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

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

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

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

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

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

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

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

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

Abstract (365 words)

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

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

1. Introduction

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

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

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

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

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

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

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

The objective of the present study was *i*) to reveal the properties of estrogen-induced transcription of nine hepatic genes (i.e., *chg*: *chgHα*, *chgHβ*, *chgL*; *vtg*: *vtgAs*, *vtgC*; *esr*: *esr1a*, *esr1b*, *esr2a*, *esr2b*), and *ii*) to evaluate the involvement of each of the four Esr subtypes in the transcriptional regulation of these estrogen-responsive genes, using our model salmonid, the cutthroat trout. To achieve this objective, the present study utilized

primary hepatocyte culture to observe and compare the estrogen-induced transcriptional properties of the nine genes. In addition, the functional property of four Esrs in driving the expression of their putative target (*chg*, *vtg* and *esr*) genes was investigated by reporter gene assays using reporter vectors containing promoter regions of the target genes.

2. Materials and methods

2.1. Experimental fish and tissue sampling

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

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

A two-year-old female cutthroat trout was used in order to clone the promoters of each gene (section 2.3). Blood was collected and immediately mixed with ethylenediaminetetraacetic acid (EDTA) to a final concentration of 17 mM. This whole

148 blood sample was stored at −80°C until used as a source for genomic DNA extraction.

149

150 *2.2. Molecular cloning of cutthroat trout esr1b, esr2a and esr2b*

151 Total RNA was extracted from the liver samples with ISOGEN (Nippon Gene,
152 Tokyo, Japan) according to the manufacturer's instructions. Concentration of total RNA
153 was measured with NanoDrop ND-1000 (Thermo Fisher Scientific). One µg total RNA
154 was reverse-transcribed by PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio,
155 Shiga, Japan), according to the manufacturer's instructions. The resulting cDNA
156 templates were stored at −30°C. Primer sets (Table 1) were designed using sequence data
157 from rainbow trout *esr1b* (NM_001124558), *esr2a* (NM_001124753) and *esr2b*
158 (NM_001124570.1) to amplify the respective open reading frames. PCR for each *esr*
159 subtype was performed using PrimeSTAR® Max DNA Polymerase (Takara Bio) in a
160 volume of 10 µl, which contained 1 µl 1st-strand cDNA, 5 µl PrimeSTAR® Max premix,
161 1 µl of each 1 µM forward and reverse primer and 2 µl nuclease-free water. The PCR
162 amplification was carried out using the following thermal parameters: 40 (*esr1b* and
163 *esr2a*) or 35 (*esr2b*) cycles at 98°C for 10 s, 55°C for 5 s and 72°C for 3 min. The PCR
164 products were separated by electrophoresis on 1% agarose gels, excised from the gels,
165 and purified by GENECLEAN Turbo Kit (MP-Biochemicals, Santa Ana, CA, USA)
166 according to the kit manual. The PCR products were subjected to an A-tailing reaction
167 (Knoche and Kephart, 1999) followed by ligation into pGEM-T Easy Vector (Promega,
168 Madison, WI, USA) according to the manufacturer's instruction. The ligated products
169 were transformed into XL1-Blue competent cells (Stratagene, La Jolla, CA, USA).
170 Recombinant clones were cultured overnight at 37°C on an agar plate containing
171 ampicillin and tetracycline, followed by selection of colonies of interest by blue-white

screening. Selected clones were grown in culture medium, and then used to extract and purify plasmid DNA by Wizard Plus SV Minipreps DNA Purification System (Promega). Purified plasmid DNAs were sequenced using BigDye terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and a 3130xl Genetic Analyzer (Thermo Fisher Scientific) according to the manufacturer's protocol.

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

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

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

196 GCF_002163495.1). PCR was carried out as follows: 5 µl of PrimeSTAR Max Premix, 1
197 µl of each 2 µM forward and reverse primer, and 0.2 µl of genomic DNA template were
198 mixed and DNA amplified at 35 cycles of 98°C for 10 s, 55°C for 10 s and 72°C for 3
199 min.
200 The PCR products were ligated into cloning vectors (pGEM-T Easy Vector),
201 transformed into XL1-Blue competent cells, and sequenced as described above.
202
203 *2.4. Computational search for putative transcription factor binding sites*
204 Two online algorithms (ConSite; http://asp.ii.uib.no:8090/cgi-bin/CONSITE/ and
205 NUBIScan; http://www.nubiscan.unibas.ch/) were used for prediction of transcription
206 factor binding sites, including both basal promoter elements and putative EREs. The
207 specifics of ConSite and NUBIScan, were described previously by Sandelin et al. (2004)
208 and Podvinec et al. (2002), respectively.
209
210 *2.5. Primary culture of hepatocytes*
211 For Experiments 1 and 2 described below, hepatocytes were isolated from one male
212 cutthroat trout for each experiment, using a two-step collagenase perfusion technique as
213 described previously (Klaunig et al., 1985) with modifications; briefly, trout (body weight,
214 BL: 420 ~ 480 g; total length, TL: 314 ~ 410 mm) were anesthetized in 2-phenoxyethanol).
215 The liver was perfused with 50 ml of Ca-free modified Hanks solution (137 mM NaCl,
216 5.4 mM KCl, 0.5 mM NaH_2PO_4, 0.42 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 5 mM glucose,
217 0.5 mM EGTA, 10 mM HEPES, pH 7.4) to remove the blood from the liver. The liver
218 was then perfused with 100 ml of the same solution without EGTA and glucose and with
219 added $CaCl_2$ (5 mM), 0.05% collagenase (Wako, Tokyo, Japan) and 0.005% trypsin

220 inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Perfusion was performed at room
221 temperature using a peristaltic pump (Iwaki, Tokyo, Japan) at a flow rate of 10 ml/min.
222 After perfusion, the hepatocytes were dispersed in ice-cold L-15 medium (Thermo Fisher
223 Scientific) supplemented with 10 mM HEPES (pH 7.4), 1% of an antibiotic + antimycotic
224 solution (final concentration: 100 units/ml penicillin, 100 µg/ml streptomycin sulfate and
225 250 ng/ml amphotericin B; Wako) and 5% fetal bovine serum (FBS, Thermo Fisher
226 Scientific). The cell suspension was filtered through a cell strainer (Ikemoto Rika, Tokyo,
227 Japan) and the filtrate centrifuged at 100 g for 90 s at 4°C. The pellet was resuspended in
228 fresh medium and re-spun at 100 g for 2 min at 4°C; altogether, the cell pellet was washed
229 three times. Cell viability was about 95% as determined by the trypan blue exclusion test.
230 Cells were plated at a density of 3×10^5 cells/well on 24-well Falcon Primaria Multiwell
231 plates (Corning, New York, NY, USA) that were coated with Matrigel (basement
232 membrane matrix, Corning) as described by Schreer et al. (2005). Cells were cultured at
233 15°C in 0.5 ml of L-15 supplemented with 10 mM HEPES (pH 7.4), 1% antibiotic +
234 antimycotic solution, 5% FBS and 10 µg/ml bovine insulin (Sigma-Aldrich). The cultured
235 cells were settled for 24 h in order to adhere to the culture plate.
236
237 *2.6. Hormone treatment*
238 *2.6.1. Experiment 1: effects of continuous treatment with E2*
239 Cells in each well were washed once with 0.5 ml of FBS- and insulin-free L-15.
240 Then, cells were treated ('initiation') with 1 µM E2 or with solvent (ethanol) only; the
241 amount of ethanol in the medium did not exceed 0.1%. Half the volume (0.25 ml) of the
242 culture medium was changed every 24 h. At 0, 24 and 72 h post initiation (hpi), the
243 medium was removed and 200 µl ISOGEN was added to each well. Following 5 min

244 incubation, the samples were stored at −80°C until use. All incubations were run in
245 triplicate.
246
247 *2.6.2. Experiment 2: effects of various doses of E2*
248 Cells were treated with various concentrations of E2 (10^{-11} ~ 10^{-6} M). Treatments
249 and sampling were performed as described in Experiment 1 with the following
250 modifications; culture medium was not changed after treatment, and all incubations were
251 replicated 4 times.
252
253 *2.7. Quantitative real-time PCR (qPCR)*
254 Primers used in qPCR for *chg*s, *vtg*s, *esr1a* and elongation factor 1-α (*ef1-α*;
255 reference gene) were designed in our previous studies (Luo et al., 2013; Mushirobira et
256 al., 2013; Nagata et al., 2018). Primer sets for *esr1b*, *esr2a* and *esr2b* (Table 1) were
257 designed in Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) to cover
258 intron/exon boundaries which were predicted from the rainbow trout genome database
259 (Accession No. GCA_900005705.1). Predicted amplicon sizes for each gene were as
260 follows: *esr1b*: 141 bp, *esr2a*: 111 bp and *esr2b*: 118 bp.
261 Total RNA was extracted from hepatocytes in ISOGEN following manufacturer's
262 instructions. The RNA (200 ng) was reverse-transcribed by SuperScript® IV VILO cDNA
263 Synthesis kit (Thermo Fisher Scientific). Some aliquots of total RNA from all E2-treated
264 groups were pooled, reverse-transcribed and used as an inter-assay control (IAC) to
265 normalize between plates.
266 All qPCR reactions were performed as described in Nagata et al. (2018) using
267 FastStart Universal SYBR Green Master (Rox) (Roche, Basel, Switzerland) and

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

2.8. Reporter gene assays

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

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

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

2.9. Statistics

All data analyses were carried out using JMP Pro 14 Software program (SAS Institute, Cary, NC, USA). Data were analyzed by two-way ANOVA with interaction, using time × dose as factors for the primary hepatocyte cultures, and promoter type × dose for the reporter gene assay. When significant effects were found ($P < 0.05$),

comparisons between groups were conducted by Student's t-test, and multiple comparisons were done with Tukey Kramer HSD test. The difference between groups was considered significant at $P < 0.05$. All the results are expressed as means ± SE. For statistical analyses, samples with values below the detection limit (100 copies/reaction mix) in qPCR were assigned this minimum detectable level.

3. Results

3.1. Molecular cloning of esr1b, esr2a and esr2b

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

3.2. Hormone Treatment

3.2.1. Experiment 1: effects of continuous treatment with E2

In *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *esr1a* mRNA levels, significant interaction effects of time × dose were observed ($P < 0.001$), as well as the significant main effects of time ($P < 0.001$) and dose ($P < 0.001$), respectively. In both *esr2* subtypes, interaction

effects of time × dose were not significant while significant main effect of time was observed ($P < 0.01$); main effect of E2 was significant only in *esr2b* ($P < 0.01$).

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

3.2.2. Experiment 2: effects of various doses of E2

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

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

366 levels in cultures exposed to 10^{-9} ~ 10^{-6} M E2 significantly decreased from 24 hpi to 72
367 hpi; moreover, significant differences between the E2-treated and the control group were
368 found only in high concentration groups (10^{-7}, 10^{-6} M E2) at 72 hpi, and the LOEC was
369 10^{-7} M..
370
371 *3.3. Molecular cloning and sequence analysis of cutthroat trout chgHα, chgHβ, chgL,*
372 *vtgAs, vtgC and esr1a promoters*
373 The *chgHa* DNA promoter sequence consisted of 2851 bp located upstream of the
374 translation initiation site and 113 bp in the transcribed region (Fig. A. 1, Accession No.
375 LC577091). A complete palindromic ERE (GGTCAnnnTGACC) was not identified in
376 the analyzed promoter sequences. One ERE-like palindrome sequence differing from the
377 consensus ERE and six ERE half sites (1/2 ERE: GGTCA or TGACC) were predicted in
378 *chgHα* at the following positions: ERE-like: -198 to -184 (AGATCTatgTGACCT);
379 1/2ERE: -2594 to -2590, -2468 to -2464, -1014 to -1010, -237 to -233, -215 to -211 and
380 -103 to -99. Other putative regulatory elements such as activator protein-1 (AP-1) and
381 specificity protein 1 (Sp1) were predicted at the following positions: AP-1: -2077 to -
382 2070 (AGACTCAC), -1198 to -1191 (ATGATTCA), -1197 to -1190 (TGATTCAT) and -
383 565 to -558 (GTGACTGA); Sp1: -985 to -976 (ACCCTCCCTA) and -69 to -60
384 (ACACACCCCA). The conserved TATA box and CAAT box were found at positions -55
385 to -41 (GTATAAAAGCAGCAA) and -103 to -88 (GTCAGCCAAGGAGGTG),
386 respectively.
387 The *chgHβ* promoter sequence was 2320 bp in length and was followed by the
388 translation initiation site and of DNA transcribed into 101 bp of exon (Fig. A. 2, Accession
389 No. LC577092). No complete palindrome ERE was found in the analyzed promoter

390 sequences. Two ERE-like sequences and eight 1/2 ERE were predicted in *chgHβ* at the
391 following positions: ERE-like: -2246 to -2228 (GGACAcactaagtcTGATC) and -1139 to
392 -1129 (GGTTAcTGACC); 1/2ERE: -2124 to -2120, -1245 to -1241, -1071 to -1067, -
393 1057 to -1053, -260 to -256, -234 to -230, -137 to -133 and -97 to –93. The *chgβ* promoter
394 contained other putative regulatory elements at the following positions: AP-1: -1354 to -
395 1347 (GTGACTGA), -1088 to -1081 (TTACTGAC) and -728 to -721 (GTGACTAA).
396 The TATA box and CAAT box were predicted at -17 to -3 (TTATAAAGGTGGCCG) and
397 -234 to -219 (AGCCTCCAATGACATG), respectively.
398 The *chgL* DNA promoter was 3636 bp in length. The promoter was upstream of the
399 translation initiation site and of 111 bp of transcribable DNA (Fig. A. 3, Accession No.
400 LC577093). No complete palindrome ERE was identified in the analyzed promoter
401 sequences. One ERE-like sequences and seven 1/2 ERE were predicted at the following
402 positions: ERE-like: -703 to -692 (GGTCAtTCACC); 1/2ERE: -3544 to -3540, -2303 to
403 -2299, -2268 to -2264, -1732 to -1728, -1616 to -1612, -1395 to -1391 and -760 to -756.
404 The AP-1 and Sp1 were at the following positions: AP-1: -3333 to -3326 (TTAATCAC),
405 -3179 to -3172 (TTAATCAC), -2538 to -2531 (ATGAGTCA), -2537 to -2530
406 (TGAGTCAC), -2480 to -2473 (ATACTCAC), -1972 to -1965 (GTGAATAA), -1719 to
407 -1712 (TGGGTCAC), -1144 to -1137 (TCAGTCAC), -765 to -758 (TTACTGAC), -703
408 to -696 (GTCATTCA) and -386 to -379 (GTGACACA); Sp1: -3559 to -3550
409 (GAGGGGTGGT), -3514 to -3505 (GAGGCAGTGA), -2109 to -2100
410 (ACACAGCCCC), -1642 to -1633 (GGGGCATGGA), -1517 to -1508 (TCCATGCCTC),
411 -95 to -86 (GGGGGCGGGT) and -94 to -85 (GGGGCGGGTT). The TATA box and
412 CAAT box were found at positions -57 to -43 (TTATAAAACTGGCCA) and -153 to -138
413 (TGTGCCCAATGGGCAG), respectively.

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

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

438 (GTTCAttggTGTCCA), -1324 to -1315 (GAGCAtccTGACC), -586 to -575
439 (GTTCAaaTGCAC), -312 to -309 (GGTCAaagaTGTTG) and -87 to -78
440 (GGTCATGTAC); 1/2ERE: -2193 to -2189, -2087 to -2083, -1273 to -1269, -1118 to -
441 1114, -712 to -708, -660 to -656 and -189 to -185. Other gene regulatory elements were
442 predicted at the following positions: AP-1: -2570 to -2563 (TTACTCAT), -2351 to -2344
443 (ATACTCAC), -1633 to -1626 (ATGAGACA), -1562 to -1555 (GTGAGCAA), -862 to -
444 855 (GTGAGTAA) and -73 to -66 (TTAGTCAT); Sp1: -2221 to -2212 (ATACTGCCCC).
445 The conserved TATA box was identified at position -48 to -34 (TTATAAAACTGGCCA).
446 A total of 6155 bp of sequence upstream of the translation initiation site and a 1041
447 bp transcribable region were isolated and squenced for the *esr1a* DNA promoter (Fig. A.
448 6, Accession No. LC577094). The promoter contained no complete palindrome ERE. The
449 presence of three ERE-like sequences and eight 1/2 ERE was predicted at the following
450 positions: ERE-like: -1107 to -1090 (GAGCAaccgaggcTTGAC), -525 to -511
451 (GGTCAagagtTGTCC) and -79 to 67(TGTCAtgtTGACC); 1/2ERE: -5609 to -5605, -
452 5509 to -5505, -5095 to -5091, -4948 to -4944, -4865 to -4861, -4086 to -4082, -3761 to
453 -3757, -3698 to -3694, -3645 to -3641, -3600 to -3596, -3287 to -3283, -3057 to -3053, -
454 2191 to -2187, -2091 to -2087, -1482 to -1478, -899 to -895, -285 to -281, -100 to -96
455 and -70 to -66. Other putative gene regulatory elements were confirmed at the following
456 positions: AP-1: -5754 to -5747 (GTGAGCCA), -5493 to -5486 (TTACTCAC), -5368 to
457 -5361 (TGTGTCAC), -5050 to -5043 (GTGACTGA), -5049 to -5042 (TGACTGAT), -
458 4213 to -4206 (TGAATGAC), -3815 to -3808 (GTGAGTTA), -3711 to -3704
459 (TGACTCCC), -2213 to -2206 (GTGAGAAA), -1126 to -1119 (TGAATGAC) and -267
460 to -260 (TTAGTCAG); Sp1: -5855 to -5846 (AGGGCAGTGT), -5808 to -5799
461 (GGGGCGGTAT), -4409 to -4400 (ACCCAGCCAG), -3604 to -3595

462 (CAGGGAGGGT), -3329 to -3320 (ACAATGCCTC), -3099 to -3090
463 (GGGGCAGGGG), -2201 to -2192 (AAGGGAGGGT), -2099 to -2090
464 (GGGGCTGTGA) and -396 to -387 (TTGGCGGGAT). The putative TATA box and
465 CAAT box were found at positions, -203 to -189 (CTATGAAAAGGGGGA) and -336 to
466 -321 (AAGGCCCAATGATAGC), respectively.

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

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

475

476 *3.4. Transactivation of estrogen-responsive gene promoters by E2 via Esr1a, Esr1b,*
477 *Esr2a and Esr2b*

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

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

remaining gene promoters in E2 culture were significantly lower than those of *chgHβ* promoter, and higher in the order of *chgL* and *esr1a*.

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

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

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

4. Discussion

The predicted cutthroat trout Esr1b, Esr2a and Esr2b polypeptide sequences showed

510 high similarities with homologous sequences of rainbow trout (Esr1b: 99.3%, Esr2a:
511 99.3%, Esr2b: 98.8%). Primary structures of cutthroat trout Esr1b, Esr2a and Esr2b
512 exhibited typical functional domains reported for vertebrate ESRs/Esrs and the motifs
513 required for DNA and ligand binding were highly conserved (data not shown). These
514 structural similarities suggest that cutthroat trout Esr1b, Esr2a and Esr2b have the basic
515 functions of vertebrate ESRs/Esrs, such as ligand (estrogen) binding and transactivation
516 of target genes (Davis et al., 2010; Menuet et al., 2002).

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

526 In Experiment 1, levels of only *esr1a* mRNA were upregulated by E2 treatment –
527 those of the other *esr*s (i.e., esr*1b*, *esr2a* and *esr2b*) were not. In rainbow trout fed with
528 E2-containing pellets for five days, Casanova-Nakayama et al. (2018) confirmed that E2
529 could stimulate hepatic *esr1b* gene expression. Osachoff et al. (2013) confirmed that 7-
530 day exposure to E2 upregulated hepatic *esr1b* mRNA levels in rainbow trout, but that 2-
531 day exposure did not. Similarly, Boyce-Derricott et al. (2009) observed no significant
532 effect of E2 on hepatic *esr1b* mRNA expression in rainbow trout by 24 h following
533 injection. These results suggest that it takes a relatively long time for E2 to upregulate

534 *esr1b* gene expression. Meanwhile, E2 did not show any significant effect on hepatic
535 *esr2a* expression in rainbow trout, regardless of the duration of the exposure period
536 (Boyce-Derricott et al., 2009; Casanova-Nakayama et al., 2018; Cleveland and Weber,
537 2015; Osachoff et al., 2013), which is in keeping with our results on primary cultured
538 hepatocytes of cutthroat trout in this study. As for *esr2b* in rainbow trout, Cleveland and
539 Weber (2015) reported downregulation of the mRNA levels by E2 treatment, similar to
540 the results for cutthroat trout in the present study, but other studies (Boyce-Derricott et
541 al., 2009; Casanova-Nakayama et al., 2018; Osachoff et al., 2013) did not document such
542 a response of *esr2b* mRNA to E2 treatment. Collectively, *esr1a* gene alone appears to
543 exhibit strong and acute upregulation by E2 stimulation, suggesting that *esr1a* is the major
544 transcriptional factor among *esr* subtypes involved in active (i.e., strong and acute)
545 synthesis of hepatic *chg* and *vtg* mRNAs in salmonids.

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

557 From 24 to 72 hpi, *chgHα*, *chgHβ*, *chgL*, *vtgAs* and *vtgC* mRNA levels increased or

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

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

582 in the presence of E2 and Esrs. In addition, AP-1 and Sp1 binding sites, which are known
583 to interact with Esr, were predicted in promoters of cutthroat trout *chg*, *vtg* and *esr1a*.
584 These binding sites possibly contribute to the transactivation of *chg*, *vtg* and *esr1a*.
585 *In silico* analysis predicted the binding sites for transcription factors; it is unclear if
586 the predicted sites are functional *in vivo* and *in vitro*. Therefore, binding sites with low or
587 no functionality for E2 responsiveness of genes were possibly identified in *esr1b*, *esr2a*
588 and *esr2b* promoters. To verify the transcriptional response of the targeted genes, *in vitro*
589 experiments, such as promoter assays, will thus be needed.
590 In reporter gene assays with teleost Esrs, several mammalian cell lines (CHO-K1,
591 HepG2, HeLa, HEK-293, CHO, etc.) have been used (Davis et al., 2010; Le Drean et al.,
592 1995; Lee Pow et al., 2016; Menuet et al., 2004; Mushirobira et al., 2018). It has been
593 shown that these cells have endogenous ESRs and other estrogen-related proteins. For
594 example, CHO-K1 expresses functional endogenous Esr2 (Thomas et al., 2003), whereas
595 HepG2 expresses the gene encoding G protein-coupled estrogen receptor 1 (*GPER1*) at
596 high levels (Transcripts Per Kilobase Million: TPM: 15.3, calculated by next-generation
597 sequences) compared to levels in other human cell lines (TPM; HeLa: 0.9, HEK-293: 1.9;
598 see The Human Protein Atlas, https://www.proteinatlas.org/). The GPER has been shown
599 to bind estrogens and initiate subsequent signaling cascades *in vitro* (Langer et al., 2010).
600 Because of high expression of endogenous estrogen receptors, the CHO derivative cells
601 (CHO and CHO-K1) and HepG2 are perhaps not best-suitable for the Esr/ESR-based
602 reporter gene assay. HeLa cells have been widely used for reporter gene assay for Esrs of
603 teleosts (Lee Pow et al., 2016; Menuet et al., 2004; Sumida and Saito, 2008). In addition,
604 HeLa expresses low levels of Esr1 (TPM: 0.1) and Esr2 (TPM: 0.4). Thus, the HeLa cell
605 line was selected for the reporter gene assays in the present study. To eliminate the

606 influence of promoter length on the transfection efficiency, reporter gene assay in this
607 study was performed using 2000 bp promoter regions of all genes.

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

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

626 In conclusion, the present study documented i) *in vitro* hepatic responses of *chg*, *vtg*
627 and *esr* gene expression to E2 stimulation within a single teleost species, and ii)
628 transactivation properties of *chg*, *vtg* and *esr* promoters by four Esr subtypes. To our
629 knowledge, this is the first report to show the different responsiveness to E2 among

hepatic estrogen-responsive genes in a single teleost species alongside the differential roles of Esr subtypes in the transactivation of promoters for those genes. We demonstrated that there were clear differences in E2-induced transactivation of *chg*, *vtg* and *esr1a* gene promoters by four discrete Esr subtypes. Differences in *chg*, *vtg* and *esr1a* gene expression in trout hepatocytes following E2 exposure possibly reflect differential transactivation properties among four Esr subtypes for these gene promoters.

Acknowledgements

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

Funding

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

Declarations of interest

None

Appendix A. Supplementary data

653 **References**

654 Amano, H., Mochizuki, M., Fujita, T., Hiramatsu, N., Todo, T., Hara, A., 2010.
655 Purification and characterization of a novel incomplete-type vitellogenin protein
656 (VgC) in Sakhalin taimen (*Hucho perryi*). Comp. Biochem. Physiol. A. Mol.
657 Integr. Physiol. 157, 41–48. https://doi.org/10.1016/j.cbpa.2010.05.006.
658 Boyce-Derricott, J., Nagler, J.J., Cloud, J.G., 2009. Regulation of hepatic estrogen
659 receptor isoform mRNA expression in rainbow trout (*Oncorhynchus mykiss*). Gen.
660 Comp. Endocrinol. 161, 73–78. https://doi.org/10.1016/j.ygcen.2008.11.022.
661 Buisine, N., Trichet, V., Wolff, J., 2002. Complex evolution of vitellogenin genes in
662 salmonid fishes. Mol. Genet. Genomics 268, 535–542.
663 https://doi.org/10.1007/s00438-002-0771-5.
664 Casanova-Nakayama, A., Von Siebenthal, E.W., Kropf, C., Oldenberg, E., Segner, H.,
665 2018. Immune-specific expression and estrogenic regulation of the four estrogen
666 receptor isoforms in female rainbow trout (*Oncorhynchus mykiss*). Int. J. Mol. Sci.
667 19. https://doi.org/10.3390/ijms19040932.
668 Cleveland B.M., Weber G.M, 2015. Effects of sex steroids on expression of genes
669 regulating growth-related mechanisms in rainbow trout (*Oncorhynchus mykiss*).
670 Gen. Comp. Endocrinol. 216, 103–115. https://doi.org/
671 10.1016/j.ygcen.2014.11.018.
672 Choi, C.Y., Habibi, H.R., 2003. Molecular cloning of estrogen receptor α and
673 expression pattern of estrogen receptor subtypes in male and female goldfish. Mol.
674 Cell. Endocrinol. 204, 169–177. https://doi.org/10.1016/S0303-7207(02)00182-X.
675 Davis, L.K., Katsu, Y., Iguchi, T., Lerner, D.T., Hirano, T., Grau, E.G., 2010.
676 Transcriptional activity and biological effects of mammalian estrogen receptor
677 ligands on three hepatic estrogen receptors in Mozambique tilapia. J. Steroid
678 Biochem. Mol. Biol. 122, 272–278. https://doi.org/10.1016/j.jsbmb.2010.05.009.
679 Filby, A.L., Tyler, C.R., 2005. Molecular characterization of estrogen receptors 1, 2a,

680 and 2b and their tissue and ontogenic expression profiles in fathead minnow
681 (*Pimephales promelas*). Biol. Reprod. 662, 648–662.
682 https://doi.org/10.1095/biolreprod.105.039701.
683 Flouriot, G., Pakdel, F., Valotaire, Y., 1996. Transcriptional and post-transcriptional
684 regulation of rainbow trout estrogen receptor and vitellogenin gene expression.
685 Mol. Cell. Endocrinol. 124, 173–183. https://doi.org/10.1016/S0303-
686 7207(96)03960-3.
687 Fujita, T., Fukada, H., Shimizu, M., Hiramatsu, N., Hara, A., 2008. Molecular cloning
688 and characterization of three distinct choriogenins in masu salmon, *Oncorhynchus*
689 *masou*. Mol. Reprod. Dev. 75, 1217–1228. https://doi.org/10.1002/mrd.20857.
690 Goudet, G., Mugnier, S., Callebaut, I., Monget, P., 2008. Phylogenetic analysis and
691 identification of pseudogenes reveal a progressive loss of zona pellucida genes
692 during evolution of vertebrates. Biol. Reprod. 78, 796–806.
693 https://doi.org/10.1095/biolreprod.107.064568.
694 Grierson, J.P., Neville, A.C., 1981. Helicoidal architecture of fish eggshell. Tissue Cell
695 13, 819–830. https://doi.org/10.1016/S0040-8166(81)80016-X.
696 Griffin, L.B., January, K.E., Ho, K.W., Cotter, K.A., Callard, G. V., 2013. Morpholino-
697 mediated knockdown of ERα, ERβa, and ERβb mRNAs in zebrafish (*Danio rerio*)
698 embryos reveals differential regulation of estrogen-inducible genes. Endocrinology
699 154, 4158–4169. https://doi.org/10.1210/en.2013-1446.
700 Halm, S., Martínez-Rodríguez, G., Rodríguez, L., Prat, F., Mylonas, C.C., Carrillo, M.,
701 Zanuy, S., 2004. Cloning, characterisation, and expression of three oestrogen
702 receptors (ERα, ERβ1 and ERβ2) in the European sea bass, *Dicentrarchus labrax*.
703 Mol. Cell. Endocrinol. 223, 63–75. https://doi.org/10.1016/J.MCE.2004.05.009.
704 Hara, A., Hiramatsu, N., Fujita, T., 2016. Vitellogenesis and choriogenesis in fishes.
705 Fish. Sci. 82, 187–202. https://doi.org/10.1007/s12562-015-0957-5.
706 Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., Thomas, P., 2000.

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

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

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

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

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

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

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

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

Le Drean, Y., Lazennec, G., Kern, L., Saligaut, D., Pakdel, F., Valotaire, Y., 1995. Characterization of an estrogen-responsive element implicated in regulation of the

734 rainbow trout estrogen receptor gene. J. Mol. Endocrinol. 15, 37–47.
735 https://doi.org/10.1677/jme.0.0150037.
736 Lee Pow, C.S.D., Yost, E.E., Aday, D.D., Kullman, S.W., 2016. Sharing the toles: An
737 assessment of japanese medaka estrogen receptors in vitellogenin induction.
738 Environ. Sci. Technol. 50, 8886–8895. https://doi.org/10.1021/acs.est.6b01968.
739 Luo, W., Ito, Y., Mizuta, H., Massaki, K., Hiramatsu, N., Todo, T., Reading, B.J.,
740 Sullivan, C. V, Hara, A., 2013. Molecular cloning and partial characterization of an
741 ovarian receptor with seven ligand binding repeats, an orthologue of low-density
742 lipoprotein receptor, in the cutthroat trout (*Oncorhynchus clarki*). Comp. Biochem.
743 Physiol. Part A 166, 263–271. https://doi.org/10.1016/j.cbpa.2013.06.026.
744 Ma, C.H., Dong, K.W., Yu, K.L., 2000. cDNA cloning and expression of a novel
745 estrogen receptor β-subtype in goldfish (*Carassius auratus*). Biochim. Biophys.
746 Acta 1490, 145–152. https://doi.org/10.1016/S0167-4781(99)00235-3.
747 Menuet, A., Le Page, Y., Torres, O., Kern, L., Kah, O., Pakdel, F., 2004. Analysis of the
748 estrogen regulation of the zebrafish estrogen receptor (ESR) reveals distinct effects
749 of ERalpha, ERbeta1 and ERbeta2. J. Mol. Endocrinol. 32, 975–86.
750 https://doi.org/10.1677/jme.0.0320975.
751 Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., Pakdel, F., 2002.
752 Molecular characterization of three estrogen receptor forms in zebrafish: binding
753 characteristics, transactivation properties, and tissue distributions. Biol. Reprod.
754 66, 1881–1892. https://doi.org/10.1095/biolreprod66.6.1881.
755 Mushirobira, Y., Mizuta, H., Luo, W., Morita, Y., Sawaguchi, S., Matsubara, T.,
756 Hiramatsu, N., Todo, T., Hara, A., 2013. Changes in levels of dual vitellogenin
757 transcripts and proteins in cutthroat trout *Oncorhynchus clarki* during ovarian
758 development. Nippon Suisan Gakkaishi 79, 175–189.
759 https://doi.org/10.2331/suisan.79.175.
760 Mushirobira, Y., Nishimiya, O., Nagata, J., Todo, T., Hara, A., Reading, B.J.,

761 Hiramatsu, N., 2018. Molecular cloning of vitellogenin gene promoters and in *vitro*
762 and in *vivo* transcription profiles following estradiol-17β administration in the
763 cutthroat trout. Gen. Comp. Endocrinol. 267, 157–166.
764 https://doi.org/10.1016/j.ygcen.2018.06.017.
765 Nagata, J., Mushirobira, Y., Nishimiya, O., Fujita, T., Hiramatsu, N., Hara, A., Todo,
766 T., 2018. Expression analysis of estradiol-17β responsive genes in the liver of
767 female cutthroat trout (*Oncorhynchus clarki*) during a reproductive cycle. Aquac.
768 Sci. 66, 91–101. https://doi.org/10.11233/aquaculturesci.66.91.
769 Nagler, J.J., Cavileer, T., Sullivan, J., Cyr, D.G., Rexroad, C., 2007. The complete
770 nuclear estrogen receptor family in the rainbow trout: Discovery of the novel ERα2
771 and both ERβ isoforms. Gene 392, 164–173.
772 https://doi.org/10.1016/j.gene.2006.12.030.
773 Nagler, J.J., Cavileer, T.D., Verducci, J.S., Schultz, I.R., Hook, S.E., Hayton, W.L.,
774 2012. Estrogen receptor mRNA expression patterns in the liver and ovary of
775 female rainbow trout over a complete reproductive cycle. Gen. Comp. Endocrinol.
776 178, 556–561. https://doi.org/10.1016/j.ygcen.2012.06.010.
777 Nelson, E.R., Habibi, H.R., 2010. Functional significance of nuclear estrogen receptor
778 subtypes in the liver of goldfish. Endocrinology 151, 1668–1676.
779 https://doi.org/10.1210/en.2009-1447.
780 Osachoff, H.L., Shelley, L.K., Furtula, V., Van Aggelen, G.C., Kennedy, C.J., 2013.
781 Induction and recovery of estrogenic effects after short-term 17β-estradiol
782 exposure in juvenile rainbow trout (*Oncorhynchus mykiss*). Arch. Environ.
783 Contam. Toxicol. 65, 276–285. https://doi.org/10.1007/s00244-013-9890-8.
784 Podvinec, M., Kaufmann, M.R., Handschin, C., Meyer, U., 2002. NUBIScan, an in
785 silico approach for prediction of nuclear receptor response elements. Mol.
786 Endocrinol. 16, 1269–1279. https://doi.org/10.1210/mend.16.6.0851.
787 Sabo-Attwood, T., Kroll, K.J., Denslow, N.D., 2004. Differential expression of

largemouth bass (*Micropterus salmoides*) estrogen receptor isotypes alpha, beta, and gamma by estradiol. Mol. Cell. Endocrinol. 218, 107–118. https://doi.org/10.1016/j.mce.2003.12.007.

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

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

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

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

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

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

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

7207(98)00191-9.
Thomas-Jones, E., Thorpe, K., Harrison, N., Thomas, G., Morris, C., Hutchinson, T., Woodhead, S., Tyler, C., 2003. Dynamics of estrogen biomarker responses in rainbow trout exposed to 17beta-estradiol and 17alpha-ethinylestradiol. Environ. Toxicol. Chem. 22, 3001–3008. https://doi.org/10.1897/03-31.
Thomas, P.B., Risinger, K.E., Klinge, C.M., 2003. Identification of estrogen receptor beta expression in Chinese hamster ovary (CHO) cells and comparison of estrogen-responsive gene transcription in cells adapted to serum-free media. J. Steroid Biochem. Mol. Biol. 86, 41–55. https://doi.org/10.1016/S0960-0760(03)00250-4.
Trichet, V., Buisine, N., Mouchel, N., Morán, P., Pendás, A.M., Le Pennec, J.P., Wolff, J., 2000. Genomic analysis of the vitellogenin locus in rainbow trout (*Oncorhynchus mykiss*) reveals a complex history of gene amplification and retroposon activity. Mol. Gen. Genet. 263, 828–37. https://doi.org/10.1007/s004380000247.
Walker, P., Germond, J.-E., Brown-Luedi, M., Givel, F., Wahli, W., 1984. Sequence homologies in the region preceding the transcription initiation site of the liver estrogen-responsive vitellogenin and apo-VLDLI genes. Nucleic Acids Res. 12, 8611–8626. https://doi.org/10.1093/nar/12.22.8611.
Westerlund, L., Hyllner, S.J., Schopen, A., Olsson, P.E., 2001. Expression of three vitelline envelope protein genes in arctic char. Gen. Comp. Endocrinol. 122, 78–87. https://doi.org/10.1006/gcen.2001.7614.

Figure captions

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

842 Hepatocytes from one trout were treated with 10^{-6} M E2. Half of the culture medium was
843 replaced by fresh medium every 24 h post initiation (hpi) of E2 treatment. At 0, 24 and
844 72 hpi, cells were harvested and mRNA levels quantified by quantitative real-time reverse
845 transcription PCR. Columns indicate mean values and vertical lines indicate standard
846 errors. Different letters denote that values are significantly different ($P < 0.05$).
847
848 Fig. 2 Effect of treatment of hepatocytes of male cutthroat trout in primary culture with
849 single doses of estradiol-17β (E2, black columns) or a control solvent (C, white columns)
850 on the transcript levels of choriogenin (*chg*: *chgHα*, *chgHβ* and *chgL*), vitellogenin (*vtg*:
851 *vtgAs* and *vtgC*) and estrogen receptor (*esr1a*). Hepatocytes from one trout were treated
852 with $10^{-11} \sim 10^{-6}$ M (-11 ~ -6 in horizontal axis) E2 for 72 h without replacement of the
853 medium. At 24 and 72 h post initiation (hpi) of E2 treatment, cells were harvested and
854 mRNA levels quantified by quantitative real-time reverse transcription PCR. Columns
855 indicate mean values and vertical lines indicate standard errors. Different letters denote
856 that values are significantly different ($P < 0.05$). Arrow heads exhibit the lowest dose that
857 induced a significant increase in the expression levels of each mRNA (LOEC) for E2.
858
859 Fig. 3 Fold-induction change in transactivation of choriogenin (*chg*), vitellogenin (*vtg)*
860 and estrogen receptor 1a (*esr1a*) promoters mediated by four Esr subtypes (Esr1a, Esr1b,
861 Esr2a and Esr2b) in the presence or absence of estradiol-17β (E2). Hela cells were co-
862 transfected with two constructs: one *esr* expression vector (*esr1a*, *esr1b, esr2a* or *esr2b*)
863 and one gene promoter (*chgHα*, *chgHβ*, *chgL*, *vtgAs, vtgC* or *esr1a*) in a reporter vector.
864 Reporter vector without a promoter (empty vector, 'vector') was used as negative control.
865 The transfected cells were treated with 1 μM E2 (closed columns) or control solvent (C,
866 open columns). The fold-induction was initially normalized to the luminescence from
867 Renilla luciferase; data were averaged from 4 replicate wells. Fold-activation was
868 represented after setting the empty vector (vehicle control) set to 1. Columns indicate

869 mean values and vertical brackets standard errors. Different letters denote significant
870 differences ($P < 0.05$).

Highlights

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

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

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

Table 1 (Continued)

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

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

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

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

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

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

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

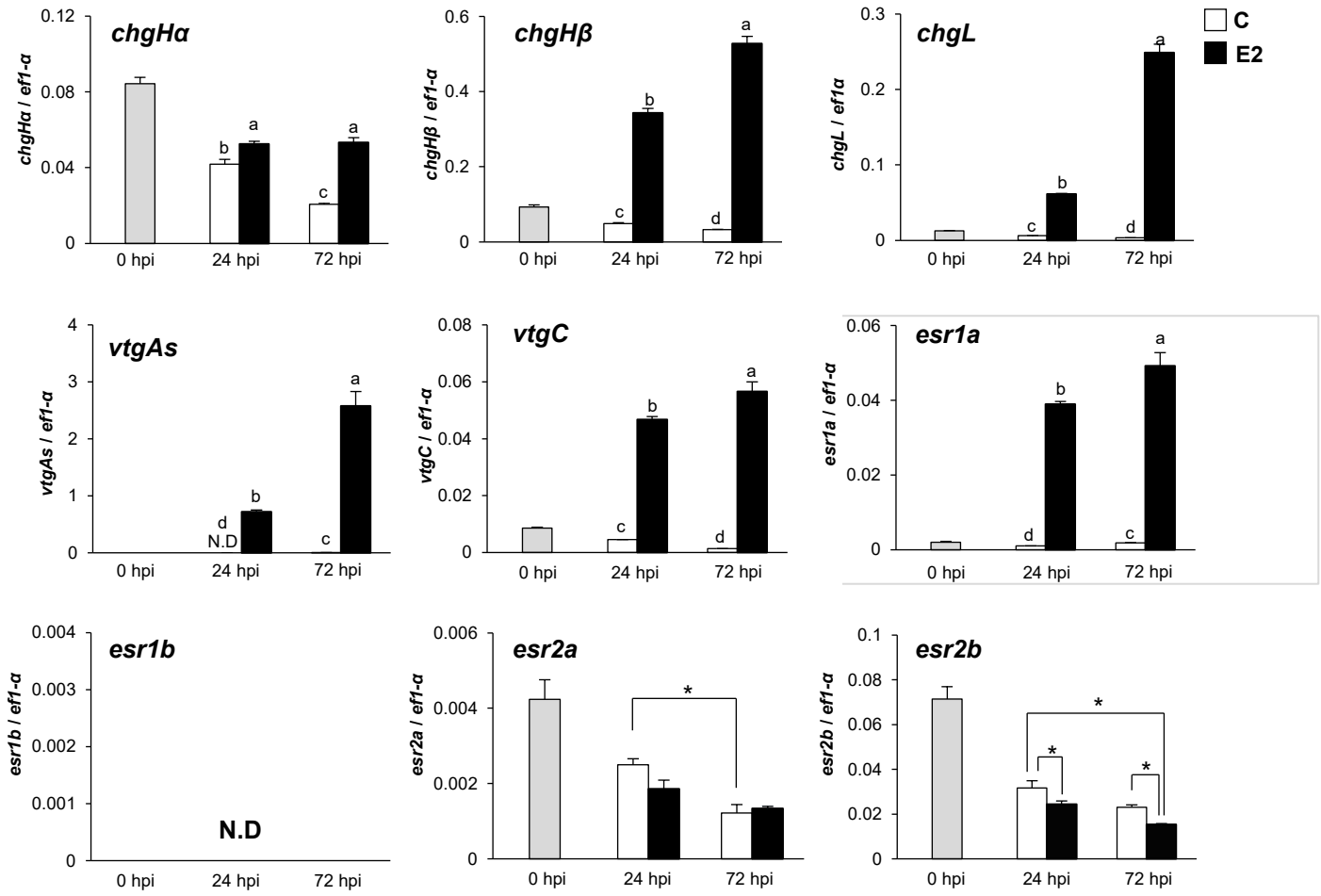
chgHα
chgHα / ef1-α
0.12
0.08
0.04
0
a
b
c
0 hpi
24 hpi
72 hpi
chgHβ
chgHβ / ef1-α
0.6
0.4
0.2
0
a
b
c
d
0 hpi
24 hpi
72 hpi
chgL
chgL / ef1α
0.3
0.2
0.1
0
a
b
c
d
0 hpi
24 hpi
72 hpi
C
E2
vtgAs
vtgAs / ef1-α
4
3
2
1
0
a
b
d
N.D
c
0 hpi
24 hpi
72 hpi
vtgC
vtgC / ef1-α
0.08
0.06
0.04
0.02
0
a
b
c
d
0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

Fig. 1

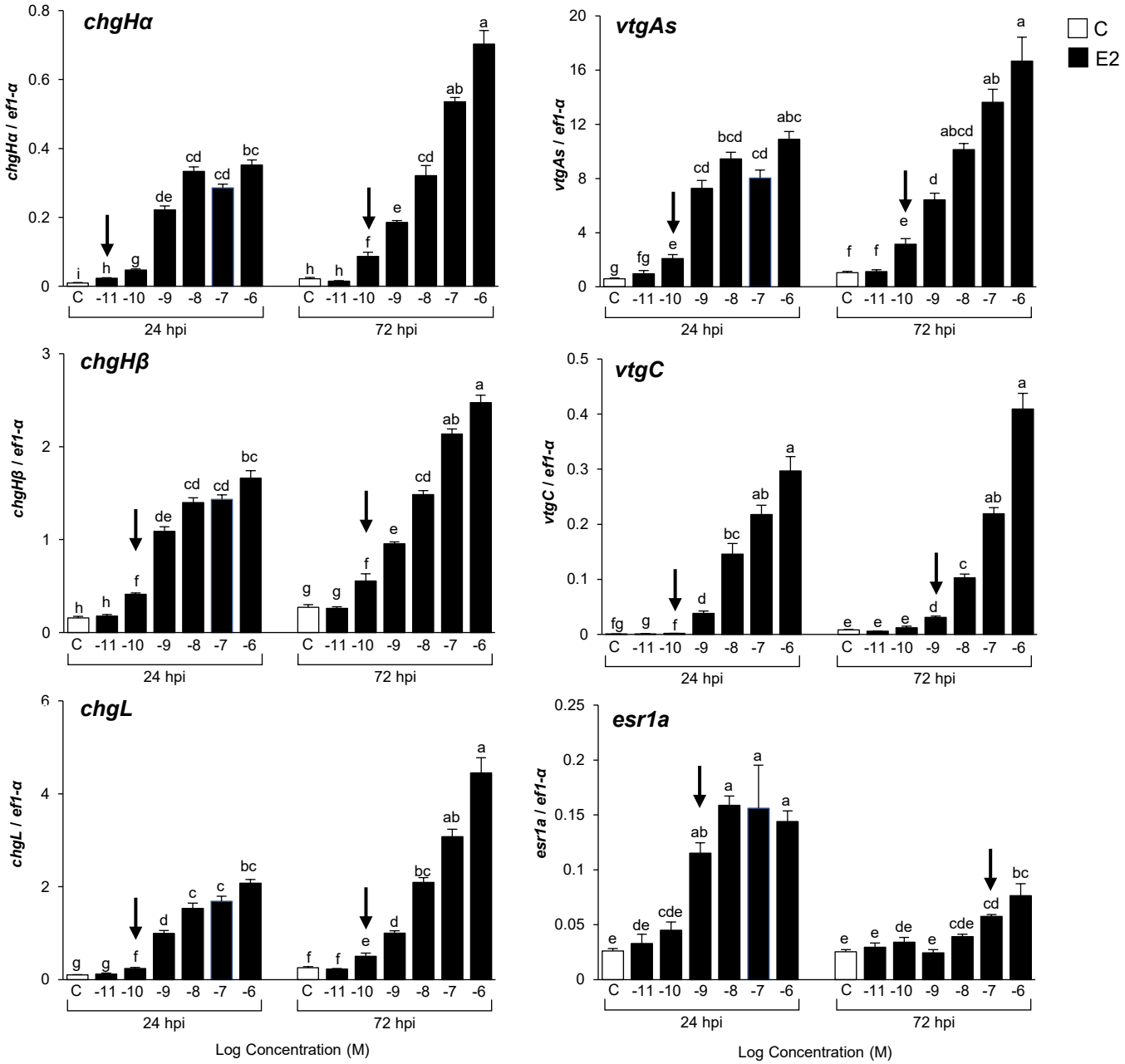
chgHα
chgHα / ef1-α
vtgAs
vtgAs / ef1-α
C
E2
chgHβ
chgHβ / ef1-α
vtgC
vtgC / ef1-α
chgL
chgL / ef1-α
esr1a
esr1a / ef1-α
C -11 -10 -9 -8 -7 -6
24 hpi
72 hpi
Log Concentration (M)

Fig. 2

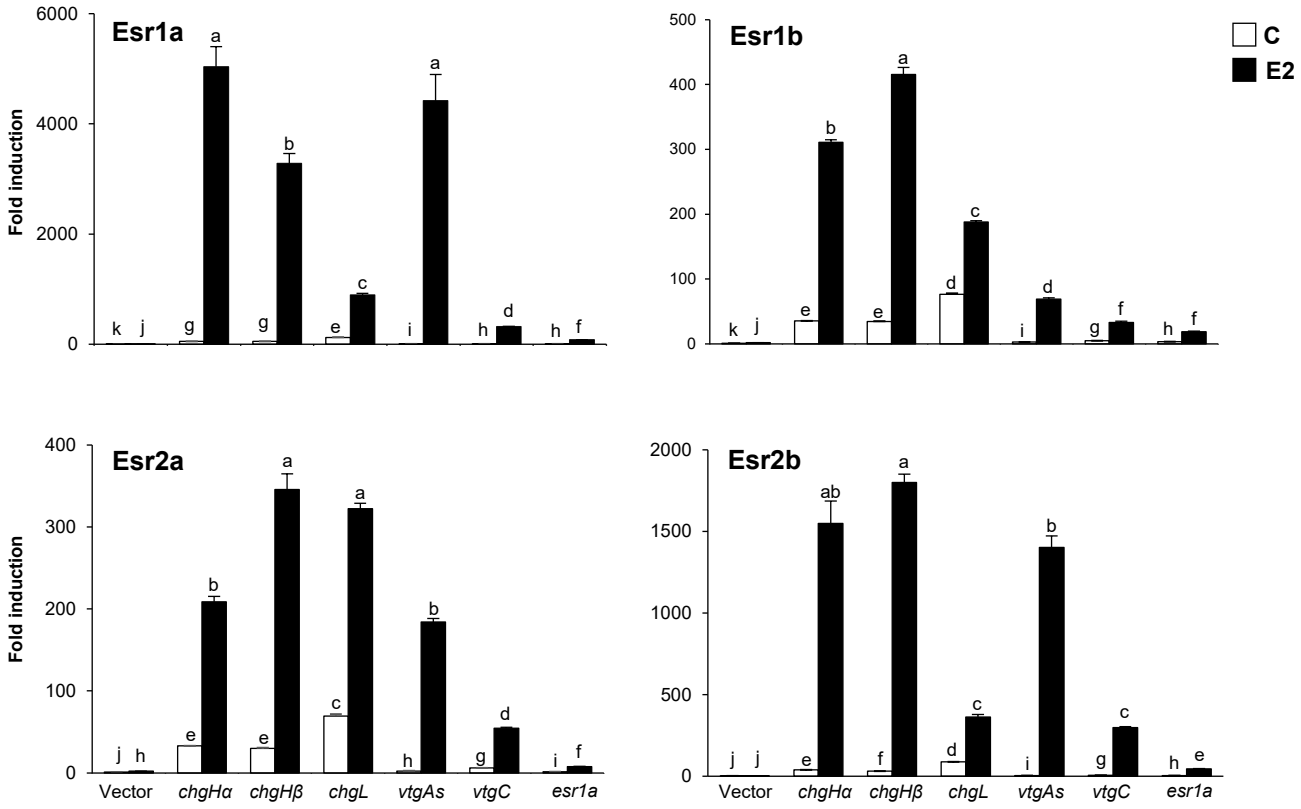
Esr1a
Esr1b
Esr2a
Esr2b
Fold induction
C
E2
Vector
chgHα
chgHβ
chgL
vtgAs
vtgC
esr1a

Fig. 3

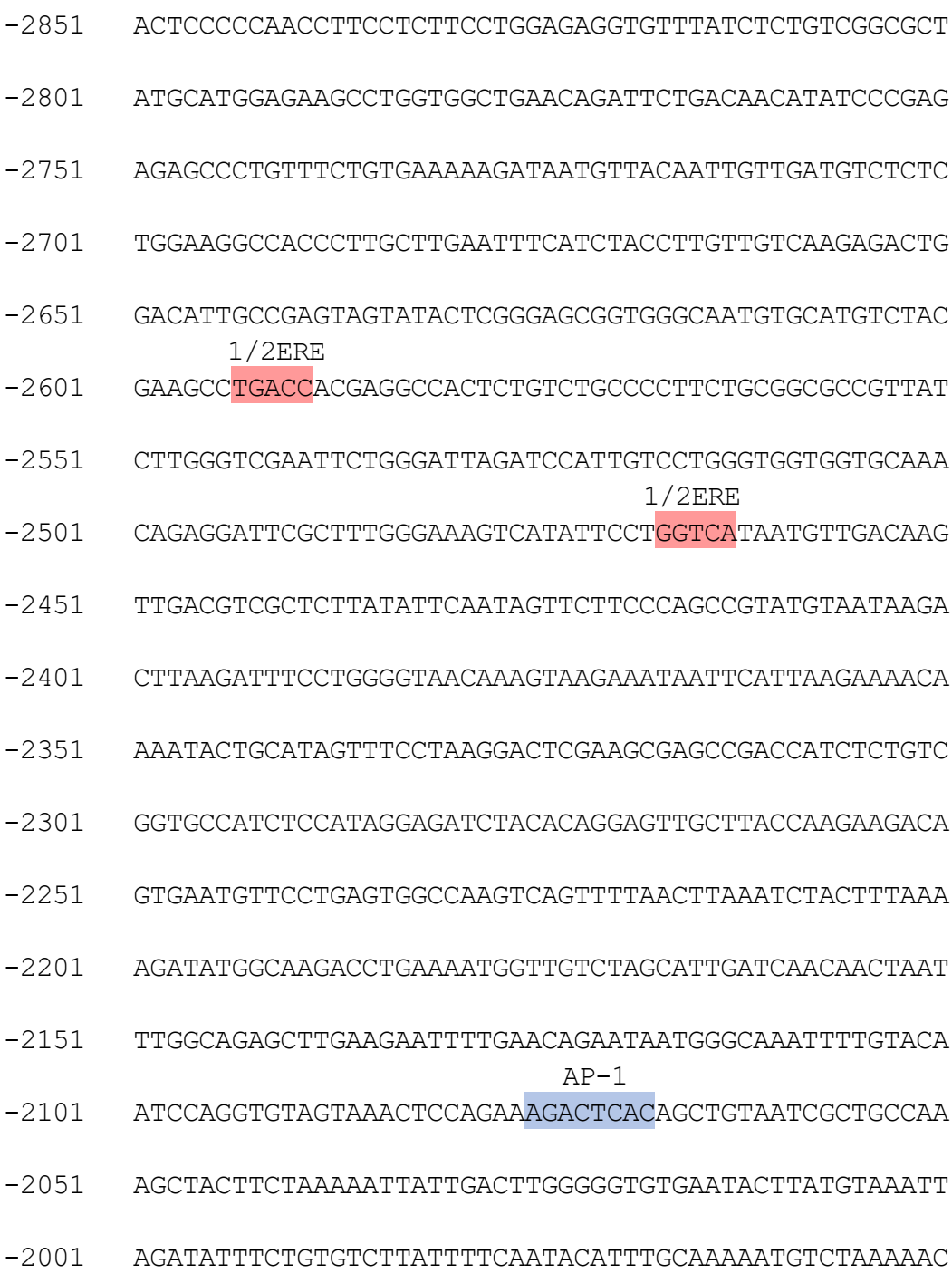

Fig. A. 1 Putative promoter sequence of the cutthroat trout choriogenin Hα gene including 5'flanking region and exon 1. The exons are indicated by open boxes. Nucleotides are numbered from the 5'-end of exon 1, negative numbers representing the 5'-flanking region. Estrogen responsive element (ERE)-like,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

Fig. A. 1 (Continued)

Fig. A. 1 (Continued)

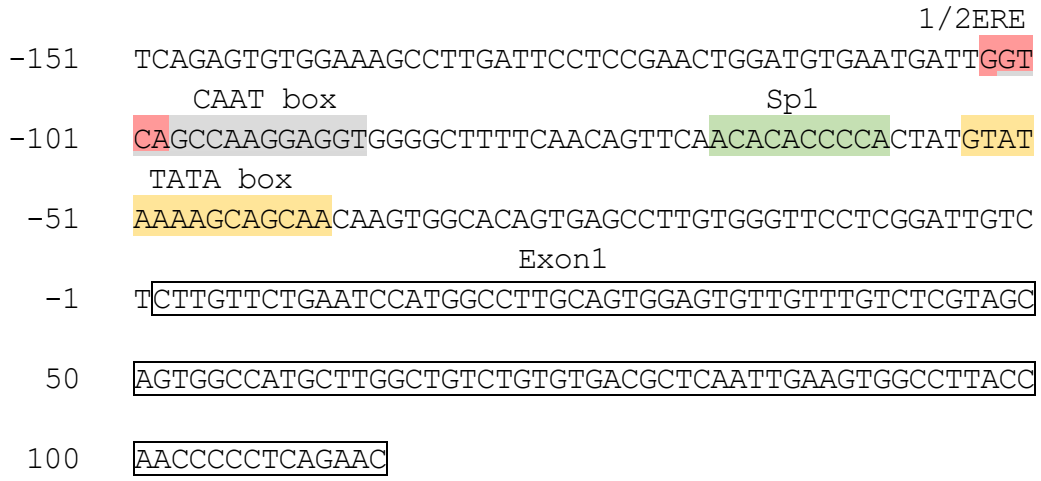

Fig. A. 1 (Continued)

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

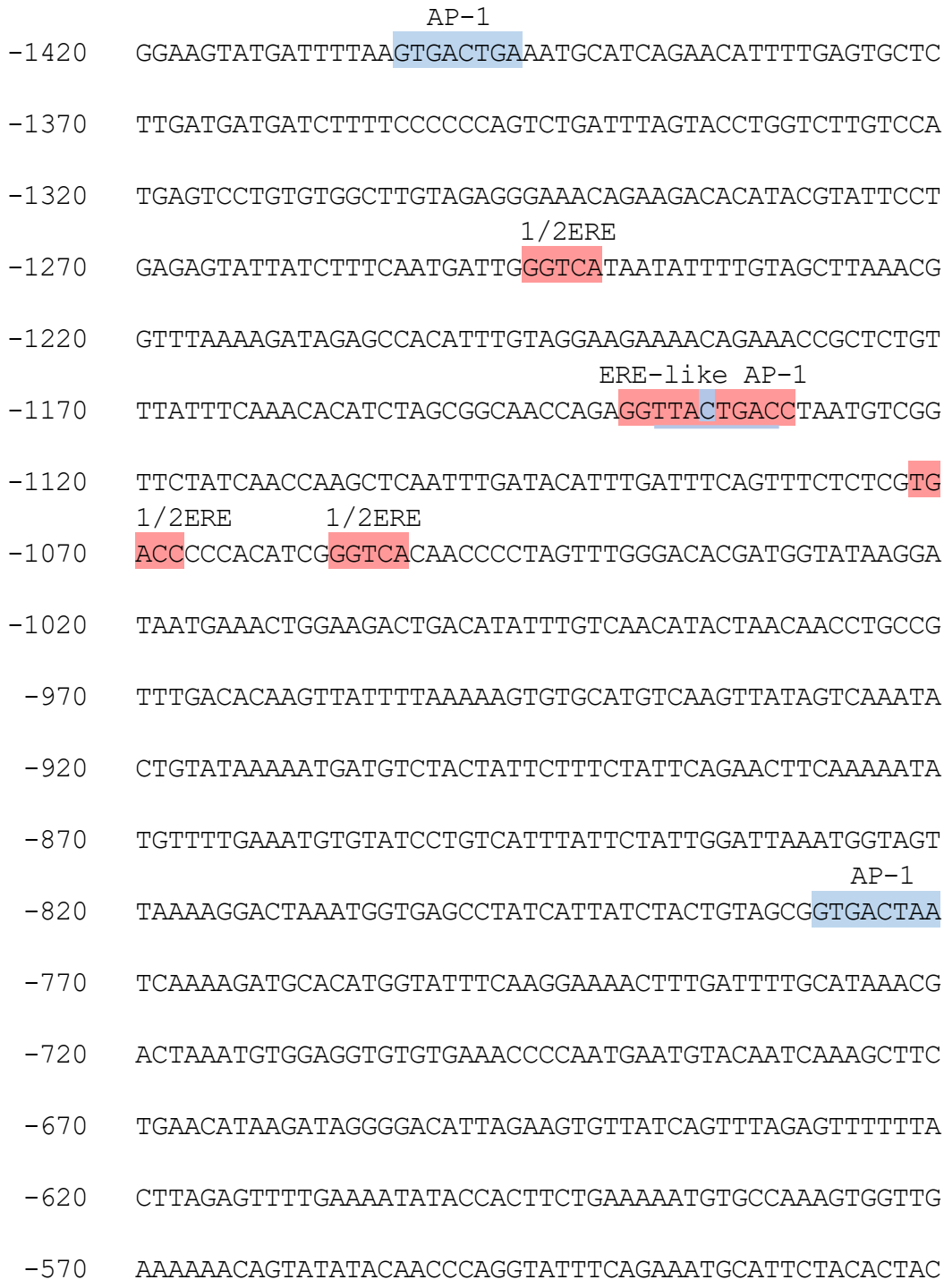

Fig. A. 2 (Continued)

Fig. A. 2 (Continued)

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

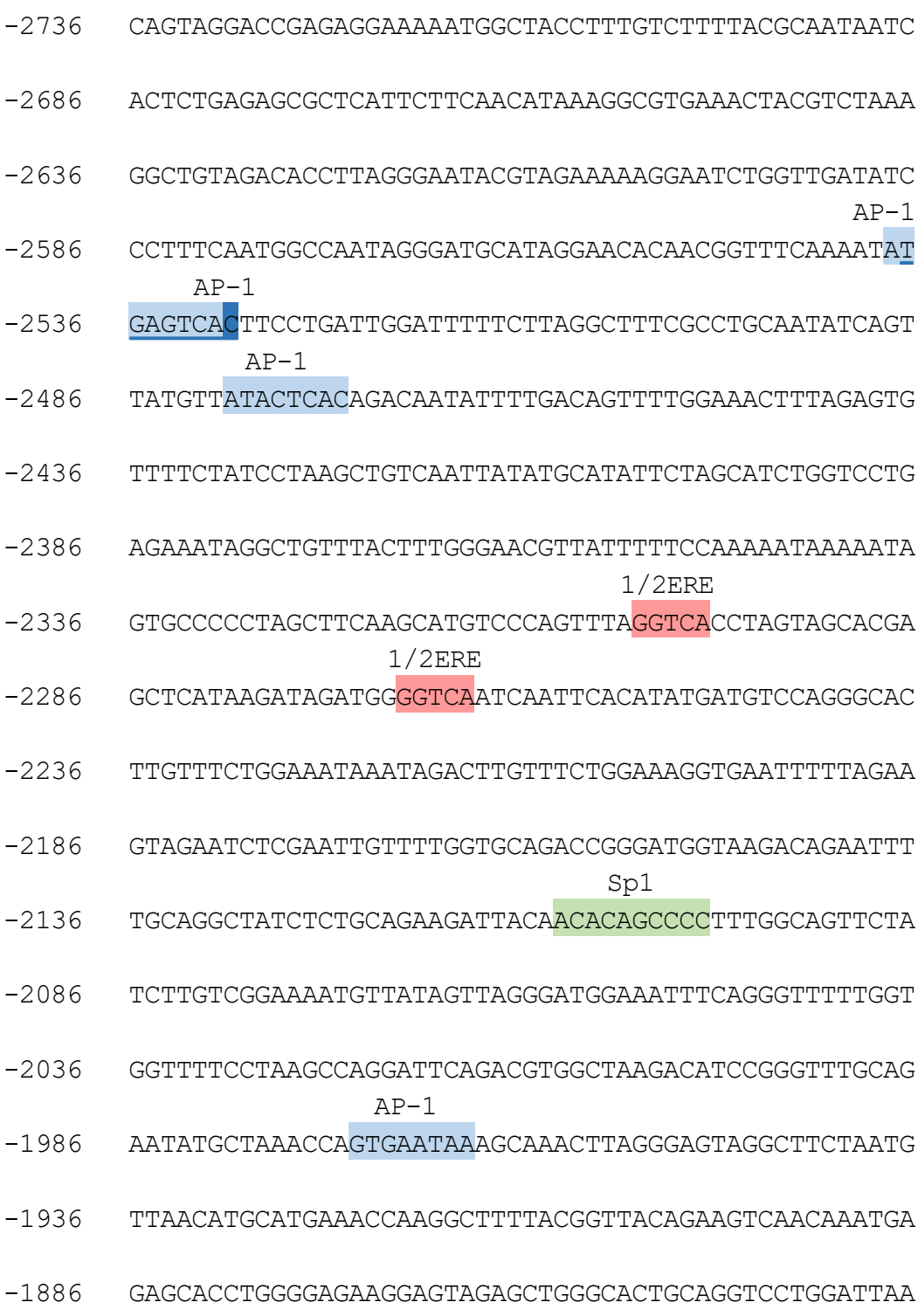

Fig. A. 3 (Continued)

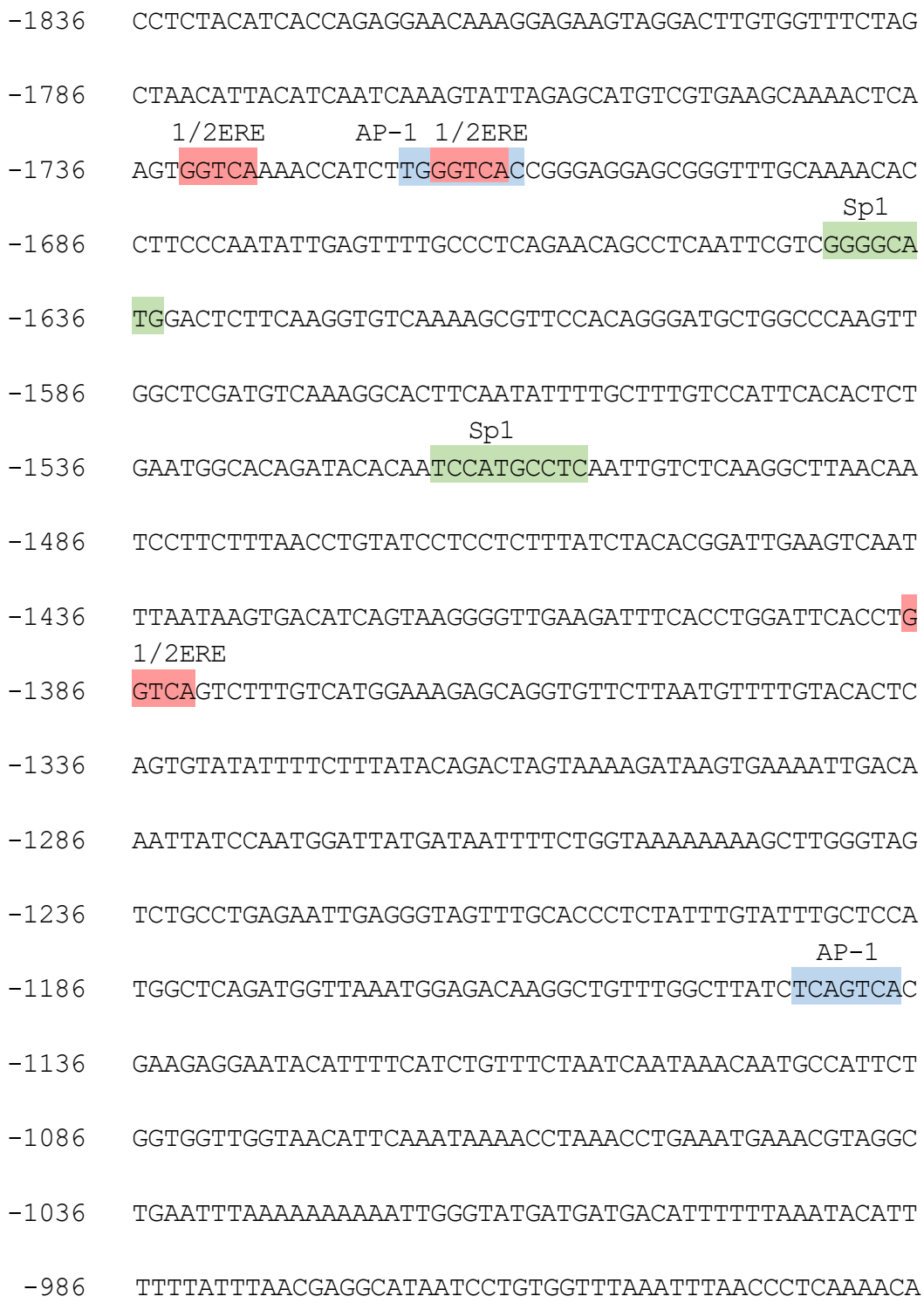

Fig. A. 3 (Continued)

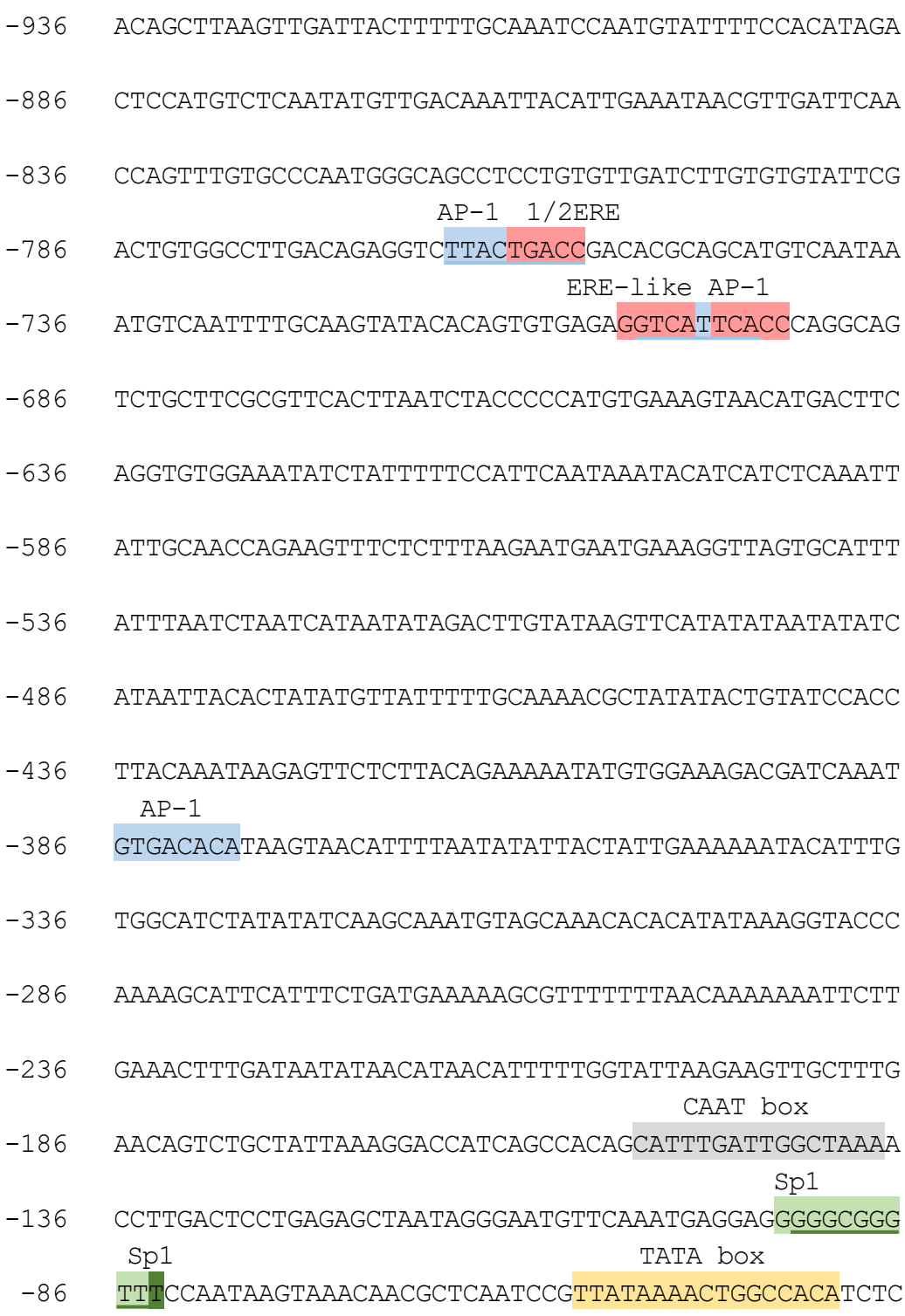

Fig. A. 3 (Continued)

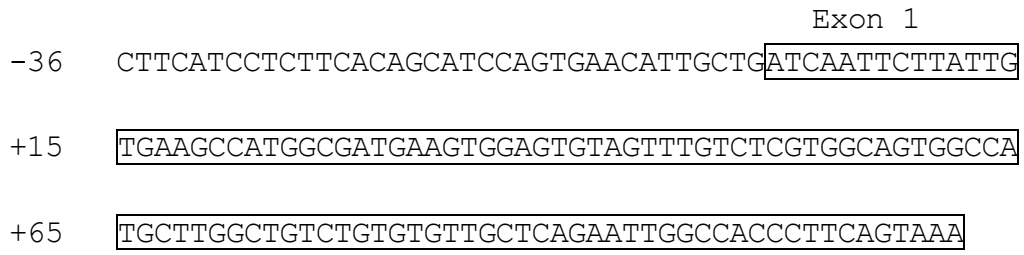

Fig. A. 3 (Continued)

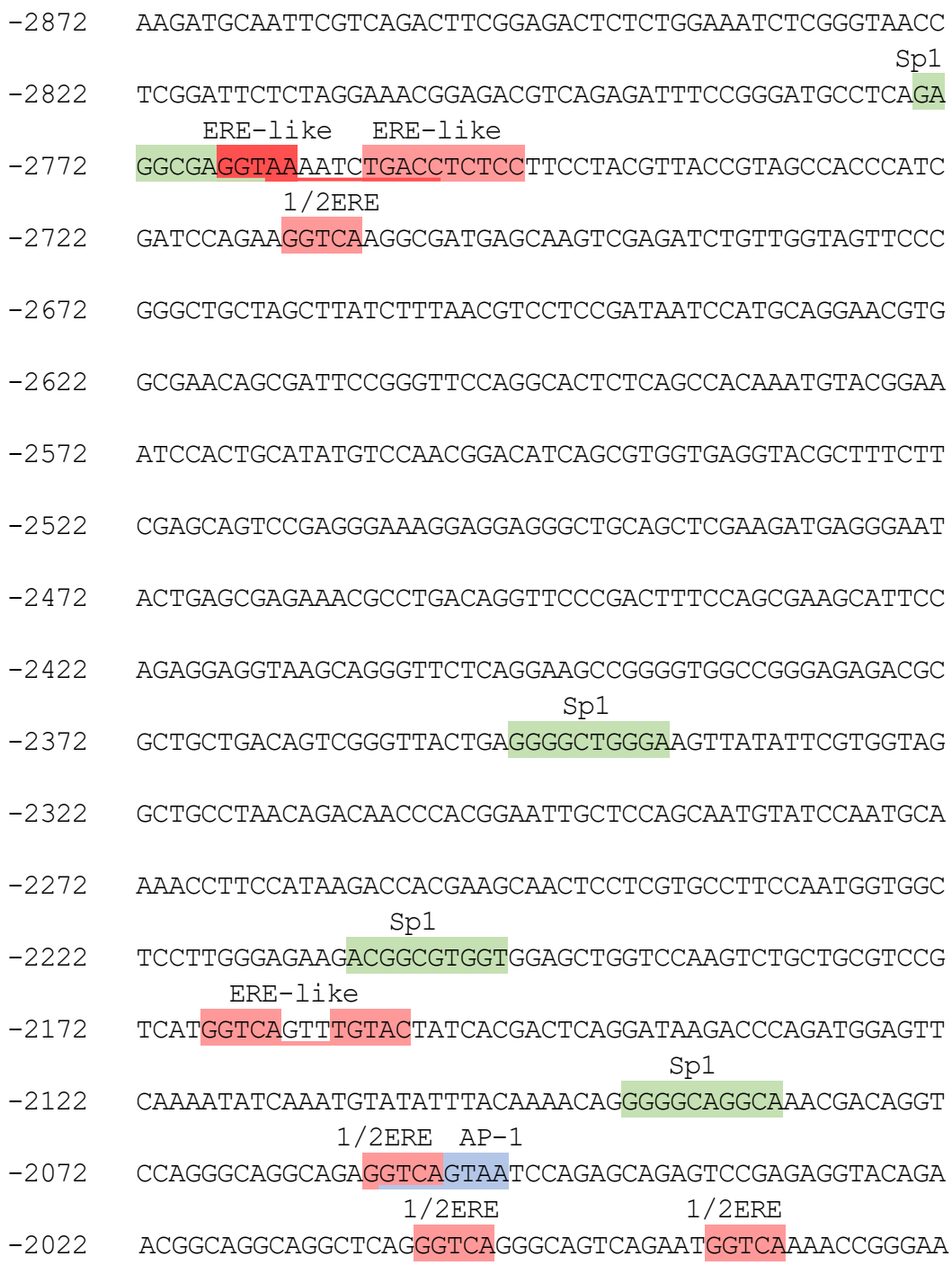

Fig. A. 4 Putative promoter sequence of the cutthroat trout vitellogenin As gene including 5'flanking region, exon 1, intron 1 and exon 2. The exons are indicated by open boxes. Nucleotides are numbered from 5'-end of exon 1, intron 1 and exon 2, with negative numbers representing the 5'flanking region.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

Fig. A. 4 (Continued)

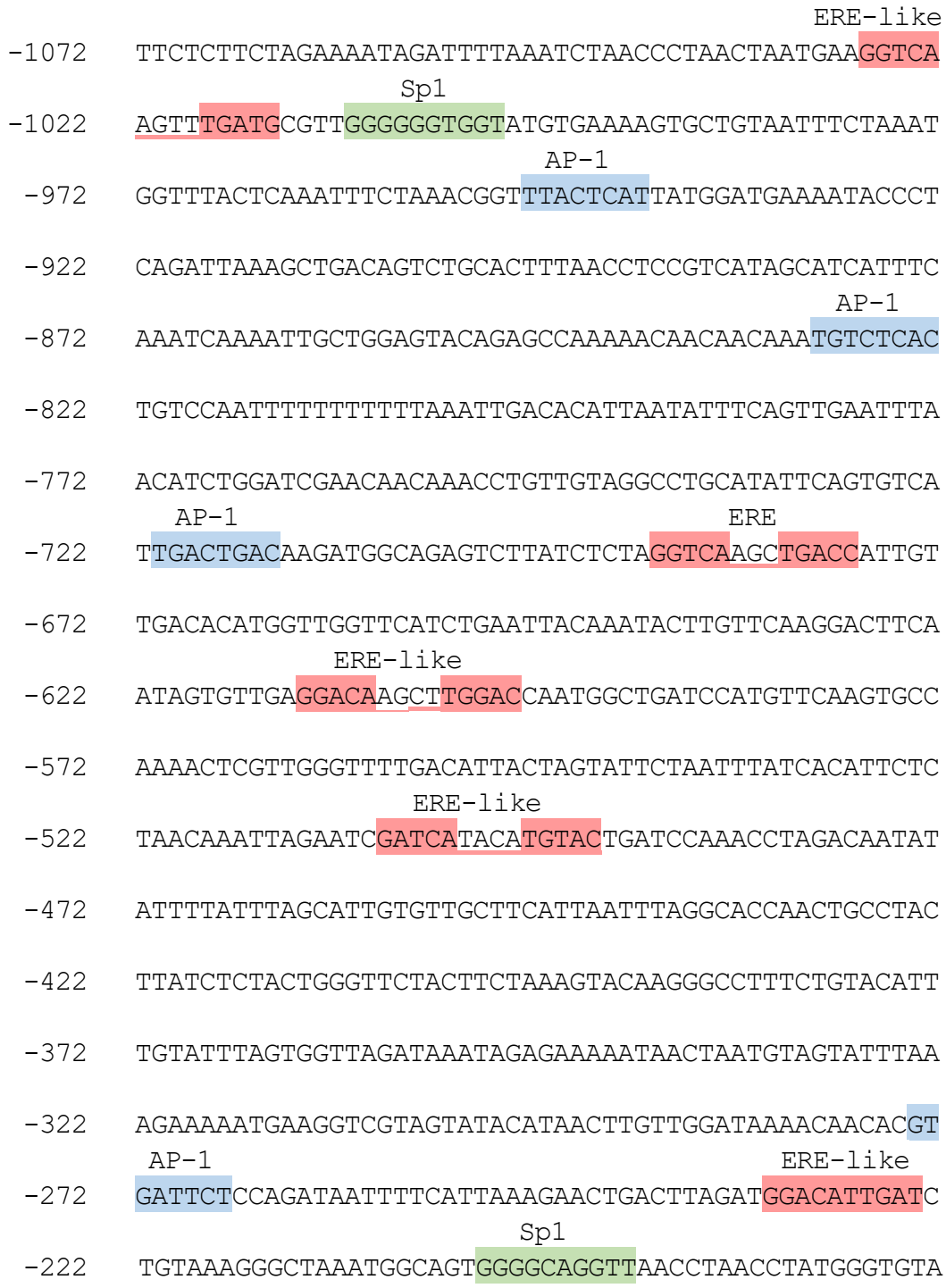

Fig. A. 4 (Continued)

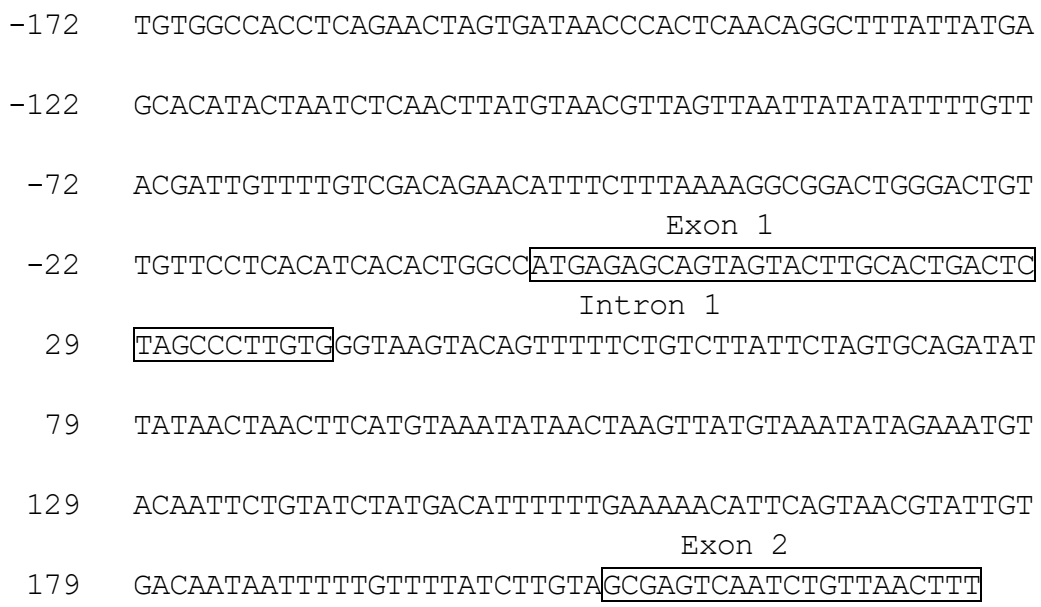

Fig. A. 4 (Continued)

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

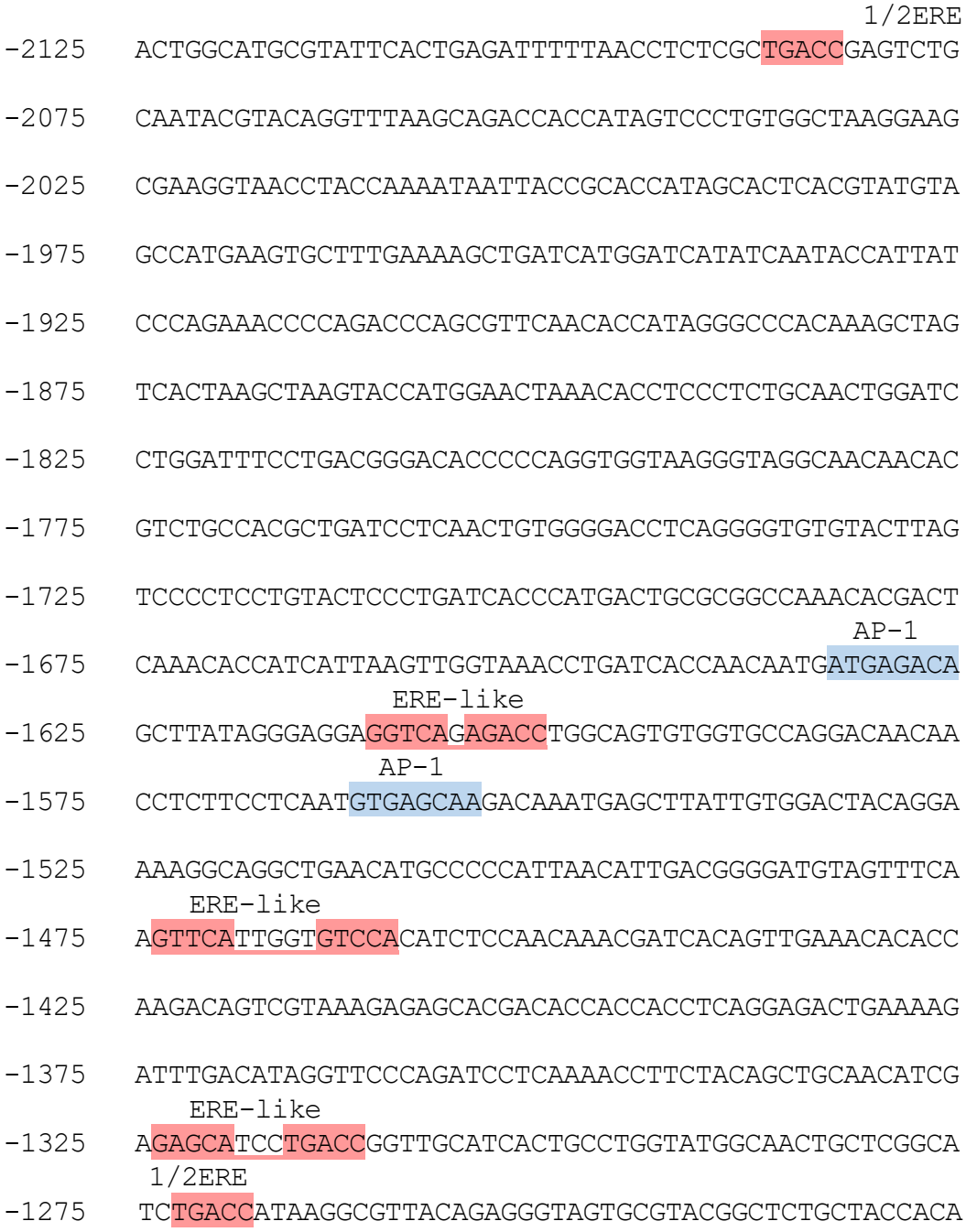

Fig. A. 5 (Continued)

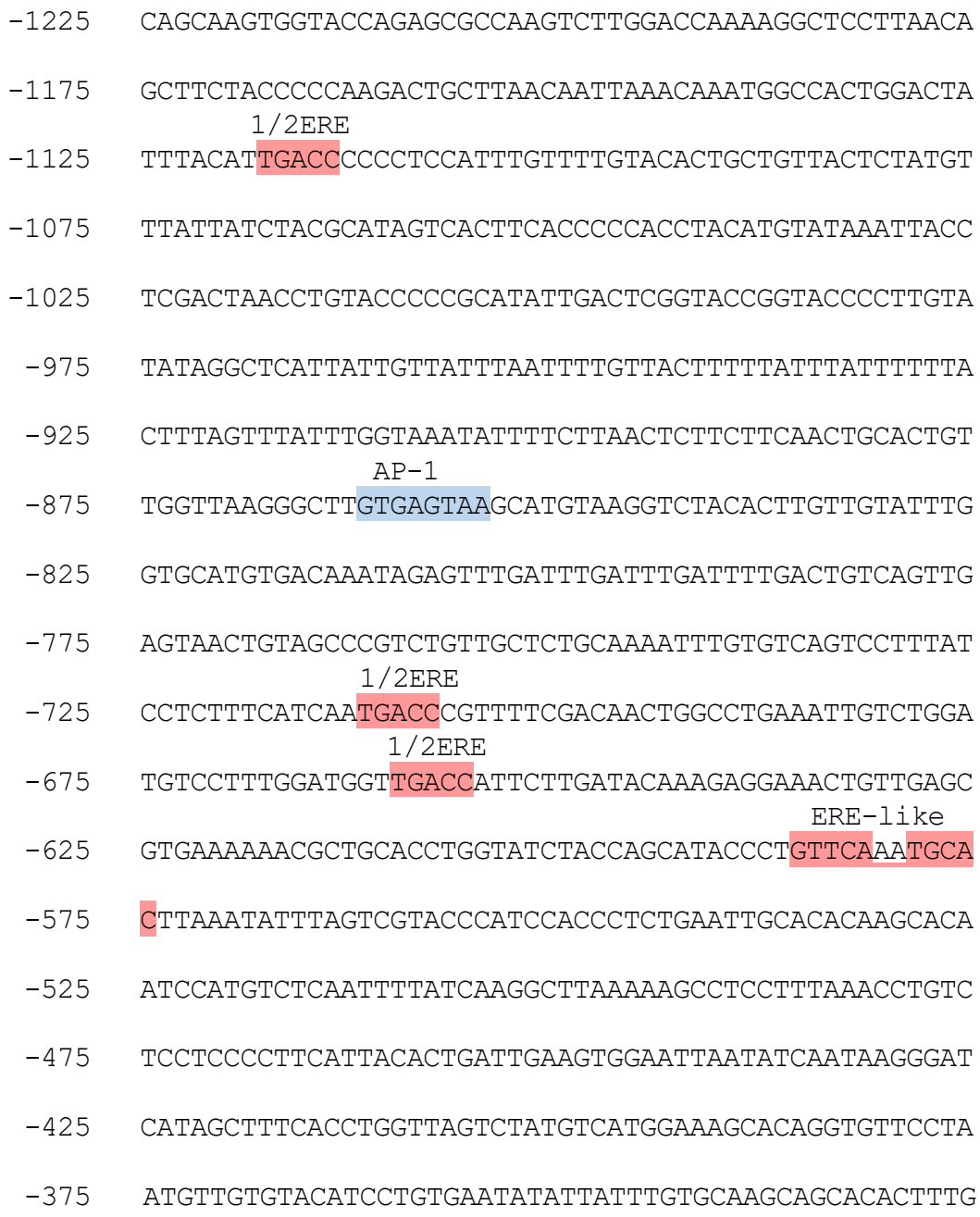

Fig. A. 5 (Continued)

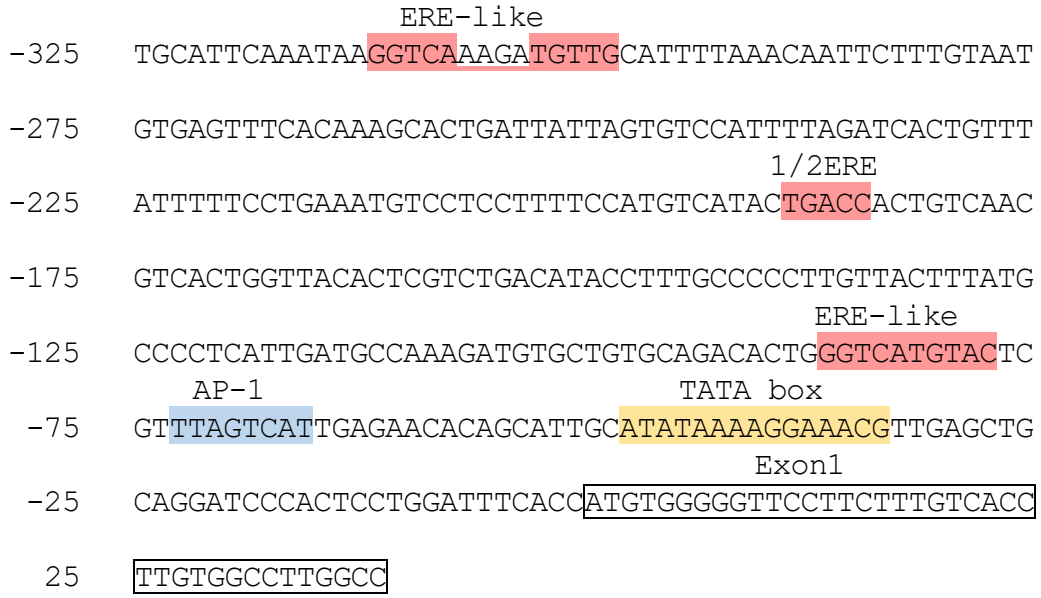

Fig. A. 5 (Continued)

-6155 CCTCGGCAAACTTAATGATGGTGTTGGAGTTAAGCTTAGCCATTCAGGTA

-6105 TGGGTGAGCAGGGAGTTCAGGAGGGGACTGAGCATGCACCCCTGAGGGGC

-6055 CCCTGTGTTGAGGATCAGCGAGGCAGATGTGTTGTTACCTACCCTTACCA

-6005 CCTGGGGGCGGCCCATCAGGAAGTCCACGATCCAGTTGCAGTGGGAGGTG

-5955 TTTAGTCCCAGGGTTCTTAGCTTGATGATGAGCTTTGAGGGTACTATGGT

-5905 GTTGAATGCTGAGCTGCAGTCAATGAATAGCAAAAGGTGTTCCTTTTGAA

Sp1

-5855 AGGGCAGTGTGGAGTGCAATAGAGATTGCAACATCTGTGGATCTGTTGGG

Sp1

-5805 GCGGTATGCAAATTGGAGTGGGTCTAGGGTTTCTGGGATAATGGTGTTGA

AP-1

-5755 TGTGAGCCATTATCAGCCTTTCAAAGCACTTCATGGCTACATATGTGAGT

-5705 TCTACGGGTTGGTAGTCATTTAAGCGTTGTTACCTTGGTGTTCTTGGGCA

1/2ERE

-5655 CAGTTCTATGGTGGTCTACTTGAAACATGTTGGTGTTACAGACTCGGTCA

-5605 GGGACAGGTTGAAAACGTCAGTGAAGACACTCGCCAATTGCATGCTCAGA

1/2ERE

-5555 GTACAAGTTCTGGTAATCCGTCTGGCCCTCTGGCCTTGTGAATGTTGACC

AP-1

-5505 TATTTAAAGGTCTTACTCACATCGGCTATGGAGAGCATGATCATACAGTC

-5455 GTTCGGAACAGCTGATGCTCTCATGCATGCTACAGTGTTGCTTGCCTCAA

AP-1

-5405 AGCAAGCATGGAAGTTATTTAGCTTGTCTGGTAGGCTTGTGTCACTGGGC

-5355 AGCTCGTGGCTGTGCTTCCCTTTTGTAGTCCGTAATAGTTTGCAGGCCCT

-5305 GCCACATCCAATGAGCGTCGGAGCCGGTGTAGTACAATTCAATCTCAGTC

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

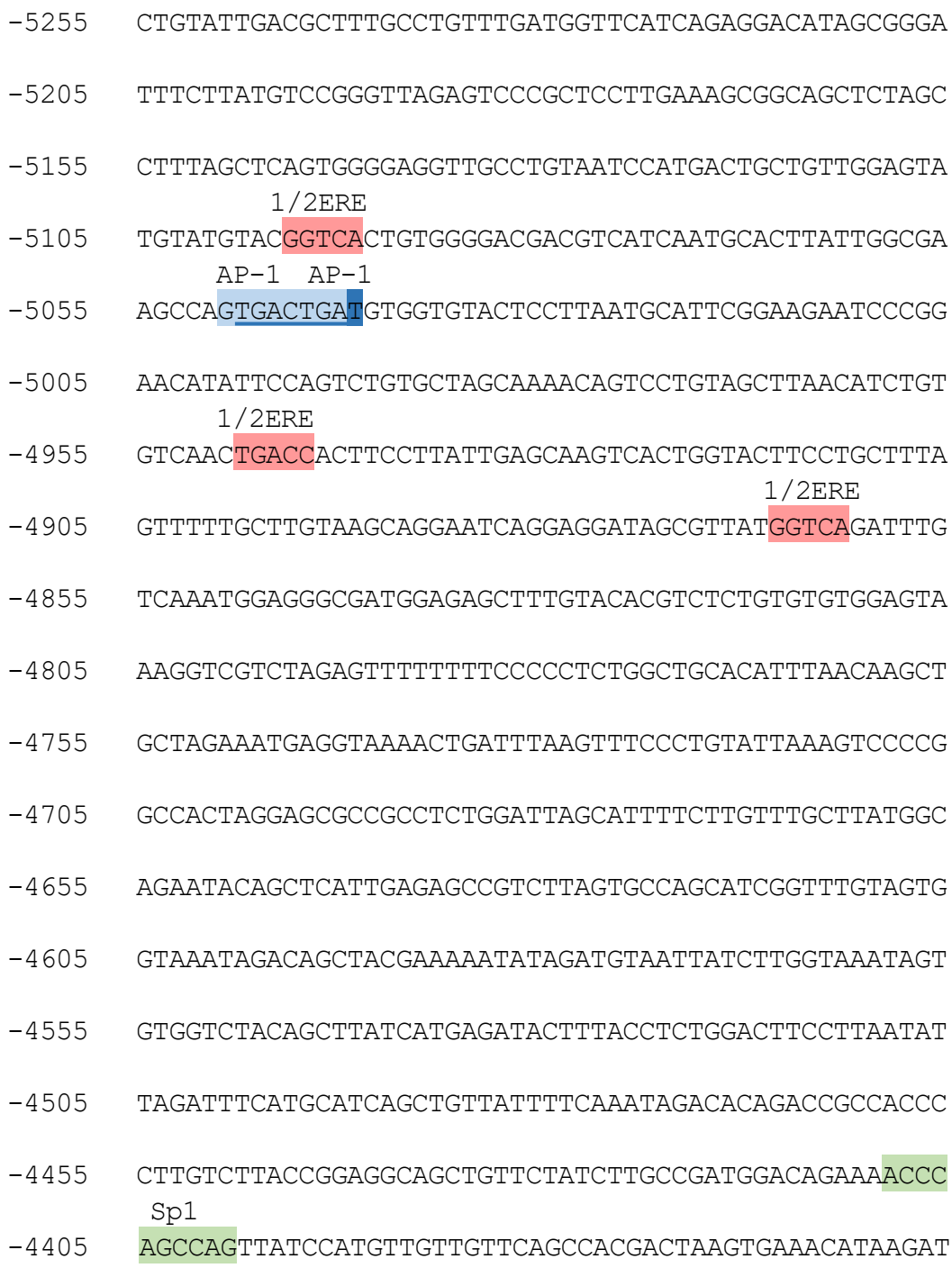

Fig. A. 6 (Continued)

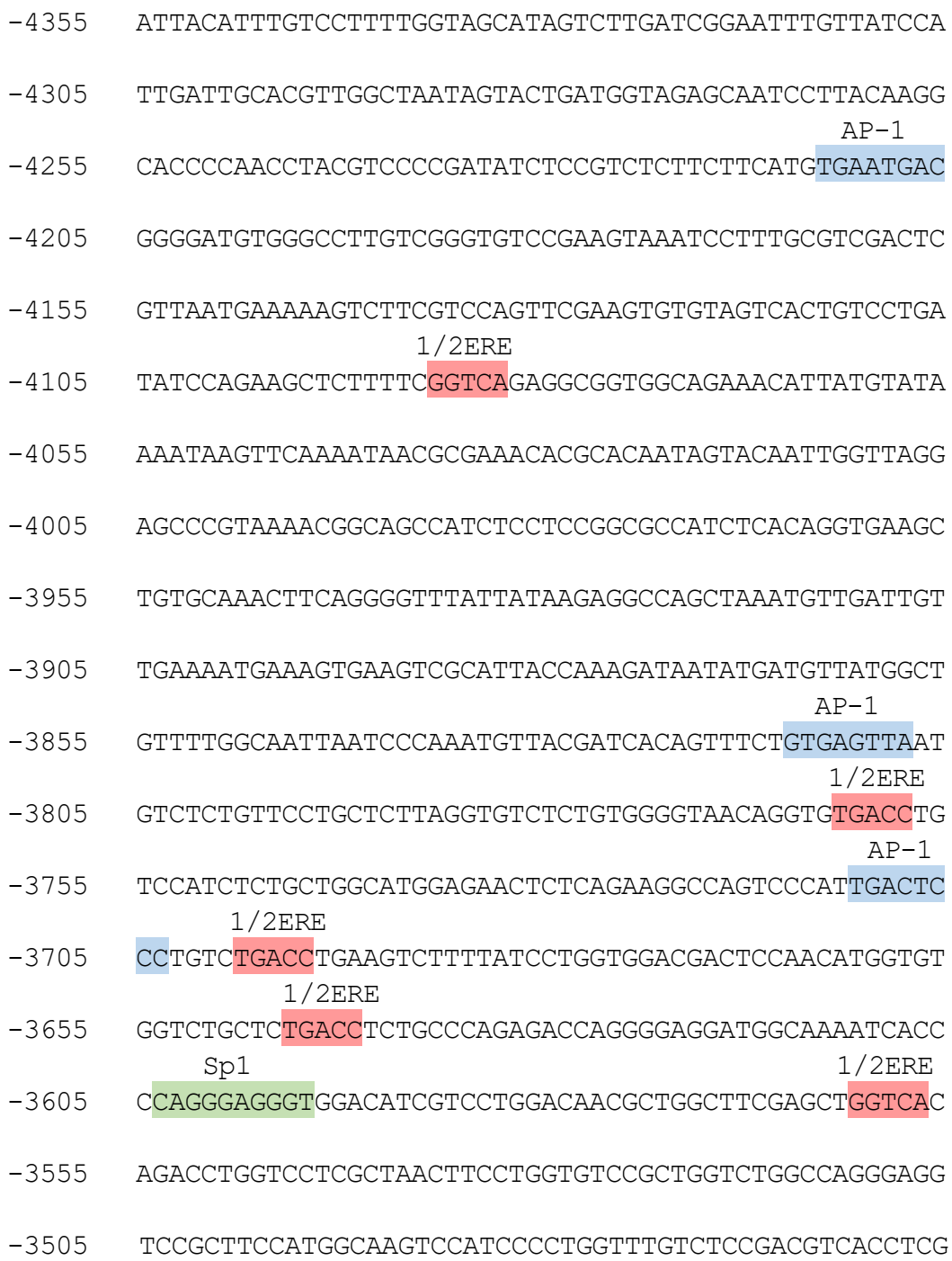

Fig. A. 6 (Continued)

-3455 CACGACTTCCAGTGGACCATCCGCCAGACCCTGGCGGCCAATCACAAGTG

-3405 GATGTCCAAGAGCGGTGTCCAGTGGCAGAGCTACGTACGGGAGGGCGTGT

Sp1

-3355 GGTCCTATCACGACCACCCGTTCTGGACAATGCCTCATGAGTACTGTGAC

1/2ERE

-3305 ATGGCGGGCGACGCGCCTGACCTCTACGCGACACTGCAGGGAGCCGACCT

-3255 GATTCTGTTTAAAGGAGACCTGAACTACAGGAAGCTGACTGGGGACAGGG

-3205 AGTGGGATCACATGGTGCCGTTTGATAGGGCACTGAGGGGATTCGGCCCA

-3155 GCTCCCCTGTGCAGTCTGAGGACTCTGAAGGCTAACATCCAGGTGGGGCT

Sp1 1/2ERE

-3105 CCAGCCGGGGCAGGGGGAGAACCTCAACACCCAGGACCCCAGCTGGATGA

-3055 CCAGCGGGAAGTACGCTGTGGTCCAGTTCTCCAGCCCTCACAGGGAACAG

-3005 TAGACCTGTCTGGGACAGGAAATGACGTCAATATGTTCATAATAAGTGGC

-2955 GTCATCATAATCAGAGGAAGTAAGGGCGTGAGCAGGGCCAGTGTACTTGT

-2905 AACCATATGCCAGGTTAGAGGTGTAGTTTGTTTTGTCGTAGAACTACCTA

-2855 ATGTATTTAAATGAGGAGAGTGGTTTAAAGGAGGATGATAGAGGATACTT

-2805 TAAAAAGAGGAGGGTAGTTAAATAGGGAGTGGCTGCAGATCCGACTTCTC

-2755 TGGACCCGTCGTCCCGACAGCTAAAGGTTCACAGCTTCTGTGAATGTGTG

-2705 GTAATCTCTACTTTACACACCATCTCTGTTCTATTTGTTCAGCTGCCCAG

-2655 AGAAGAAGCTACTCTGGCGAAAGGTGCTTGTTTAATAAAGTTAGATCAAA

-2605 CTCGAATCATTGTAAGATTACATAAAGATAATATTCTATAGAACCAAATA

Fig. A. 6 (Continued)

-2555 AAATAGCATAGTATAACATTACTGTATGACAAAAAACACCTAATTTCTTA

-2505 TATTTTAGGATGATTGTGCTCATCGTCACATTTTTCTCTCATGCAGTCTA

-2455 AACTCAACAGTGCACGCCACTCAGCAAAGGTATGCTACAGTTTAATACCA

-2405 TCTGCAAAAAAATGTGCTAAGGTACATAACTAATTCAAAACACTGTAGCC

-2355 TACTTCAAAACAACACTGTAGCCTACACTCAATAATATTTAAATGCATAT

-2305 GCCCATGAAACTGATTGCAAATGGTTGGCGTGCAGATTCTGCATGTATGT

AP-1

-2255 TTCACCGGTAAAACTTGGAAGAATTATCATTCACGATTGACAGTGAGAAA

Sp1 1/2ERE

-2205 TGCGAAGGGAGGGTGACCAGAAGTTTGGGTGTGCGTAGAGTTAAAGAAAC

-2155 ACATGCACAGAACGTGAGTGGGATCTTCAGCTCTGGGGAAAGGGATCCAG

Sp1 1/2ERE

-2105 TGGGATGGGGCTGTGACCTCCGGATCCCCAGCCTCTGCCATTTAAATAAT

-2055 GATCAACTGCTGGTTAGCAATCCATCCTGGCATAACTACAAAACAATGAC

-2005 GTTTACTTCTCCCATTTAACAAATGTGTTGACGGTTGTTTACCATCATTC

-1955 AGTACTAGGGTCGGGCTCTACCTCGATTCCAGTTCAAATCAGTTAATTTA

-1905 GAAACATTGAAATTCCAAGTCATGAATTGAAACATGCTTATCTGATCTTA

-1855 AGTGACTTTTTTCCAGACATATTATGTAATTTATGTAATGTTTTTGTCTC

-1805 CCCAACAGCGACTACACAAAATATAGATTTGAGACACATTTCATTCATTA

-1755 TCAGAGGCAATAAGATGCAAGGTGCAATTTGAACATTTACATTTTAAGTT

-1705 GGCCTGTCGTAAATAGTTTAGAATTGTTATTTTAAGTTGAGCCCTTATGT

Fig. A. 6 (Continued)

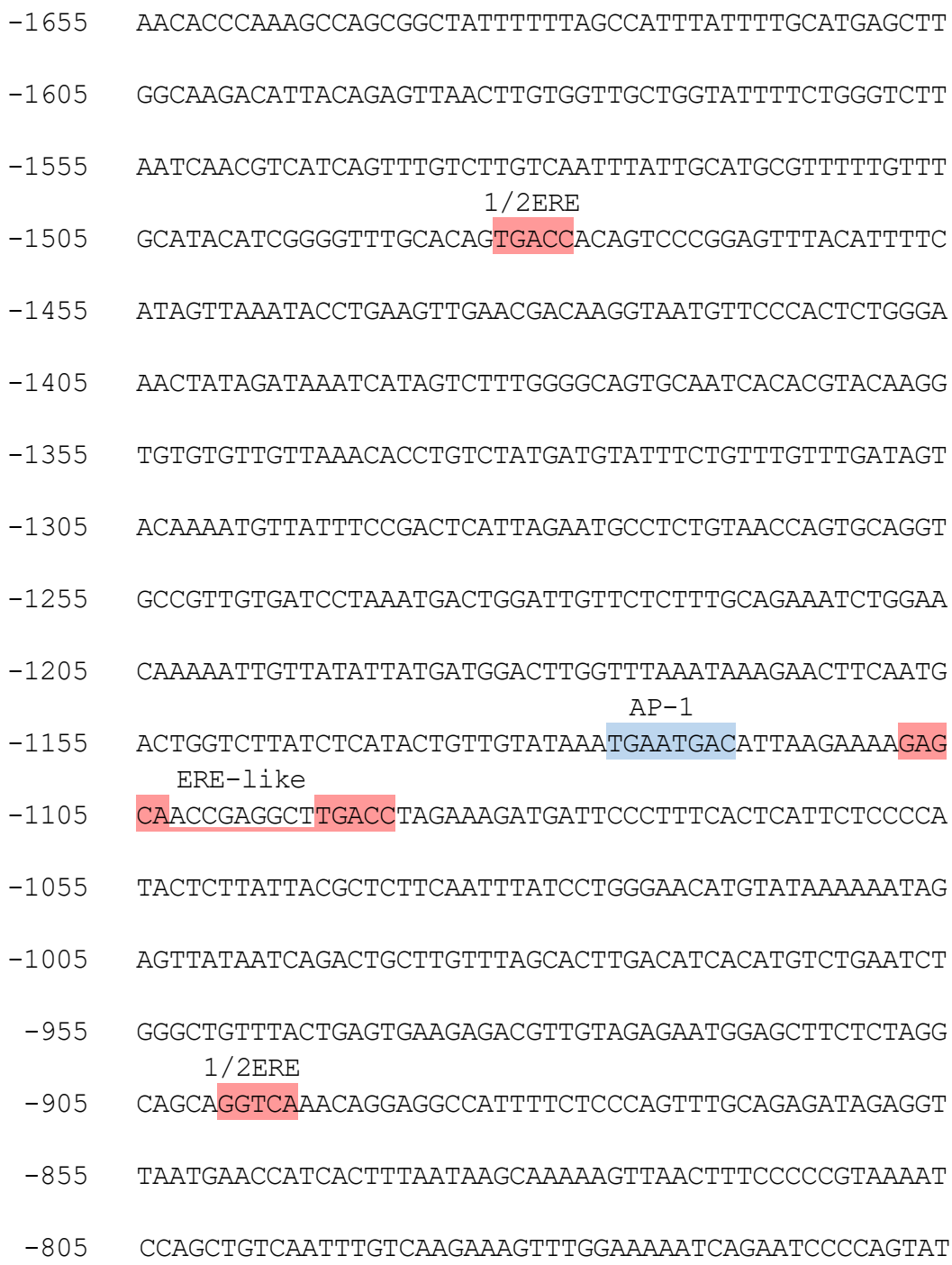

Fig. A. 6 (Continued)

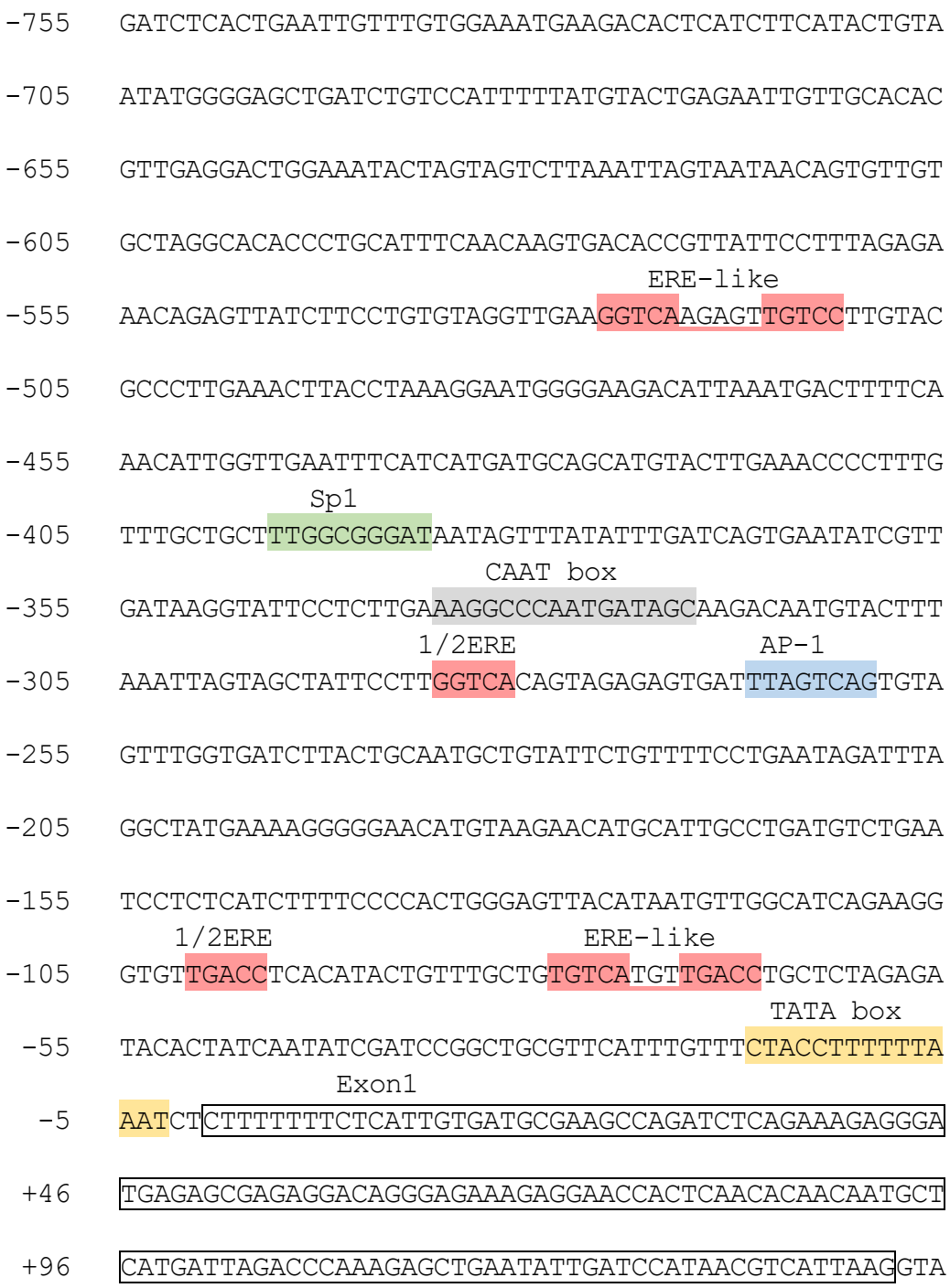

Fig. A. 6 (Continued)

Intron1

+146 AGTACTGTATATATGACGTCTTTGTGTGTATTTCATGTAATGGGTTCCTA

+196 TTTTATTGCTTCCTTCTCGTGGAATTGTTTTGTATAATTTTTCCATATTT

+246 GCACATAAATCCACTGATAGACACATTTAATAATGATTTAACTTCTTAGA

+296 AGAGTGAGTCAGAAAGGAGTGATATTCTGGGCCACGGCAGTACATGTAAC

+346 TAAATGACTGATAAGCATTCAGAGAGGAAGTCACACCAGGGTGTAACTCT

+396 CCTTGGAGCCTAGCAGCGTCCTCTAAAAGCTTTTCAGGGAAGAGAAAAAT

+446 AAGCTGCATTTTAGAAAAAAATGTAAGGGGACAGGGGAGTTGTTGGAGTG

+496 GTTGCCTCTTTCTTTCGTTCTCTCACTCTCCTTCTCTCTTGCTCTCTATC

+546 TCGAAATATCTGTCACTTGTTGTCCTTTCCTCCACCCCTCTCTCCCACAG

+596 CAGTGTCGATAGTTAAGGGAAAAGAGAAAAGCTCTGCCTCTCAAAAATAA

+646 GTGGGAGGGAGGAATGTTTTTGGGCTGTCTCCAGGCGGAACCTGGGAGGG

+696 AAAGAGAGAGCAAGGAGGGACGAGAAAAGAGAGAGAGAGAACCTAGTGAA

+746 TGCCTCTTCCCTTCCTCTTCCCAACAGCCAGTATTGAGTTGCTTAGCACG

+796 GGCTGTTAAGGAAGAAACAGAGCAAGAGAGGGACGAGAGAAAAGAGAGAG

+846 AGAAGACAGAACAGAGCCCTTCTCCCCTCCCACCCCTTAGTGAGCCAGTC

1/2ERE

+896 TAAACCAAGCTGCTTGTCACTGCTGTTGTTCTGTGAATGTGATGCTGGTC

+946 AGACAGTCCCATACGCAGATTTCCAAACCTCTCGGAGCTCCTCTCAGATC

Exon2

+996 CCGAACGACCCTGGAGAGCCACGTCATCTCCACCCCAAAACTCTCACCAC

Fig. A. 6 (Continued)

+1046 AGCAGCCGACCACCCCCAACAGCAACATGTACCCTGAGGAGACACGCGGA

+1096 GGTGGTGGGGCGGCCGCCTTTAACTACCTGGACGGAGGGTATGACTACAC

+1146 AGCCCCTGCCCAA

Fig. A. 6 (Continued)