chgL* > *vtgAs* > *vtgC*. These results suggest that each Er subtype has differential transactivation properties. Therefore, the characteristics and/or the amount of each Er subtype possibly reflect the *chg* and *vtg* expression levels *in vivo* and *ex vivo*. These complex mechanisms appeared to cause the difference in results between *ex vivo* and *in vitro* (the sensitivity of genes to E2 etc.). However, the protein levels of each Er subtype in the hepatocytes remain unclear. Thus, further studies that analyze each Er subtype at the protein level, are required to confirm the roles of each Er subtype on the expression of *chg*, *vtg* and *erα1* genes.

The promoters of *chgL* and *vtgC* for cutthroat trout were transactivated by Erα1 in the presence of E2 compared to the solvent controls and the non-promoter construct groups, but the increases were not significant. These promoters seem to be weakly transactivated by E2-Erα1 complexes, unlike *chgHα, chgHβ* and *vtgAs* promoters. In the analysis of shorter *vtgC* promoter for cutthroat trout than the promoter used in the

present study (Mushirobira et al., 2018), the fold activation levels of *vtgC* promoter in E2 treatment groups were significantly higher (13 fold) than those in the solvent control group cells under expression of Erα1 in CHO-K1 cells. In the present study, the fold activation levels of *vtgC* promoter were 42 fold. In the previous report (Mushirobira et al., 2018), the multiple comparison test (Tukey-Kramer HSD test) were performed among eight groups (E2 treated and untreated groups of non-promoter construct, *vtgAs* promoter 1, *vtg* promoter 2 and *vtgC* promoter). Meanwhile, in the present study, the analysis applied to 14 groups (E2 treated and untreated groups of non-promoter construct, *chgHα, chgHβ, chgL, vtgAs*, *vtgC* and *erα1* promoter). Possibly, the differences of statistic results between the two experiments may be due to the sample set that applied to the multiple comparison test.

In presence of E2, *erα1* promoter was transactivated by all *er* subtypes in the presence of E2 compared to the solvent controls (Erα1: 10 Fold, Erα2: 5.7 Fold, Erβ1: 5.8 Fold, Erβ2: 13 Fold), but the increases were not significant. The transactivation properties of *erα1* promoters have been reported in rainbow trout and zebrafish (Le Drean et al., 1995; Menuet et al., 2004). In the analysis of rainbow trout *erα1* promoter in CHO cells under expression of Erα1, the 2.5 kb *erα1* promoter that contains the 5'-flanking region, exon1 and partial intron1, the reporter activities in E2-treated promoter containing groups are 2.5-fold higher than those in solvent control groups. Also, in *erα* promoter of zebrafish, E2 treatment increases reporter activities by up to 10 times under expression of either Erα1 or Erβ1 or Erβ2 in CHO, HeLa, and HepG2 cells. Thus, *erα1* promoter of cutthroat trout does not seem to show significant increase because the fold activation levels of teleost *erα* were generally quite low.

It has been considered that various transcriptional factors are involved in the transactivation of estrogen responsive gene by Er (Björnström and Sjöberg, 2005). In addition, it has been shown that the interactions of Er, co-activator and/or co-repressor are important on the gene regulation by E2/Er (Björnström and Sjöberg, 2005). These co-activator and co-repressor are thought to be involved in tissue- and/or cell-type specific regulation of gene expression. Furthermore, other endocrine factors as described in Chapter III are also thought to be involved in the expression of *chg*s, *vtg*s and *erα1* mRNAs. Therefore, future works should examine the role of these factors in the expression of *chg*, *vtg* and *erα1* mRNAs.

In conclusion, the present study has shown the transactivation properties of all four Er subtypes for *chg*, *vtg*, and *erα1* promoters within the single species. Each Er subtype transactivated *chg*, *vtg*, and *erα1* promoters differentially. Thus, the characteristics of each Er subtype and/or the amount of each Er subtype are likely result in the differencial *chg*, *vtg* and *erα1* expressions *in vivo* and *ex vivo*. The *chg*, *vtg*, and *erα1* expressions are possibly regulated by complex interaction of Er subtypes and their promoters.

Chapter VI. Summary and conclusion

Estrogen, estradiol-17β (E2) in most cases, induces the expression of precursor proteins of chorion (choriogenin, Chg) and yolk proteins (vitellogenin, Vtg) in the liver of many teleosts including salmonids. These E2 actions are mediated by nuclear estrogen receptors (Er). The Er is a transcriptional factor that belongs to a nuclear receptor superfamily. After E2 binds to Er in the target cell, the complex of E2 and Er forms a homodimer that binds to estrogen responsive elements (ERE), present in the promoter regions of targeted gene. Then, expression of the target gene is induced. Multiple Chg and Vtg subtypes (Chg: ChgHα, ChgHβ, ChgL; Vtg: VtgAs, VtgC), as well as four Er subtypes (Erα1, Erα2, Erβ1, Erβ2), have been identified in salmonids. Among Ers, Erα1 subtype alone appeared to be induced by E2 in the liver. However, differences in the transcriptional response to E2 stimulation among these hepatic E2-responsive genes remain unclear.

This study aimed at the clarification of molecular mechanisms underlying regulation of multiple hepatic estrogen-responsive genes. Thus, the transcriptional responses of the six hepatic estrogen-responsive genes, *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *erα1*, to E2 were examined by *in vivo*, *ex vivo* and *in vitro* experiments using cutthroat trout (*Oncorhynchus clarki*), a model salmonid species for basic research on teleost ovarian follicle growth in our laboratory.

1. Changes in *chgs*, *vtgs* and *erα1* mRNAs in the liver during a reproductive cycle of female cutthroat trout

The cDNAs of *chgHα*, *chgHβ* and *chgL* were isolated and sequenced from liver of female fish. The sequences *of chgHα, chgHβ and chgL* contained open reading frames encoded 532, 517 and 441 amino acids, respectively. The encoded cutthroat trout ChgHα, ChgHβ and ChgL proteins had ZP domains and consensus furin cleavage sites (CFCS) that are typical of ZP protein family. The similarities of ChgHα and ChgHβ and ChgL between cutthroat trout and rainbow trout were 93.3%, 97.7% and 99.3%, respectively.

Levels of hepatic *chg* and *erα1* mRNA during a reproductive cycle of female fish were quantified by quantitative real-time reverse transcription PCR (qPCR). Levels of hepatic *chg* mRNAs increased with the progress of oocyte growth, as well as the levels of serum E2 and hepatic *vtg* mRNAs. In the ovulated fish, *chg* mRNAs remained at high levels while the levels of serum E2 and hepatic *vtg* mRNAs decreased. Hepatic *erα1* mRNA levels exhibited a peak in August (at the beginning of vitellogenesis) before levels of E2, *chg* and *vtg* mRNAs started to increase.

The levels of *chg* mRNAs were highly correlated with those of serum E2 levels (*chgHα*: R^2 = 0.78; *chgHβ*: R^2 = 0.92; *chgL*: R^2 = 0.75) between January (perinuclear stage) and December (vitellogenic stage), like *vtg* mRNAs were (*vtgAs*: R^2 = 0.91, *vtgC*: R^2 = 0.83). These results suggest that *chg* and *vtg* mRNAs are expressed under strong E2 regulation during the periods from perinuclear stage to vitellogenic stage. Unlike *chg*s and *vtg*s mRNAs, *erα1* mRNA did not show any correlation with serum E2 levels.

Collectively, mRNAs of *chg*s, *vtg*s and *er α 1* were different from each other in response patterns to E2 during reproductive cycle of female fish, suggesting that expressions of these genes are potentially regulated through E2 stimulation by different

mechanisms.

2. Transcriptional responses of *chg*s, *vtg*s and *erα1* to E2 administration in the liver of cutthroat trout

Mature male and immature fish were administrated a single intraperitoneal injection of E2 at one of three doses (low: 50 μg/kg body weight; middle: 500 μg/kg body weight; and high: 5000 μg/kg). At 2 and 5 days post injections (dpi), hepatic *chg*, and *erα1* mRNA levels were quantified by qPCR.

At both 2 dpi and 5 dpi, mRNA levels of three *chg* subtypes in the E2 treatment groups tended to increase in dose-dependent manners, like *vtg* mRNAs do. At 2 dpi and 5 dpi, the significant increase in *chgHα* mRNA levels occurred at high-dose groups compared to control groups. The *chgHβ* and *chgL* mRNA in middle- and high-dose at 2 dpi, as well as in high-dose at 5 dpi, showed significant increases versus their corresponding control groups. From 2 dpi to 5 dpi, mRNA levels of three *chg* subtypes significantly increased in high-dose groups, like *vtgAs* mRNAs do.

At 2 dpi, levels of *erα1* mRNA exhibited the highest levels in the middle-dose group at 2 dpi, while those in the high-dose group increased, but were not significantly higher than those in the control group. At 5 dpi, *erα1* mRNA levels in the both of middle and high-dose groups were significantly greater than those in the control groups. From 2 dpi to 5 dpi, *erα1* mRNA levels did not show no significant change, as well as *vtgC* mRNA.

Collectively, expression of mRNAs of *chg*s, *vtg*s and *er α 1* were different from each other in response to E2 *in vivo*, suggesting that their expression are under different E2 regulation. In particular, *erα1* mRNA reach high levels in shorter period in comparison

with the results obtained for *chg* and *vtgAs* mRNA expressions. The above mentioned characteristics of *erα1* mRNA expression to exogenous E2 stimulation possibly predict the increase of Erα1 protein to be responsible for the *chg* and *vtg* gene expressions prior to or at early period of the vitellogenesis.

3. Transcriptional responses of *chg*s, *vtg*s and *erα1* to E2 in primary cultured hepatocytes of cutthroat trout

At first, primary cultured hepatocytes prepared from male trout were continuously treated with 10^{-6} M E2 by daily replacement of the culture medium. At 24 and 72 hrs post initiation (hpi) of the treatments, mRNA levels of all *chg*, *vtg* and *er* subtypes were quantified by qPCR.

In E2 treatment groups, *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *erα1* mRNA levels were significantly higher than those in control groups at 24 hpi and 72 hpi. These mRNAs levels kept high levels or significantly increased from 24 hpi to 72 hpi. Meanwhile, *erα2* mRNA was undetectable and both *erβ1* and *erβ2* mRNAs did not show any estrogen responses at both sampling points.

In the next, effects of various E2 dose on expression of these estrogen-responsive genes were examined. Primary cultured hepatocytes prepared from male trout were treated with or without E2 (10^{-11} ~ 10^{-6} M) for different periods (24 and 72 hrs), and the culture media were not replaced during the whole experimental period. At 24 hpi and 72 hpi after treatments, *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *erα1* mRNA levels were quantified by qPCR.

The mRNA levels of *chg*s and *vtg*s at 24 hpi and 72 hpi showed dose-dependent

increases. The lowest doses inducing a significant increase in the expression levels (LOECs) of *chg* and *vtg* mRNAs for E2 were determined as *chgHβ* (10^{-10} M) < *chgHα*, *chgL*, *vtgAs* (10^{-9} M) < *vtgC* (10^{-8} M). From 24 hpi to 72 hpi, *chgHα*, *chgHβ*, *chgL*, *vtgAs* and *vtgC* mRNA levels in high-dose-E2 treatment groups increased. Meanwhile, *erα1* mRNA levels increased in a dose-dependent manner at 24 hpi and the LOEC was 10^{-9} M. However, *erα1* mRNA levels in high-dose groups (10^{-9} ~ 10^{-6} M E2) decreased from 24 hrs to 72 hpi; the levels in all E2 treatment groups did not show any significant increase compared to the control groups at 72 hpi.

Collectively, these results indicated that hepatic expressions of *chg*, *vtg* and *erα1* mRNAs are under strict E2 regulation. Hepatic expressions of these genes appeared to be different in response to E2 regarding the sensitivities and the durational changes, suggesting that these genes are differentially regulated by E2.

4. Molecular cloning and characterization of *chg*s, *vtg*s and *erα1* gene promoters in cutthroat trout

The promoter regions of *chg* and *erα1* genes were cloned from cutthroat trout genome. The previous reported promoter regions of *vtgAs* promoter 1 and *vtgC* were also obtained with the extended lengths and analyzed in this study.

The *chgHα* DNA promoter sequence consisted of 2851 bp located upstream of the translation initiation site. No complete palindrome ERE was identified in the analyzed promoter sequences. One ERE-like palindrome sequences differing from the consensus ERE and six ERE half sites (1/2 ERE) were predicted in *chgHα* promoters. The binding sites for activator proteins-1 (AP-1) and specificity protein-1 (Sp1), which

interact with Er, were found in the promoters, as well as the TATA box and CAAT box.

The cloned *chgHβ* DNA promoter sequence was 2270 bp. No complete palindrome ERE was found in the promoter sequences. Two ERE-like and eight 1/2 ERE sequences were predicted in the promoter. In addition to the binding sites for AP-1, TATA box and CAAT box were found in the promoters.

The *chgL* DNA promoter sequence consisted of 3636 bp. No complete palindrome ERE was identified in the analyzed promoter sequences. One ERE-like and seven 1/2 ERE sequences were predicted in the promoters. The binding sites for AP-1 and Sp1 were found in the promoters, as well as TATA box and CAAT box.

The 2872 bp *vtgAs* DNA promoter were cloned. The consensus palindrome canonical ERE was identified in *vtgAs* promoter. Eight ERE-like and six 1/2 ERE sequences were predicted in the promoter. TATA box and CAAT box and the binding sites for AP-1 and Sp1 were found in the promoters.

The *vtgC* promoter sequence was 3025 bp. No complete palindrome ERE was identified in the analyzed promoter sequences. Six ERE-like sequences and eight 1/2 ERE were predicted in the promoter. TATA box and the binding sites for AP-1 and Sp1 were found in the promoters. The *erα1* DNA promoter sequence consisted of 6155 bp. No complete palindrome ERE was identified in the analyzed promoter sequences. Two ERE-like sequences and eight 1/2 ERE were predicted in the promoter. The binding sites for AP-1 and Sp1 were found in the promoters, as well as TATA box and CAAT box.

The reporter plasmid containing either one of *chg*s, *vtg*s and *erα1* promoters (2 kb in size) and the expression plasmid containing *erα1* were co-transfected into HeLa cells.

The transactivation levels of the reporter gene were quantified in presence or absence of E2.

The luciferase reporter activity in all promoter-containing construct groups was higher in presence of E2 (10^{-6} M) than that of solvent control group (non-E2 controls) and non-promoter construct group. Among promoters, the reporter activities in *chgHα*, *chgHβ* and *vtgAs* promoters showed the significant increase in comparison with the activities in the non-E2 and the non-promoter controls. The reporter activities in E2 treated groups were determined as *chgHα* ≈ *vtgAs* > *chgHβ* >*chgL* ≈*vtgC* ≈ *erα1*. Further treatments of cells with E2 (10^{-14} M ~ 10^{-5} M) indicated that the half-maximal concentration effective concentrations (EC_{50}: the concentration of E2 that induces 50% reporter activity) for *vtgAs*, *chgHα* and *chgHβ* promoters were quite similar (*vtgAs*: 1.82×10^{-8} M, *chgHα*: 2.75×10^{-8} M, *chgHβ*: 3.98×10^{-8} M). In addition, the LOECs of *vtgAs*, *chgHα* and *chgHβ* promoter for E2 were identical (10^{-8} M).

The reporter plasmid containing either one of *chg*, *vtg* or *erα1* promoters and the expression plasmid containing either one of *erα2*, *erβ1* or *erβ2* were co-transfected into HeLa cells. The cells were incubated in presence or absence of E2.

In presence of E2 (10^{-6} M), Erα2, Erβ1 and Erβ2 significantly transactivated all promoter-containing construct groups compared to solvent control groups (non-E2 controls) and non-promoter construct groups. The reporter activities in E2 treated groups were determined as *chgHβ* > *chgHα* > *chgL* > *vtgAs* > *vtgC* in *erα2*, *chgHβ* ≈ *chgL* > *chgHα* ≈ *vtgAs* > *vtgC* in *erβ1* and *chgHβ* > *chgHα* ≈ *vtgAs* > *chgL* ≈ *vtgC* in *erβ2*.

Collectively, these results indicated that all Er subtypes differentially transactivated

the *chg*s, *vtg*s and *erα1* promoters in the presence of E2 *in vitro*, suggesting that the differences in the transactivation functions of Er subtypes affect *chg*, *vtg* and *erα1* expressions *in vivo* and *ex vivo*.

Conclusion

The conclusions of the molecular mechanisms underlying regulation of hepatic estrogen-responsive genes in cutthroat trout emerging from the present study are expressed diagrammatically in Fig. 25. Expression of *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *erα1* mRNAs exhibit positive response to E2 in hepatocytes. Therefore, hepatic expressions of these estrogen-responsive genes are expected to be up-regulated associating with gradual increase in circulating E2 levels from the beginning of and through the vitellogenic period. The up-regulation of these genes by E2 are mediated by four Er subtypes (Erα1, Erα2, Erβ1, Erβ2) in hepatocytes. Among the subtypes, Erα1 strongly transactivates *chgHα*, *chgHβ* and *vtgAs* genes, but weakly activates *chgL*, *vtgC* and *erα1* genes. The responses of these genes to E2 are regulated by the amount of each Er subtypes and/or characteristics, such as binding properties with E2 and interaction with other transcription factors. Thus, these genes show differential responses to E2 *in vivo* and *ex vivo*. Of the Er subtypes, the only Erα1 has the ability to positively response to E2. Therefore, Erα1 protein seems to be a major regulator of *chg*, *vtg* and *erα1* gene expression, contributing to their active expression in hepatocytes of vitellogeninc female trout, while Erα2, Erβ1 and Erβ2 are minor Ers. However, the presented model has still not been completed yet, because the mechanisms underlying the regulation of *chg*s, *vtg*s and *erα1* genes include unclear but important parts as follows: 1) protein

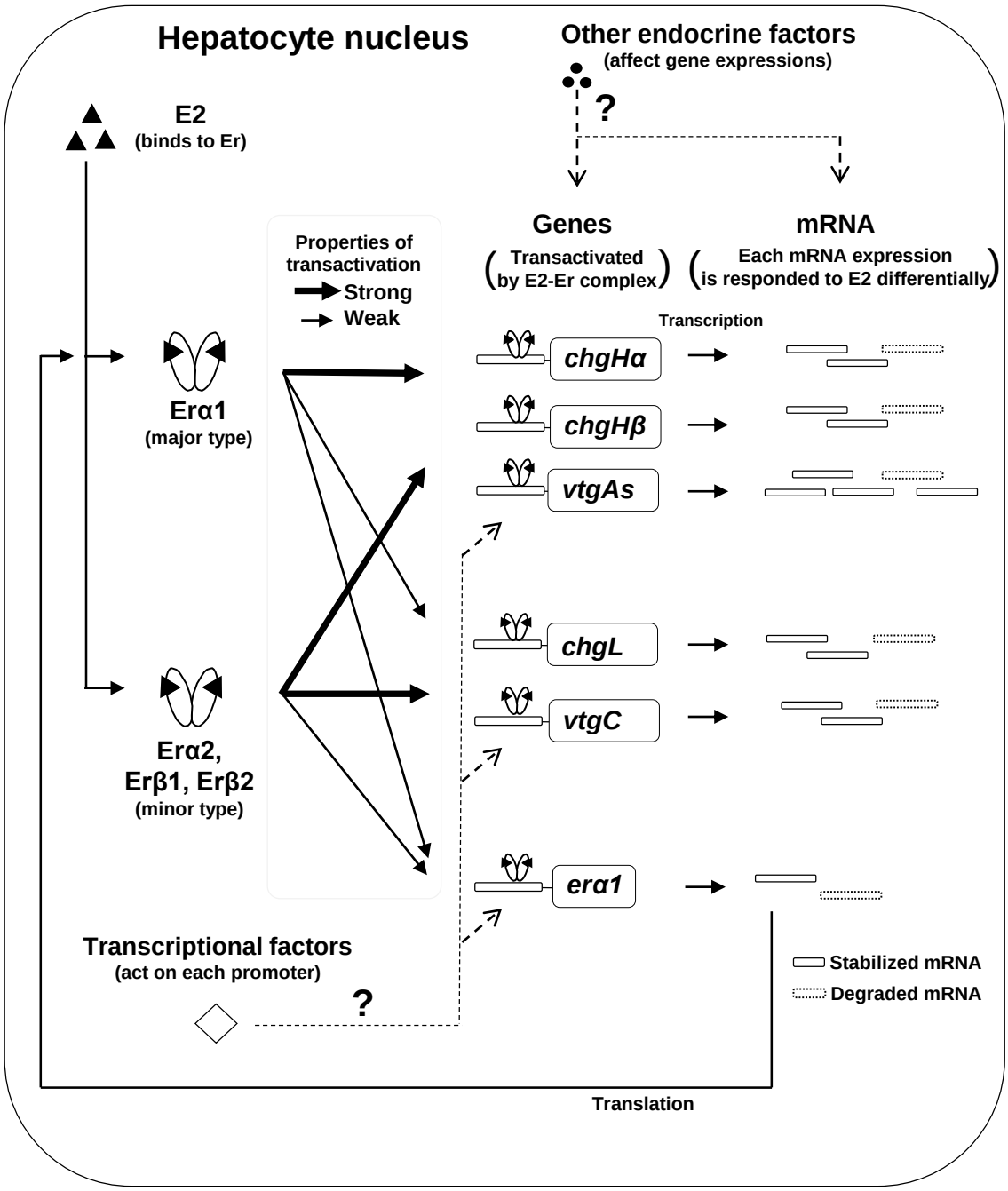

Fig. 25 A scheme summarizing the molecular mechanisms underlying regulation of hepatic estrogen responsive genes in cutthroat trout.
E2: estradiol-17β, Er: estrogen receptor, *chg*: choriogenin, *vtg:* vitellogenin.

levels of Er subtypes in hepatocytes, 2) effects of E2 on the rates of synthesis and clearance for *chg*s, *vtg*s and *erα1* mRNAs, 3) the roles of other endocrine factors and transcription factors in the expression of *chg*, *vtg* and *erα1* genes.

Finally, the present study provided novel knowledge on the molecular mechanisms underlying regulation of hepatic estrogen-responsive genes, using a model salmonid species, the cutthroat trout. To our knowledge, this study is a first report that revealed differences in hepatic expressions of multiple estrogen-responsive genes *in vivo* and *ex vivo*, and in transcriptional activities of their promoters, as well as differential transactivation properties of Er subtypes *in vitro*. In addition, it should be emphasized that these results were obtained from a single species for the first time. Future studies toward clarifying subjects with respect to the regulation of hepatic estrogen-responsive genes, will provide more clear insights underlying the molecular mechanisms on the formation of chorion and yolk; such mechanisms appear to directly relate with qualities of eggs and offsprings, and thus important to improve the egg production and viability of progeny for efficient aquaculture.

Acknowledgements

First, I wish to express special gratitude to Associate Professor Takashi Todo, Division of Marine Life Science, Faculty of Fisheries Sciences, Hokkaido University, for his invaluable guidance throughout the present study, technical supports, critical reading of the manuscript and constant encouragements throughout the present study

I would like to express my gratitude to Professor Shinji Adachi, Professor Yasuaki Takagi, Emeritus Professor Akihiko Hara and Associate Professor Naoshi Hiramatsu, Division of Marine Life Science, Faculty of Fisheries Sciences, Hokkaido University, for their invaluable guidance throughout the present study and critical reading of the manuscript.

I wish to express my thanks to Associate Professors Munetaka Shimizu, Shigeho Ijiri and Kazuhiro Ura, Division of Marine Life Science, Faculty of Fisheries Sciences, Hokkaido University, for their important and technical suggestions.

I am also grateful to Professor Etsuro Yamaha, Technical staffs Shizuo Kimura and Eisuke Takahashi, Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, for the maintenance of fish during this study.

Thanks are also to Dr. Osamu Nishimiya, South Ehime Fisheries Research Center, Ehime University and Dr. Yuji Mushirobira, Institute for East China Sea Research, Organization for Marine Science and Technology, Nagasaki University for the technical support and their previous efforts in the present study.

I also want to thank Mrs. Kaori Hiramatsu, Laboratory of Aquaculture Biology, Division Marine Life Science, Faculty of the Fisheries Sciences, Hokkaido University for her help and encouragement.

Appreciates are further extended to all my friends, graduate and undergraduate students of Fish Reproductive Physiology Laboratory from 2013 to 2018, Hokkaido University, for their encouragements, technical suggestions, sampling and friendship.

Especially, I appreciate to elder students, Dr. Meiqin Wu and Dr, Hiroko Mizuta, for their previous efforts, such as technical support and development, as well as maintenance of experimental laboratory and fish, that contribute largely for me to conduct and prepare this thesis.

Finally, I appreciate to my private friends and family for their constant support and encouragement throughout my life.

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