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1 **Hepatic estrogen-responsive genes relating to oogenesis in cutthroat trout**  
2 **(*Oncorhynchus clarki*): the transcriptional induction in primary cultured**  
3 **hepatocytes and the *in vitro* promoter transactivation in responses to estradiol-17 $\beta$**   
4

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22 **Abstract (365 words)**

23 Estradiol-17 $\beta$  (E2) regulates transcription of estrogen-responsive genes *via* estrogen  
24 receptors (Esr). In many teleost species, choriogenin (*chg*), vitellogenin (*vtg*) and *esr*  
25 genes are transactivated by E2 in the liver. This study aimed i) to compare expression  
26 properties of all subtypes of these genes (*chg*: *chgHa*, *chgH $\beta$* , *chgL*; *vtg*: *vtgAs*, *vtgC*; *esr*:  
27 *esr1a*, *esr1b*, *esr2a*, *esr2b*) in response to estrogen stimulation, and ii) to confirm how  
28 each of four Esr subtypes is involved in the transcriptional regulation of these estrogen-  
29 responsive genes in cutthroat trout hepatocytes. In hepatocytes in primary culture, all *chg*  
30 and *vtg* subtype mRNA levels, and those of *esr1a*, were increased by E2 treatment ( $10^{-6}$   
31 M) at 24 and 72 h post initiation (hpi), but *esr1b*, *esr2a* and *esr2b* mRNA levels were not.  
32 Treatment of hepatocytes with various concentrations of E2 ( $10^{-11}$  ~  $10^{-6}$  M) induced dose-  
33 dependent increases in the levels of all *chg* and *vtg* subtype mRNAs at 24 and 72 hpi. At  
34 both time points, the lowest dose that induced a significant increase in the expression  
35 levels of mRNAs (LOEC) for E2 differed among the genes; LOECs were estimated as  
36  $10^{-11}$  M for *chgHa* at 24 hpi, as  $10^{-9}$  M for *vtgC* at 72 hpi, and as  $10^{-10}$  M for other mRNAs  
37 at both 24 and 72 hpi. Meanwhile, the levels of *esr1a* mRNA exhibited a dose-dependent  
38 increase at 24 and 72 hpi, but the LOEC shifted from  $10^{-9}$  M at 24 hpi to  $10^{-7}$  M at 72 hpi  
39 because of a decrease in mRNA levels at treatment groups exposed to high concentrations  
40 of E2. All Esr subtypes transactivated *chg*, *vtg* and *esr1a* promoters in the presence of E2  
41 *in vitro*. The activation levels indicated that promoter activity of *chgHa*  $\geq$  *vtgAs* > *chgH $\beta$*   
42 > *chgL*  $\geq$  *vtgC*  $\geq$  *esr1a* when mediated by Esr1a, *chgH $\beta$*  > *chgHa* > *chgHL* > *vtgAs*  $\geq$  *vtgC*  
43  $\geq$  *esr1a* by Esr1b, *chgH $\beta$*   $\geq$  *chgL* > *chgHa*  $\geq$  *vtgAs* > *vtgC* > *esr1a* by Esr2a, and *chgH $\beta$*   
44  $\geq$  *chgHa*  $\geq$  *vtgAs* > *chgL*  $\geq$  *vtgC* > *esr1a* by Esr2b. Collectively, different Esr subtypes  
45 were distinctly different in their ability to transactivate estrogen-responsive target genes,  
46 resulting in differential expression of *chg*, *vtg* and *esr1a* genes in the estrogen-exposed  
47 hepatocytes.

48

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

50

## 51 **1. Introduction**

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

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

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

76 various vertebrates, including teleosts. The presence of two forms of ESR, designated as  
77 ESR1 and ESR2, has been confirmed in most vertebrates, while most teleosts exhibit at  
78 least three distinct subtypes of Esrs, i.e., Esr1 (also designated as Er $\alpha$ ), Esr2a (also known  
79 as Ery or Er $\beta$ 1) and Esr2b (also known as Er $\beta$  or Er $\beta$ 2) (Choi and Habibi, 2003; Halm et  
80 al., 2004; Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2004; Nagler et al., 2007).  
81 Of the *esr* subtypes, hepatic *esr1* (*esr1a* in salmonids) expression is high in the liver of  
82 vitellogenic females (Nagler et al., 2012; Sabo-Attwood et al., 2004) and induced by E2  
83 treatment (Boyce-Derricott et al., 2009; Filby and Tyler, 2005; Sabo-Attwood et al., 2004).  
84 In rainbow trout (*Oncorhynchus mykiss*), an additional *esr1* subtype (*esr1b*) has been  
85 identified (Nagler et al., 2007), considered to be a minor subtype in terms of its mRNA  
86 levels in the liver of vitellogenic females and E2-treated fish (Boyce-Derricott et al., 2009;  
87 Nagler et al., 2012). So far, the functionality of Esr1b protein (ligand-binding and  
88 estrogen-dependent transactivation qualities) has not been confirmed yet. As for Esr2  
89 subtypes, recent studies using zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*)  
90 have demonstrated their possible involvement in the expression of *vtg* (Griffin et al.,  
91 2013; Nelson and Habibi, 2010). Other than these studies, both on cyprinids, functional  
92 information on teleost Esr subtypes pertaining to the regulation of hepatic expression of  
93 estrogen-responsive genes important for oogenesis (i.e., *chg*, *vtg* and *esr1* etc.) has been  
94 quite limited.

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

100 ChgH $\beta$  (Fujita et al., 2008; Hyllner et al., 2001; Westerlund et al., 2001). Meanwhile,  
101 highly evolved acanthomorph species are likely to express three Vtg subtypes, VtgAa  
102 (previously termed VtgA), VtgAb (previously termed VtgB), and VtgC (Hiramatsu et al.,  
103 2005). Salmonids, on the other hand, express multiple copies of salmonid-type A Vtg  
104 (VtgAs) alongside a VtgC orthologue (Buisine et al., 2002; Mushirobira et al., 2018;  
105 Trichet et al., 2000).

106 As described above, E2 regulates the expression of oogenesis-related genes, such as  
107 *chg*, *vtg* and *esr* subtypes, in the liver of teleosts during vitellogenesis. However, it is  
108 unclear how E2 can differentially regulate the expression profiles of these genes.  
109 Resolving this issue leads to further our understanding of oocyte development, and of the  
110 molecular mechanisms underlying the transcriptional regulation of multiple genes by E2.  
111 To date, many studies have demonstrated responses of *chg*, *vtg* and *esr1* genes to E2  
112 stimulation. Because each of these studies used different species and/or did not consider  
113 the multiplicity of *chg*, *vtg* and *esr*, it has been difficult to obtain an integrated view of  
114 transcriptional regulation of these genes in response to E2 stimulation; it is therefore  
115 needed to perform such a study in a single species and include all subtypes of the genes  
116 of interest. Recently, the two promoters of *vtgAs* (1 and 2) and one promoter of *vtgC* for  
117 cutthroat trout (*Oncorhynchus clarki*) have been cloned and analyzed (Mushirobira et al.,  
118 2018).

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

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

128

## 129 **2. Materials and methods**

### 130 *2.1. Experimental fish and tissue sampling*

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

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

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

148 blood sample was stored at  $-80^{\circ}\text{C}$  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  $\mu\text{g}$  total RNA  
154 was reverse-transcribed by PrimeScript II 1<sup>st</sup> strand cDNA Synthesis Kit (Takara Bio,  
155 Shiga, Japan), according to the manufacturer's instructions. The resulting cDNA  
156 templates were stored at  $-30^{\circ}\text{C}$ . 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  $\mu\text{l}$ , which contained 1  $\mu\text{l}$  1<sup>st</sup>-strand cDNA, 5  $\mu\text{l}$  PrimeSTAR® Max premix,  
161 1  $\mu\text{l}$  of each 1  $\mu\text{M}$  forward and reverse primer and 2  $\mu\text{l}$  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  $98^{\circ}\text{C}$  for 10 s,  $55^{\circ}\text{C}$  for 5 s and  $72^{\circ}\text{C}$  for 3 min. The PCR  
164 products were separated by electrophoresis on 1% agarose gels, excised from the gels,  
165 and purified by GENE CLEAN 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  $37^{\circ}\text{C}$  on an agar plate containing  
171 ampicillin and tetracycline, followed by selection of colonies of interest by blue-white



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

177

### 178 *2.3. Molecular cloning of chg, vtg and esr1a gene promoters*

179 Genomic DNA was extracted from whole blood as described in Mushirobira et al.  
180 (2018). A genome walking library was used for amplification of *chgH $\beta$* , *chgL* and *esr1a*  
181 gene promoters. Four genome walking libraries were made from genomic DNA using the  
182 GenomeWalker Universal Kit (Takara Bio) according to manufacturer's protocol. Gene  
183 specific primers (GSP, sense primer) were designed within the coding region of each gene  
184 (Table 1). Primary PCR was carried out after mixing 5  $\mu$ l of PrimeSTAR Max Premix  
185 (Takara Bio), 0.2  $\mu$ l of adaptor primer (AP, antisense primer) 1, 0.2  $\mu$ l of 10  $\mu$ M GSP (for  
186 *chgH $\beta$* : chgH $\beta$ -GW(Genome Walker)-R1, for *chgL*: chgL-GW-R1, for *esr1a*: esr1a-GW-  
187 R1), and 0.2  $\mu$ l of genomic DNA template; 7 cycles of 98°C for 10 s and of 72°C for 3  
188 min were then run, followed by 32 cycles of 98°C for 10 s and of 68°C for 3 min. Nested  
189 PCR was carried out using 5  $\mu$ l of PrimeSTAR Max Premix, 0.2  $\mu$ l of AP1, 0.2  $\mu$ l of 10  
190  $\mu$ M GSP-2 (for *chgH $\beta$* : chgH $\beta$ -GW-R2, for *chgL*: chgL-GW-R2, for *esr1a*: esr1a-GW-  
191 R2), and 0.2  $\mu$ l of the PCR product from the first round of amplification; after running 5  
192 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  
193 s and at 68°C for 3 min.

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

196 GCF\_002163495.1). PCR was carried out as follows: 5  $\mu$ l of PrimeSTAR Max Premix, 1  
197  $\mu$ l of each 2  $\mu$ M forward and reverse primer, and 0.2  $\mu$ l 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.iu.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<sub>2</sub>PO<sub>4</sub>, 0.42 mM Na<sub>2</sub>HPO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>, 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<sub>2</sub> (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  $3 \times 10^5$  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  $-80^{\circ}\text{C}$  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 ( $10^{-11} \sim 10^{-6}$  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 *chgs*, *vtgs*, *esr1a* and elongation factor 1- $\alpha$  (*efl- $\alpha$* ;  
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

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

274

## 275 2.8. Reporter gene assays

276 The reporter plasmid containing either one of six promoters (*chgHa*, *chgH $\beta$* , *chgL*,  
277 *vtgAs*, *vtgC* and *esr1a*) and the expression plasmid containing either one of four *esr*  
278 subtypes (*esr1a*, *esr1b*, *esr2a*, *esr2b*) were co-transfected into HeLa cells, and the  
279 transactivation of the reporter gene was induced in the presence or absence of 1  $\mu$ M E2  
280 ( $10^{-6}$  M).

281 Construction of plasmid vectors for reporter gene assays was done using In-Fusion  
282 HD Cloning Kit (Takara Bio) according to the manufacturer's instructions. Primer sets  
283 (Table 1) for In-Fusion cloning were designed using the Primer Design tool for In-  
284 Fusion® HD Cloning Kit ([http://www.takara-](http://www.takara-bio.co.jp/infusion_primer/infusion_primer_form.php)  
285 [bio.co.jp/infusion\\_primer/infusion\\_primer\\_form.php](http://www.takara-bio.co.jp/infusion_primer/infusion_primer_form.php)). The promoter regions (2 kb in  
286 size) of *chgHa*, *chgH $\beta$* , *chgL*, *vtgAs*, *vtgC* and *esr1a* in pGEM T-Easy vector were  
287 subcloned into pGL4.10[*luc2*] Vector (Promega). The open reading frames of *esr1b*, *esr2a*  
288 and *esr2b* were subcloned into pcDNA3.1(+) Vector (Thermo Fisher Scientific). The  
289 *Esr1a* expression plasmid, pcDNA3.1-*Esr1a*, has been described previously (Mushirobira  
290 et al., 2018). The subcloned plasmids were purified for transfection using PureLink  
291 HiPure Plasmid Filter Midiprep Kit (Thermo Fisher Scientific).

292 Reporter gene assays were carried out as described previously (Mushirobira et al.,  
293 2018) with some modifications. HeLa cells were seeded in 24-well Falcon Primaria  
294 Multiwell plates at  $3 \times 10^4$  cells/well in phenol-red free Dulbecco's Modified Eagle  
295 Medium (DMEM; Sigma-Aldrich) supplemented with 10% charcoal/dextran treated FBS  
296 (Hyclone, Logan, UT, USA). The cells were pre-incubated for 24 h at 37°C under 95%  
297 air, 5% CO<sub>2</sub> and 100% humidity. After pre-incubation, the cells were transfected with 400  
298 ng of either the promoter-harboring pGL4.10 (pGL4.10-*chgHa*, pGL4.10-*chgHβ*,  
299 pGL4.10-*chgL*, pGL4.10-*vtgAs*, pGL4.10-*vtgC* and pGL4.10-*esr1a*) or the empty  
300 pGL4.10 (negative promoter-construct control), 200 ng of the *esrs*-harboring pcDNA3.1  
301 (pcDNA3.1-*esr1a*, pcDNA3.1-*esr1b*, pcDNA3.1-*esr2a* and pcDNA3.1-*esr2b*), and 100  
302 ng of pRL-TK Vector (internal control to normalize for variation in transfection efficiency  
303 between wells, Promega) using X-tremeGENE HP DNA Transfection reagent (Roche)  
304 according to the manufacturer's instructions. Four h after commencement of transfection  
305 at 37°C, the cells were treated with 1 μM E2 or vehicle (ethanol). The amount of ethanol  
306 in the medium did not exceed 0.1%. After a further 40 h of incubation at 37°C, the cells  
307 were collected to measure luciferase activities using Dual-Luciferase Reporter Gene  
308 Assay System (Promega) by the Luminescanner-JNR (ATTO, Tokyo, Japan). All  
309 incubations were run in quadruplicate.

310

### 311 2.9. Statistics

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

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

321

### 322 **3. Results**

#### 323 *3.1. Molecular cloning of *esr1b*, *esr2a* and *esr2b**

324 Cutthroat trout *esr1b*, *esr2a* and *esr2b* cDNAs (*esr1b*: 1782 bp, *esr2a*: 2324 bp,  
325 *esr2b*: 2354 bp) encoding the cutthroat trout Esr1b, Esr2a and Esr2b were isolated and  
326 sequenced (GenBank accession no. *esr1b*: LC577088, *esr2a*: LC577089, *esr2b*:  
327 LC577090). The open reading frames of *esr1b*, *esr2a* and *esr2b* encoded 556, 594 and  
328 606 amino acids, respectively. The deduced cutthroat trout Esr1b, Esr2a and Esr2b  
329 proteins exhibited domain features typical of the estrogen receptor (A/B, C, D, E, F  
330 domain). The Esr1b, Esr2a and Esr2b sequences shared high similarity (Esr1b: 99.3%,  
331 Esr2a: 99.3% Esr2b: 98.8%) with the homologous sequences of rainbow trout (GenBank  
332 Accession No. Esr1b: NP\_001118030.1, Esr2a: NP\_001118225.1, Esr2b:  
333 NP\_001118042.1).

334

#### 335 *3.2. Hormone Treatment*

##### 336 *3.2.1. Experiment 1: effects of continuous treatment with E2*

337 In *chgHa*, *chgH $\beta$* , *chgL*, *vtgAs*, *vtgC* and *esr1a* mRNA levels, significant interaction  
338 effects of time  $\times$  dose were observed ( $P < 0.001$ ), as well as the significant main effects  
339 of time ( $P < 0.001$ ) and dose ( $P < 0.001$ ), respectively. In both *esr2* subtypes, interaction

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

342 Levels of *chgHa*, *chgH $\beta$* , *chgL*, *vtgAs*, *vtgC* and *esr1a* mRNA in E2-treated  
343 hepatocytes were significantly higher than those in the corresponding control group, both  
344 at 24 hpi and 72 hpi (Fig. 1). In the E2 culture, *chgH $\beta$* , *chgL*, *vtgAs*, *vtgC* and *esr1a* mRNA  
345 levels significantly increased from 24 hpi to 72 hpi, while *chgHa* mRNA levels did not.  
346 Levels of *esr1b* mRNA were undetectable in all groups at 24 hpi and 72 hpi. Treatment  
347 with E2 did not affect mRNA levels of *esr2a* at 24 hpi and 72 hpi when compared to  
348 levels in the corresponding control groups. Levels of *esr2b* mRNA in E2 exposure groups  
349 were significantly lower than those in the corresponding control groups at 24 and 72 hpi.  
350 In cultures supplemented with E2, *esr2b* mRNA levels decreased from 24 to 72 hpi.

351

### 352 3.2.2. Experiment 2: effects of various doses of E2

353 Exposure of hepatocytes to various doses of E2 yielded significant interaction effects  
354 of time  $\times$  dose in all mRNAs ( $P < 0.001$ : *chgHa*, *chgH $\beta$* , *chgL*, *vtgC*, *esr1a*;  $P < 0.01$ :  
355 *vtgAs*), in addition to the main effect of each factor ( $P < 0.001$ ).

356 In hepatocytes cultured for 24 and 72 h, *chgHa*, *chgH $\beta$* , *chgL*, *vtgAs* and *vtgC*  
357 mRNAs levels were upregulated by E2 treatments in a dose-dependent manner (Fig. 2).  
358 The lowest doses that induced a significant increase in the expression level (LOEC) for  
359 *chgs* and *vtgs* were  $10^{-10}$  M at both sampling points, except for those of *chgHa* at 24 hpi  
360 ( $10^{-11}$  M) and *vtgC* at 72 hpi ( $10^{-9}$  M). The *chgHa*, *chgH $\beta$*  and *chgL* mRNAs levels in the  
361 high E2 concentration groups (*chgHa*, *chgH $\beta$* , *chgL* and *vtgAs*:  $10^{-7}$ ,  $10^{-6}$  M E2; *vtgC*:  $10^{-6}$   
362 M E2) increased from 24 hpi to 72 hpi; treatment means of all target gene mRNAs,  
363 excluding that encoding *vtgAs*, exhibited significant differences. At the 24 hpi sample  
364 collection, *esr1a* mRNA levels were upregulated by E2 treatments in a dose-dependent  
365 manner and yielded an LOEC *esr1a* of  $10^{-9}$  M E2. Unlike other target genes, *esr1a* mRNA



366 levels in cultures exposed to  $10^{-9} \sim 10^{-6}$  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 chgHa, chgH $\beta$ , 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: GGTC A or TGACC) were predicted in  
378 *chgHa* 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 *chgH $\beta$*  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 (TACTGAC) 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 (TACTGAC), -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.

414 The *vtgAs* promoter sequence (2872 bp) was located upstream of the translation  
415 initiation site and 225 bp of the transcribed gene (Fig. A. 4, Accession No. LC577095).  
416 Unlike the other tested promoters, *vtgAs* promoter contained the consensus palindrome  
417 ERE at position -690 to -678 (GGTCAagcTGACC). Putative sequences of seven ERE-  
418 like sequences and six 1/2 ERE were found at the following positions: ERE-like: -2767  
419 to -2754 (GGTGAaatcTGACCT), -2758 to -2749 (TGACCTCTCC), -2168 to -2156  
420 (GGTCAggtTGATG), -1027 to -1014 (GGTCAagttTGATG), -612 to -599  
421 (GGACAagctTGGAC), -509 to -496 (GATCAatacTGATC) and -233 to -224  
422 (TGACCTCTCC); 1/2ERE: -2713 to -2709, -2058 to -2054, -2005 to -2001, -1987 to -  
423 1983, -1197 to -1193 and -1175 to -1171. The putative AP-1 and Sp1 sites were identified  
424 at the following positions: AP-1: -2057 to -2050 (GTCAGTAA), -1760 to -1753  
425 (TGAATGAC), -1746 to -1739 (TGTCTCAC), -1582 to -1575 (GTGTGTCA), -1434 to  
426 -1427 (GTGAGTGA), -1167 to -1160 (CTGAGTAA), -948 to -941 (TACTCAT), -830  
427 to -823 (TGTCTCAC), -721 to -714 (TGACTGAC) and -274 to -267 (GTGATTCT);  
428 Sp1: -2774 to -2765 (GAGGCGAGGT), -2349 to -2340 (GGGGCTGGGA), -2209 to -  
429 2200 (ACGGCGTGGT), -2092 to -2083 (GGGGCAGGCA), -1092 to -1083  
430 (ACCCTGCCCA), -1009 to -1000 (GGGGGGTGGT) and -201 to -192  
431 (GGGGCAGGTT). The predicted TATA box and CAAT box were found at -47 to -33  
432 (CTTTAAAAGGCGGAC) and -189 to -174 (CCTAACCTATGGGTGT), respectively.

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

438 (GTTCA<sup>ttg</sup>gTGCCA), -1324 to -1315 (GAGCA<sup>ttc</sup>TGACC), -586 to -575  
439 (GTTCA<sup>aa</sup>TGCAC), -312 to -309 (GGTCA<sup>aaga</sup>TGTTG) 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 (T<sup>t</sup>ACTCAT), -2351 to -2344  
443 (A<sup>t</sup>ACTCAC), -1633 to -1626 (A<sup>t</sup>GAGACA), -1562 to -1555 (G<sup>t</sup>GAGCAA), -862 to -  
444 855 (G<sup>t</sup>GAGTAA) and -73 to -66 (T<sup>t</sup>AGTCAT); Sp1: -2221 to -2212 (A<sup>t</sup>ACTGCCCC).  
445 The conserved TATA box was identified at position -48 to -34 (T<sup>t</sup>TATAAAACTGGCCA).

446 A total of 6155 bp of sequence upstream of the translation initiation site and a 1041  
447 bp transcribable region were isolated and sequenced 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 (GAGCA<sup>accgagc</sup>TTGAC), -525 to -511  
451 (GGTCA<sup>aagagt</sup>TGTCC) and -79 to 67(TGTCA<sup>tgt</sup>TGACC); 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 (G<sup>t</sup>GAGCCA), -5493 to -5486 (T<sup>t</sup>ACTCAC), -5368 to  
457 -5361 (T<sup>t</sup>GTGTCAC), -5050 to -5043 (G<sup>t</sup>GACTGA), -5049 to -5042 (T<sup>t</sup>GACTGAT), -  
458 4213 to -4206 (T<sup>t</sup>GAA<sup>t</sup>GAC), -3815 to -3808 (G<sup>t</sup>GAGTTA), -3711 to -3704  
459 (T<sup>t</sup>GACTCCC), -2213 to -2206 (G<sup>t</sup>GAGAAA), -1126 to -1119 (T<sup>t</sup>GAA<sup>t</sup>GAC) and -267  
460 to -260 (T<sup>t</sup>AGTCAG); Sp1: -5855 to -5846 (A<sup>g</sup>GGCAGTGT), -5808 to -5799  
461 (G<sup>g</sup>GGC<sup>g</sup>GTAT), -4409 to -4400 (A<sup>c</sup>CCAGCCAG), -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 *chgHa*, *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 ( $P < 0.001$ : *chgHa*, *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 *chgHa* and *vtgAs* promoters were significantly higher than  
485 those in HeLa cells provided with the *chgHβ* promoter. The reporter activities of the

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

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

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

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

507

#### 508 **4. Discussion**

509 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 al., 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 *chgs*, *vtgs* 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 *esrs* (i.e., *esr1b*, *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., *chgHa*, *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 ( $10^{-11} \sim 10^{-6}$  M)  
549 on the expression of these six genes. Transcript levels of *chgs* and *vtgs* 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 *chgHa* 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 *chgHa* 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, *chgHa*, *chgHβ*, *chgL*, *vtgAs* and *vtgC* mRNA levels increased or



558 were maintained at high values in high-dose E2 cultures, both in Experiments 1 (culture  
559 with replacement of medium) and 2 (culture without replacement of medium). Meanwhile,  
560 *esr1a* mRNA levels in high-dose E2 incubations increased in Experiment 1 but decreased  
561 in Experiment 2 from 24 hpi to 72 hpi. Thus a difference in the way of E2 supplementation  
562 possibly caused the opposite *esr1a* response to E2 between the two experiments, similar  
563 to a study using primary cultured hepatocytes of rainbow trout (Flouriot et al., 1996). In  
564 said study, high *esr1a* mRNA levels were maintained from 24 to 72 h following  
565 supplementation with  $10^{-6}$  M E2 and replacement of E2-containing culture medium every  
566 24 h, but levels decreased when the medium was not replaced – those findings are in good  
567 agreement with the results of Experiment 1 and Experiment 2 in our study on cutthroat  
568 trout. It has been shown that hepatocytes of *Xenopus laevis* in primary culture rapidly  
569 metabolize E2 in culture medium (Tenniswood et al., 1983). Although such analysis was  
570 not performed in the present study, rapid metabolism of E2 in the medium could thus be  
571 a cause of the time-dependent decrease of *esr1a* mRNA expression. Adhesion of E2 to  
572 the wall of the plate could be another potential cause for this decrease.

573 Numbers of ERE, ERE-like, 1/2 ERE, AP-1 and Sp1 sites in *chgHa*, *chgHβ*, *chgL*,  
574 *vtgAs*, *vtgC* and *esr1a* promoters were different, suggesting these promoters have  
575 differential transcriptional properties. An ERE consensus sequence was identified only in  
576 the *vtgAs* promoter while all promoters had ERE-like and 1/2 ERE sequences. In teleosts,  
577 ERE-like and 1/2 ERE sites elicit a significant increase in estrogen-dependent synthesis  
578 of reporter protein (Le Drean et al., 1995; Menuet et al., 2004; Teo et al., 1998). Thus,  
579 aside from the consensus ERE, ERE-like and 1/2 ERE sites are likely to be responsible  
580 for the E2-induced expression of *chg*, *vtg* and *esr1a* genes. This was also supported by  
581 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  $chgHa \geq vtgAs > chgH\beta > chgL > vtgC > esr1a$ , while that through *esr1b* was  $chgH\beta$   
620  $> chgHa > chgL > vtgAs > vtgC \geq esr1a$ . 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

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

636

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647

### 648 **Declarations of interest**

649 None

650

### 651 **Appendix A. Supplementary data**

652

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836

### 837 **Figure captions**

838 Fig. 1 Effects of continuous treatment of primary cultured hepatocytes of male cutthroat  
839 trout with estradiol-17 $\beta$  (E2, black columns) or a control solvent (C, white columns) on  
840 the transcript levels of choriogenin (*chg*: *chgHa*, *chgH $\beta$*  and *chgL*), vitellogenin (*vtg*:  
841 *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-17 $\beta$  (E2, black columns) or a control solvent (C, white columns)  
850 on the transcript levels of choriogenin (*chg*: *chgHa*, *chgH $\beta$*  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  $\sim$  -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-17 $\beta$  (E2). Hela cells were co-  
862 transfected with two constructs: one *esr* expression vector (*esr1a*, *esr1b*, *esr2a* or *esr2b*)  
863 and one gene promoter (*chgHa*, *chgH $\beta$* , *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  $\mu$ M 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 ( $P < 0.05$ ).

1 **Highlights**

- 2 • Choriogenin, vitellogenin and estrogen receptor-1a genes were estrogen-responsive
- 3 • These estrogen responsive genes responded differentially to estradiol-17 $\beta$  (E2)
- 4 • 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	GCCATCTCACCCAGAAACT	Forward	Cloning for <i>esr1b</i>
esr1b-R	ACTCTCACCTCCACAAATGTCA	Reverse	Cloning for <i>esr1b</i>
esr2a-F	CACGGATGGATTGCTACTCC	Forward	Cloning for <i>esr2a</i>
esr2a-R	GAACAGTGCTAATGCCTGAAAGG	Reverse	Cloning for <i>esr2a</i>
esr2b-F	CACTGATGACAGGCTTGGCAG	Forward	Cloning for <i>esr2b</i>
esr2b-R	AATGGTCACAGACACTGATAAAG	Reverse	Cloning for <i>esr2b</i>
esr1b-F-qPCR	AGCCTCCCCAGCCAGTCTATC	Forward	qPCR for <i>esr1b</i>
esr1b-R-qPCR	TGAGCCTGACCCTGACTCCAC	Reverse	qPCR for <i>esr1b</i>
esr2a-F-qPCR	TCCATTGTCTCTGCACCATCG	Forward	qPCR for <i>esr2a</i>
esr2a-R-qPCR	TTCTCAGAGGCTTACTGCTCTC	Reverse	qPCR for <i>esr2a</i>
esr2b-F-qPCR	TCCAAACGAGGCCTGTCATTC	Forward	qPCR for <i>esr2b</i>
esr2b-R-qPCR	TCTTCATGCTAGAGAGGTGCTG	Reverse	qPCR for <i>esr2b</i>
chgH $\alpha$ -promoter-F	ACTCCCCAACCTTCCTCTT	Forward	Cloning of <i>chgHa</i> promoter
chgH $\alpha$ -promoter-R	GTTCTGAGGGGGTTGGTAAGG	Reverse	Cloning of <i>chgHa</i> promoter
chgH $\beta$ -GW-R1	CCCTGGTTTTTCCAAGTAAATCTGAG	Reverse	Genome walking for <i>chgH<math>\beta</math></i>
chgH $\beta$ -GW-R2	GAACAGTGCTAATGCCTGAAAGG	Reverse	Genome walking for <i>chgH<math>\beta</math></i>
chgL-GW-R1	CTGACGATTGGGTCTGAAGGGTTGCTG	Reverse	Genome walking for <i>chgL</i>
chgL-GW-R2	TTTACTGAAGGGTGGCCAATTCTGAG	Reverse	Genome walking for <i>chgL</i>
esr1a-GW-R1	CTGACGATTGGGTCTGAAGGGTTGCTG	Reverse	Genome walking for <i>esr1a</i>
esr1a-GW-R2	TTTACTGAAGGGTGGCCAATTCTGAG	Reverse	Genome walking for <i>esr1a</i>
vtgAs-promoter-1-F	AAGATGCAATTCGTCAGACTTCG	Forward	Cloning of <i>vtgAs</i> promoter 1
vtgAs-promoter-1-R	AAAGTTAACAGATTGACTCGCTACA	Reverse	Cloning of <i>vtgAs</i> promoter 1
vtgC-promoter-F	ATCAAACCATGCAATAATCTGAGTC	Forward	Cloning of <i>vtgC</i>
vtgC-promoter-R	GGCCAAGGCCACAAGGT	Reverse	Cloning of <i>vtgC</i>
pcDNA3.1-linealize-F	GTTTAAACCCGCTGATCA	Forward	Subcloning of <i>esr1b</i> , <i>esr2a</i> and <i>esr2b</i>
pcDNA3.1-linealize-R	GCTAGCCAGCTTGGGTCT	Reverse	Subcloning of <i>esr1b</i> , <i>esr2a</i> and <i>esr2b</i>
esr1b-pGL3.1-infusion-F	CCCAAGCTGGCTAGCACCATGTACCCTG	Forward	Subcloning of <i>esr1b</i>
esr1b-pGL3.1-infusion-R	TCAGCGGGTTTAAACTCATGGAATGGG	Reverse	Subcloning of <i>esr1b</i>
esr2a-pGL3.1-infusion-F	CCCAAGCTGGCTAGCACCATGTCACAAT	Forward	Subcloning of <i>esr2a</i>
esr2a-pGL3.1-infusion-R	TCAGCGGGTTTAAACTCACCCTGTCTT	Reverse	Subcloning of <i>esr2a</i>
esr2b-pGL3.1-infusion-F	CCCAAGCTGGCTAGCACCATGGCATGTT	Forward	Subcloning of <i>esr2b</i>
esr2b-pGL3.1-infusion-R	TCAGCGGGTTTAAACTTACTGAGGTACA	Reverse	Subcloning of <i>esr2b</i>

Table 1 (Continued)

pGL4.10-linearize-F	CTGTTGGTAAAGCCACCATGGAAG	Forward	Subcloning of <i>chgHa</i> , <i>chgHβ</i> , <i>chgL</i>
pGL4.10-linearize-R	GCGAGCTCAGGTACCGGC	Reverse	Subcloning of <i>chgHa</i> , <i>chgHβ</i> , <i>chgL</i>
chgH $\alpha$ -pGL4.10-infusion-F	GGTACCTGAGCTCGCGATATTTCTGTGTC	Forward	Subcloning of <i>chgHa</i> promoter
chgH $\alpha$ -pGL4.10-infusion-R	TGGCTTTACCAACAGAGACAATCCGAGG	Reverse	Subcloning of <i>chgHa</i> promoter
chgH $\beta$ -pGL4.10-infusion-F	GGTACCTGAGCTCGCTCATAATGCATCAA	Forward	Subcloning of <i>chgHβ</i> promoter
chgH $\beta$ -pGL4.10-infusion-R	TGGCTTTACCAACAGAAGAATAATCCG	Reverse	Subcloning of <i>chgHβ</i> promoter
chgL-pGL4.10-infusion-F	GGTACCTGAGCTCGCATCCGGGTTTGCAG	Forward	Subcloning of <i>chgL</i> promoter
chgL-pGL4.10-infusion-R	TGGCTTTACCAACAGCAGCAATGTTTAC	Reverse	Subcloning of <i>chgL</i> promoter
vtgAs-promoter-1-pGL4.10	GGTACCTGAGCTCGCGGGCAGTCAGAATG	Forward	Subcloning of <i>vtgAs</i> promoter 1
vtgAs-promoter-1-pGL4.10	TGGCTTTACCAACAGGGCCAGTGTGATGT	Reverse	Subcloning of <i>vtgAs</i> promoter 1
vtgC-promoter-pGL4.10	GGTACCTGAGCTCGCCCGCACCATAGCAC	Forward	Subcloning of <i>vtgC</i> promoter
vtgC-promoter-pGL4.10	TGGCTTTACCAACAGGGTGAAATCCAG G	Reverse	Subcloning of <i>vtgC</i> promoter
esr1a-pGL4.10-infusion-F	GGTACCTGAGCTCGCCTTCTCCATTTAAC	Forward	Subcloning of <i>esr1a</i> promoter
esr1a-pGL4.10-infusion-R	TGGCTTTACCAACAGAGATTTAAAAAAG	Reverse	Subcloning of <i>esr1a</i> 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
<i>chgH α</i>	—	1	6	2	4
<i>chgH β</i>	—	2	8	—	3
<i>chgL</i>	—	1	7	7	11
<i>vtgAs</i>	1	7	6	7	10
<i>vtgC</i>	—	6	7	1	6
<i>esr1a</i>	—	3	19	9	11

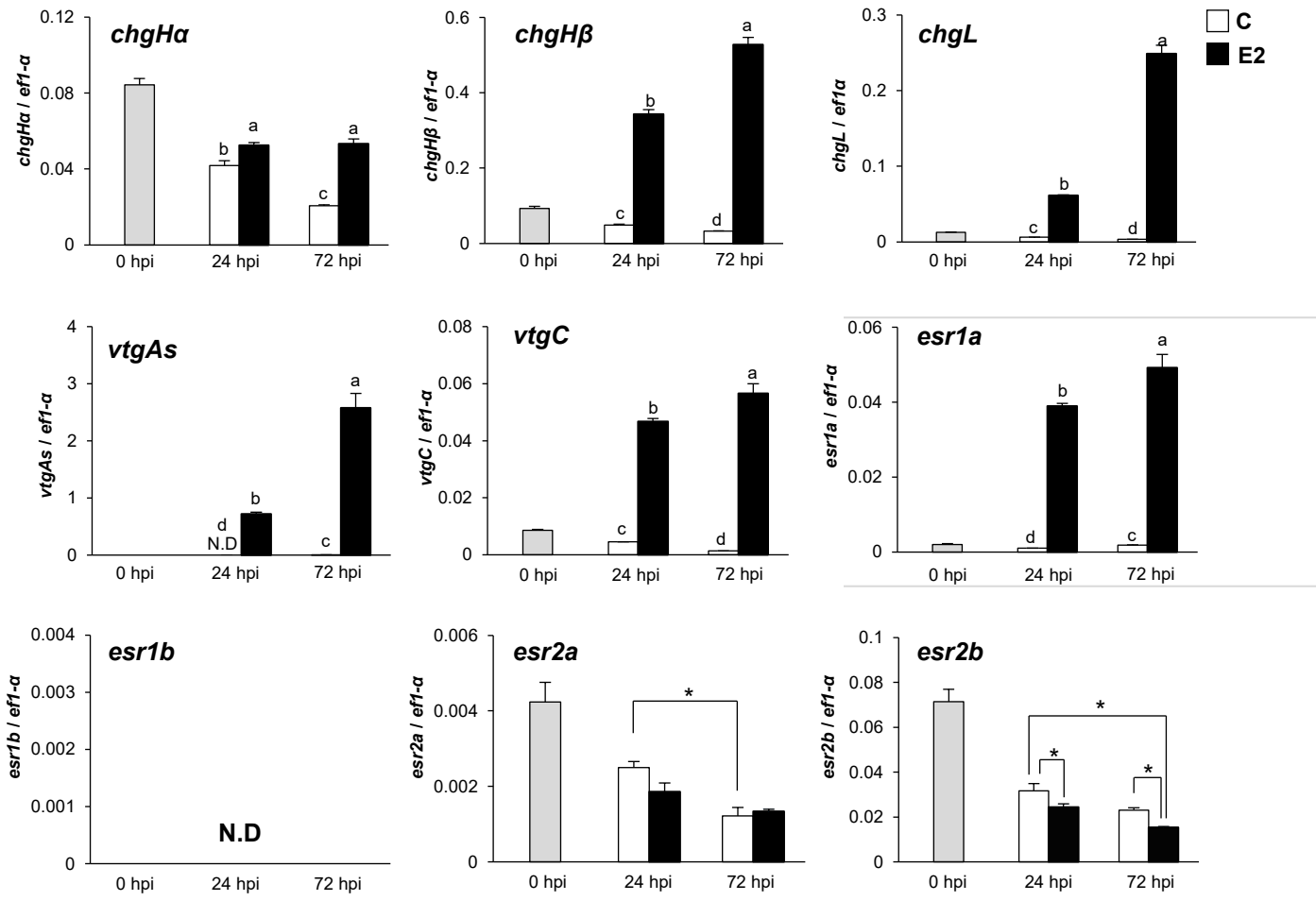
ERE: Estrogen responsive element; ERE-like: Incomplete ERE; 1/2 ERE: ERE half-site; Sp1: Specificity Protein-1;

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*  $\alpha$ : NM\_001124273.1; *chgH*  $\beta$ : 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/CONSIT/> 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
<i>chgH</i> $\alpha$	-	4	4	-	2
<i>chgH</i> $\beta$	-	2	7	-	2
<i>chgL</i>	-	2	4	2	3
<i>vtgAs</i>	1	4	4	2	4
<i>vtgC</i>	-	7	5	-	3
<i>esr1a</i>	-	2	5	1	-
<i>esr1b</i>	-	2	3	-	3
<i>esr2a</i>	-	4	7	1	2
<i>esr2b</i>	-	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



**Fig. 1**

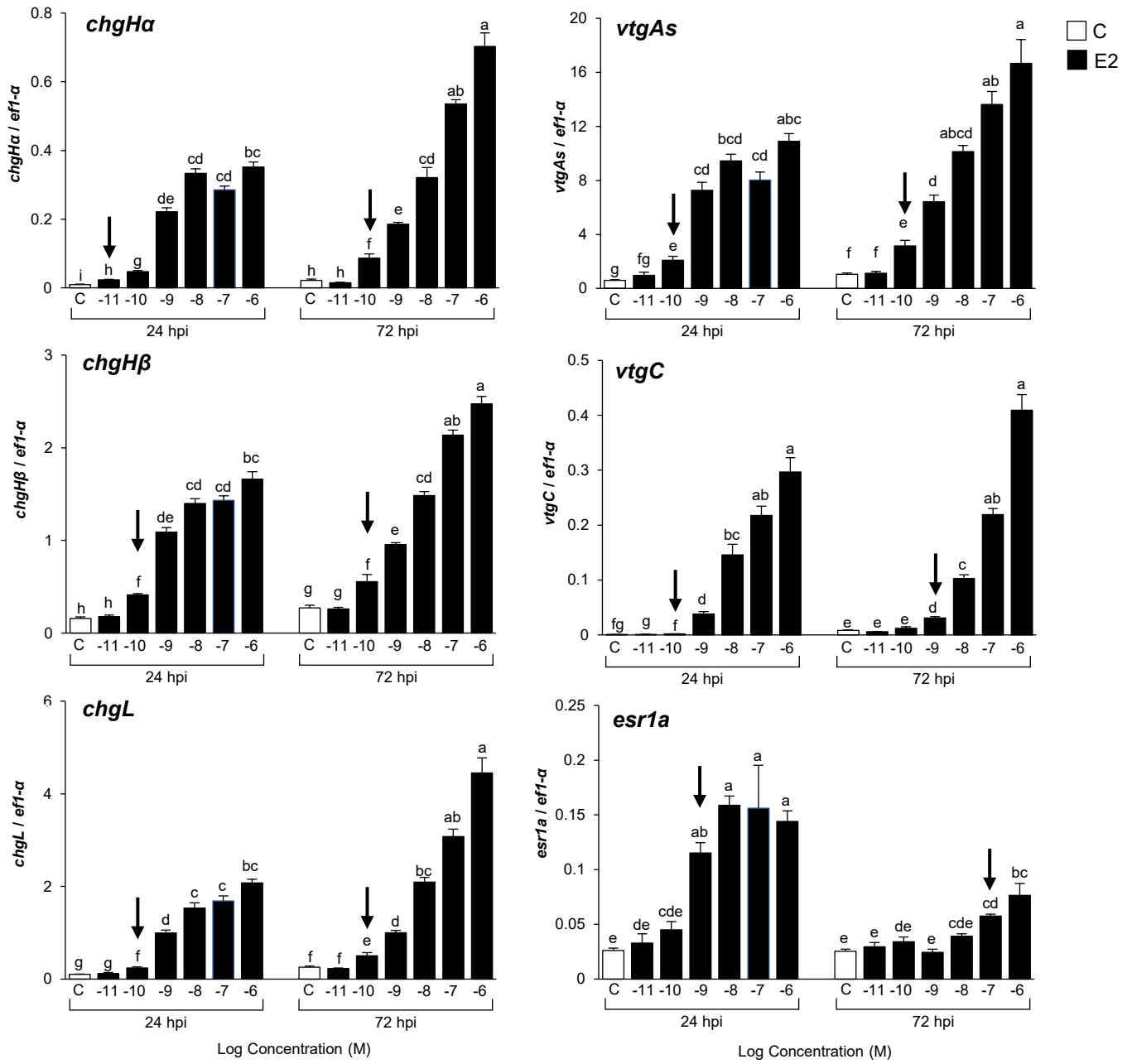
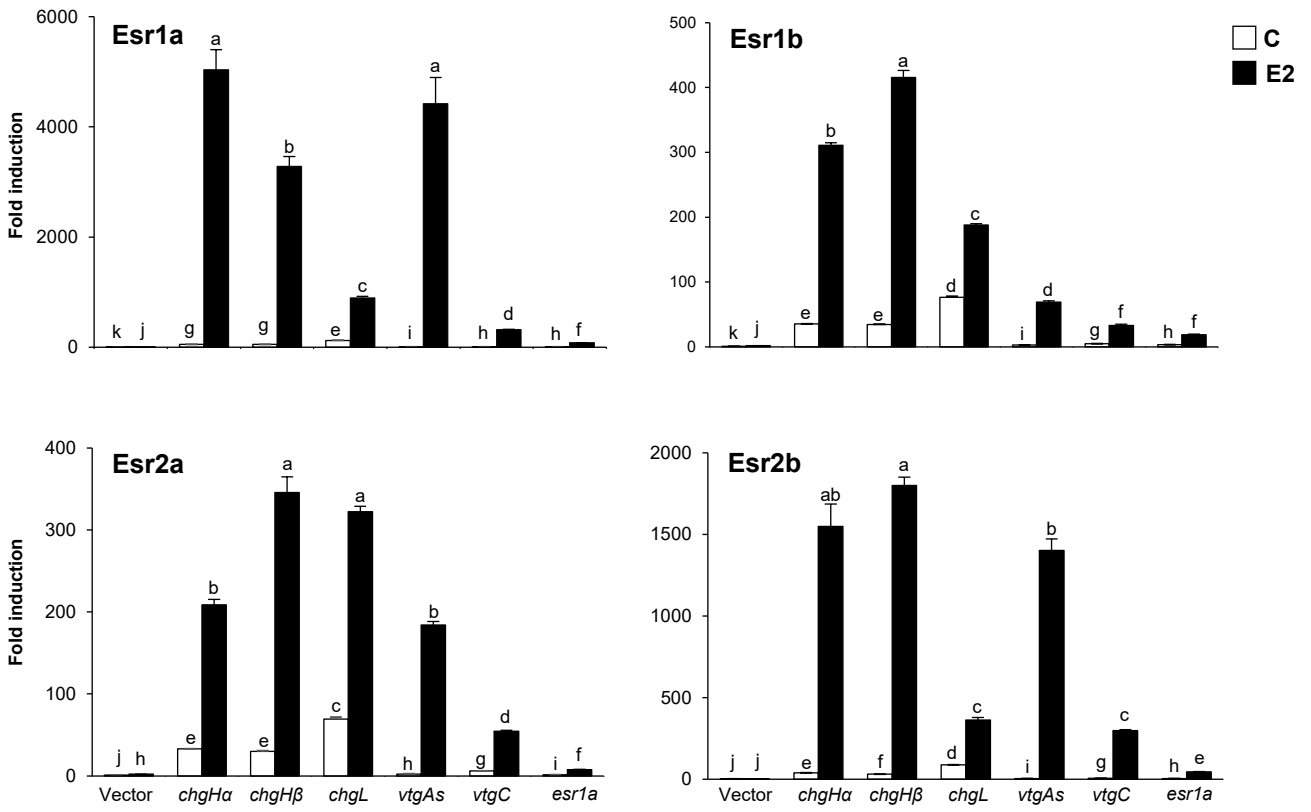


Fig. 2



**Fig. 3**



-1951 ATGTTTCCACTTTGTCATTATGGGGTATTGTGTGTAGATTAATAAAAACT  
-1901 GTAACACAACAAAATGTGGAATTAGTCCATGGGTATGAATACTTTCTGAA  
-1851 GGCCTGTATGTACTTGTCCCTGCTTACAAATATCTGGGCATCTGGATAG  
-1801 ATGAAAAGCTGTCTTTAAAAAAGCATATTGATGAGTTAGTTAAGAATCTG  
-1751 AGAATAAAAAATGGGCTTTACTATAGAAATACAGTAAGTCCTGCCTCTCG  
-1701 CTAAATAGTAGAAAGCAGATTATTCAGTCGACATTACTATCGGTCCCTAGA  
-1651 CCATGGCGAAATCATATATATGAACGCAGCTGACACTTCATTAAAGCCGG  
-1601 TAGATGCAGGTGATCATAATGCACTGTGCTTTATTACGGGCAACAATTTT  
-1551 AATACTCATCACTGCATTTTCTAGTAGAAAAGTTGGTTGGTCCTCTTTGAT  
-1501 GTCACCTAGGTTGATACATTACTATGTTTTTCATTTATAAAGCCATTTTAC  
-1451 AAAAAGTCCCCTGTACCTAACATCATTACTGAACTTTTGACATGAGTTA  
-1401 CCAAACCTGGTCTCATGAATGGCTAACTCTGGAAATTCCTTTTGTCTCTA  
-1351 ATTAGTTAGGTAAATCAGTTTTTTTTATGATTGTGTTTTTCTTTCTGCTTG  
-1301 CATTTTGTGTTTAGATTTGTGTATTTCCCTGTAAAATTATCCCTGATAACA  
-1251 TAAAGGTAAATAGTATAAAAAAGTTTTGTAGGAATGCTGTTATTTTCTT  
AP-1 AP-1  
-1201 TGAATGATTCATTTGGTCGTTGGTAAACATTATAGGAAGAACCTACTACT  
-1151 GAAAGAGACACGAGAAACCACATTGAACTTTGCAAAAGCCACCTAAACA  
-1101 AGTCTAAATCCTGTGAAAATGTTCTGTGGACCACTGAAACAAAATTAGAG

Fig. A. 1 (Continued)

1/2ERE

-1051 CTATTTGGCAATACAGATCAGTGCTATGTTTACAGATGACCAACTGAAGC

Sp1

-1001 TTTAAAAGAAAAGAACCACCCTCCCTACAATCAAACATGGGGAGGTTTGAT

-951 AATGCTGTGGGGTTGTTTTGCTTCCTCTGGTACTGAGGACCTTGAACATG

-901 TGAAAGACGTAGTAGATTATCAATGTGTTTTTGGTAAACTAGTGGGTGTC

-851 TGTTGAAGGTTGTGGGTCTTCCATCAGGACAAAAACACAAACACACATCA

-801 ATAAACAACAGGAATGGTTAAAAATGAACACTGGACTGTTCTGGAGCGGC

-751 CAGCGATGAGTTCAGATCAGAATCCCATCAAAAACCTATGGTGAGATATT

-701 TTTCACAGATAACTCCAGTACCTGAAAATAAGTACCTGGATGTTTCATATG

-651 TTCTTCAGATTTTGTGTATGAGATCTGAAAACAGTAGTTAGTGGGAAGGCA

AP-1

-601 CCACTCAAACATTGAAGAATTAGAGCAGTTTGAAGAGTGAAGAGTG

-551 GGACAAATTGCCATTAGAAAGGTGCAGCAAGCTCATTGATGGCAACAAGA

-501 AGCATTTTCTGGTGGTTCTGCAATGCTGACAATCCAATAACAACATGTCT

-451 AGACTGAAGTTTTTTTTTAAATGTCAACTTATTTTTGAAGAAATAGGGAAT

-401 TATTTAAGAAAAGTGAAAGGCAATATATTTATCTATATTAGAAATAGAAT

-351 ACAATAGATACATTTGTGTCTATTATTATTTATAAAATGTTTACTTCTTG

-301 AGTGTAGTTCTGCACACCGTGTGTACCTGCAAATAAACATTTGTTAACA

1/2ERE

1/2ERE

-251 AATACAATGTTACGGTCACAAAGACCTTCAACTCTGGTCAAAGGCATGTC

ERE-like

-201 AGAGATCTATGTGACCTGAACATTGCGTTTGTCAATAAATGTTTGGGACT

Fig. A. 1 (Continued)



1/2ERE

-151 TCAGAGTGTGGAAAGCCTTGATTCCCTCCGAACTGGATGTGAATGATTGGT

CAAT box

Sp1

-101 CAGCCAAGGAGGTGGGGCTTTTCAACAGTTCAACACACCCCACTATGTAT

TATA box

-51 AAAAGCAGCAACAAGTGGCACAGTGAGCCTTGTGGGTTCTCGGATTGTC

Exon1

-1 TCTTGTTCTGAATCCATGGCCTTGACAGTGGAGTGTTGTTTGTCTCGTAGC

50 AGTGGCCATGCTTGGCTGTCTGTGTGACGCTCAATTGAAGTGGCCTTACC

100 AACCCCTCAGAAC

Fig. A. 1 (Continued)



AP-1

-1420 GGAAGTATGATTTTAAAGTGACTGAAATGCATCAGAACATTTTGAGTGCTC

-1370 TTGATGATGATCTTTTCCCCCAGTCTGATTTAGTACCTGGTCTTGTCCA

-1320 TGAGTCCTGTGTGGCTTGTAGAGGGAAACAGAAGACACATACGTATTCCT

1/2ERE

-1270 GAGAGTATTATCTTTCAATGATTGGGTCA TAATATTTTGTAGCTTAAACG

-1220 GTTTAAAAGATAGAGCCACATTTGTAGGAAGAAAACAGAAACCGCTCTGT

ERE-like AP-1

-1170 TTATTTCAAACACATCTAGCGGCAACCAGAGGTTACTGACCTAATGTCGG

-1120 TTCTATCAACCAAGCTCAATTTGATACATTTGATTTTCTCTCTCGTG

1/2ERE      1/2ERE

-1070 ACCCCCACATCGGGTCA CAACCCCTAGTTTGGGACACGATGGTATAAGGA

-1020 TAATGAAACTGGAAGACTGACATATTTGTCAACATACTAACAACCTGCCG

-970 TTTGACACAAGTTATTTTAAAAAGTGTGCATGTCAAGTTATAGTCAAATA

-920 CTGTATAAAAATGATGTCTACTATTCTTTCTATTTCAGAACTTCAAAAATA

-870 TGTTTTGAAATGTGTATCCTGTCATTTATTCTATTGGATTAAATGGTAGT

AP-1

-820 TAAAAGGACTAAATGGTGAGCCTATCATTATCTACTGTAGCGGTGACTAA

-770 TCAAAGATGCACATGGTATTTCAAGGAAAACCTTTGATTTTGCATAAACG

-720 ACTAAATGTGGAGGTGTGTGAAACCCCAATGAATGTACAATCAAAGCTTC

-670 TGAACATAAGATAGGGGACATTAGAAGTGTATCAGTTTAGAGTTTTTTTA

-620 CTTAGAGTTTTGAAAATATAACCACTTCTGAAAATGTGCCAAAGTGGTTG

-570 AAAAAACAGTATATAACAACCCAGGTATTTTCAGAAATGCATTCTACTACTAC

Fig. A. 2 (Continued)

-520 ACTATACACTGAGTATACAAAACATTAGAACACCTTGCTACTATTGAGTT  
 -470 GCACCCTCTTTTGGCCCTCAGAAAAGAAAAAACTGTAATATATACAAAAT  
 -420 TGTCTAATTATCTATTTATAATGCGTAAATCATTTTGAATTGTTTCTGAC  
 -370 TATCAAATAAATATATAAATAAATATATATGTATATTGTCACATACAAC  
 -320 AACTAGGAGCAAATTAGATACTGACAGAACTGGCAAAGCCTCCAATGACA  
                   1/2ERE  1/2ERE  
 -270 TGACGTGTCGGTCACAAAGACCTTTCTCAAGGCCATGACCGAAATGTTGG  
 -220 ATGTTTCGCAAATGTTACACAACCTGTTTTGAATAGTGTGCTACTAAAGGAA  
                                   CAAT box                                  1/2ERE  
 -170 CATTAGCCAGGGCATTGATTGGCTAAAACCTTGACCCCTCAGAAAAGGA  
                                   1/2ERE  
 -120 ACGGTCA GATAAAGAGGGGGCGGATTTCCAATAAGTAAACAACGCTCCAT  
                                   TATA box  
 -70 CTGTTATAAAGGTGGCCGCAATTCGTTTCATCCTCATCACAGCATCCAGTG  
   Exon 1  
 -20 AACATTGCGGATTAGTTCTTGTAGCGAAGCCATTGCGATGAAGTGGAGTG  
 +81 CAGTTTGTCTAGTGGCAGTGGCCACGCTTGGCTGGCTGTGTGATGCTCAG  
 +131 ATTTACTTGGAAAAACCAGGG

Fig. A. 2 (Continued)



-2736 CAGTAGGACCGAGAGGAAAAATGGCTACCTTTGTCTTTTACGCAATAATC  
 -2686 ACTCTGAGAGCGCTCATTCTTCAACATAAAGGCGTGAAACTACGTCTAAA  
 -2636 GGCTGTAGACACCTTAGGGAATACGTAGAAAAAGGAATCTGGTTGATATC  
 AP-1  
 -2586 CCTTTCAATGGCCAATAGGGATGCATAGGAACACAACGTTTTCAAATAT  
 AP-1  
 -2536 GAGTCACTTCCTGATTGGATTTTCTTAGGCTTTCGCCTGCAATATCAGT  
 AP-1  
 -2486 TATGTTATACTCACAGACAATATTTTGACAGTTTTGGAACTTTAGAGTG  
 -2436 TTTTCTATCCTAAGCTGTCAATTATATGCATATTCTAGCATCTGGTCCTG  
 -2386 AGAAATAGGCTGTTTACTTTGGGAACGTTATTTTTCCAAAAATAAAAATA  
 1/2ERE  
 -2336 GTGCCCCCTAGCTTCAAGCATGTCCCAGTTTAGGTCACTAGTAGCACGA  
 1/2ERE  
 -2286 GCTCATAAGATAGATGGGTCAATCAATTCACATATGATGTCCAGGGCAC  
 -2236 TTGTTTCTGGAAATAAATAGACTTGTCTGAAAGGTGAATTTTTAGAA  
 -2186 GTAGAATCTCGAATTGTTTTGGTGCAGACCGGGATGGTAAGACAGAATTT  
 Sp1  
 -2136 TGCAGGCTATCTCTGCAGAAGATTACAACACAGCCCCTTTGGCAGTTCTA  
 -2086 TCTTGTGCGAAAATGTTATAGTTAGGGATGGAAATTCAGGGTTTTTGGT  
 -2036 GGTTTTCTAAGCCAGGATTCAGACGTGGCTAAGACATCCGGGTTTGCAG  
 AP-1  
 -1986 AATATGCTAAACCAGTGAATAAAGCAAACCTTAGGGAGTAGGCTTCTAATG  
 -1936 TTAACATGCATGAAACCAAGGCTTTTACGGTTACAGAAGTCAACAAATGA  
 -1886 GAGCACCTGGGGAGAAGGAGTAGAGCTGGGCACTGCAGGTCCTGGATTAA

Fig. A. 3 (Continued)







Exon 1

-36   CTTCATCCTCTTCACAGCATCCAGTGAACATTGCTGATCAATTCTTATTG

+15   TGAAGCCATGGCGATGAAGTGGAGTGTAGTTTGTCTCGTGGCAGTGGCCA

+65   TGCTTGGCTGTCTGTGTGTTGCTCAGAATTGGCCACCCTTCAGTAAA

Fig. A. 3 (Continued)

-2872 AAGATGCAATTCGTCAGACTTCGGAGACTCTCTGGAAATCTCGGGTAACC  
Sp1  
 -2822 TCGGATTCTCTAGGAAACGGAGACGTCAGAGATTTCCGGGATGCCTCAGA  
ERE-like ERE-like  
 -2772 GGCGAGGTAAATCTGACCTCTCCTTCCTACGTTACCGTAGCCACCCATC  
1/2ERE  
 -2722 GATCCAGAAGGTCAAGGCGATGAGCAAGTCGAGATCTGTTGGTAGTTCCC  
 -2672 GGGCTGCTAGCTTATCTTTAACGTCCTCCGATAATCCATGCAGGAACGTG  
 -2622 GCGAACAGCGATTCCGGGTTCCAGGCACTCTCAGCCACAAATGTACGGAA  
 -2572 ATCCACTGCATATGTCCAACGGACATCAGCGTGGTGAGGTACGCTTTCTT  
 -2522 CGAGCAGTCCGAGGGAAAGGAGGGGCTGCAGCTCGAAGATGAGGGAAT  
 -2472 ACTGAGCGAGAAACGCCTGACAGGTTCCCGACTTTCCAGCGAAGCATTCC  
 -2422 AGAGGAGGTAAGCAGGGTTCTCAGGAAGCCGGGGTGGCCGGGAGAGACGC  
Sp1  
 -2372 GCTGCTGACAGTCGGGTTACTGAGGGGCTGGGAAGTTATATTCGTGGTAG  
 -2322 GCTGCCTAACAGACAACCCACGGAATTGCTCCAGCAATGTATCCAATGCA  
 -2272 AAACCTTCCATAAGACCACGAAGCAACTCCTCGTGCCTTCCAATGGTGGC  
Sp1  
 -2222 TCCTTGGGAGAAGACGGCGTGGTGGAGCTGGTCCAAGTCTGCTGCGTCCG  
ERE-like  
 -2172 TCATGGTCAGTTTGTACTATCACGACTCAGGATAAGACCCAGATGGAGTT  
Sp1  
 -2122 CAAAATATCAAATGTATATTTACAAAACAGGGGGCAGGCAACGACAGGT  
1/2ERE AP-1  
 -2072 CCAGGGCAGGCAGAGGTCAAGTATCCAGAGCAGAGTCCGAGAGGTACAGA  
1/2ERE 1/2ERE  
 -2022 ACGGCAGGCAGGCTCAGGGTCAGGGCAGTCAGAATGGTCAAAACCGGGAA

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

-1972 AACAGGGACTAGAGTGAAAACAGGAGTACGTGAAAACCACTAGTAGGCTT  
 -1922 GACGAGACAAGACGAACTGGTAACAGACAAACAGAGAACACAGGTATAAA  
 -1872 TGCACCAGGGATAATGGGGAAGATGGGCGACACCTGGAAGGGGGTAGAGA  
 -1822 CAAGCACAAAGACAGGAGAAACAGATCAGGGTGTGACGGATAGGGTATTT  
 -1772 ATGGATGTGTTATGAATGACTGAAGGTGTCTCACCTTCAAACAAAGTATTA  
 AP-1 AP-1  
 -1722 CAGTATTACATGAGGTGTCAATAAATAGTTTTTTAATATTCTGCTAATTT  
 -1672 ATGAAGGTTCTCTCATGATCCACAGGTTATTGTAGGGTATGGGAGATATT  
 AP-1  
 -1622 TAAATGGAGAGATGGACCTCCAAAGCTTGTGTTTGGGTGTGTGTGTCAGA  
 -1572 GCCAAGATGATTTGGAGTAGTCCAACCTAAGACGACCTCACCAGGTATTC  
 -1522 CTTTTCTTTTCCCATGCTGGAGACCAGGGCTTGTTCACAACCTGTTATAA  
 AP-1  
 -1472 CGGTGCCAACATTTTGTGGCTGATCTTTCAGCGGGGAGTGAGTGAATGT  
 -1422 GTCAAAGACCTGACTGGGCCCAGCACTGCACGGTATGGCTCGTTATGTTG  
 -1372 TTGTGTGTGGGTTTGTGTACTGAGGAACATGTAACCTGGTTGCAGTAAAA  
 -1322 GCCACTTTC AATTTCCCTTATGTTGGGAGGTTTCATCAGGGTAAGTGTTTC  
 -1272 TTGGTGTAAGTCGTCCCGAACACAGCACATATCAGCCTGGGATATCAGC  
 1/2ERE 1/2ERE  
 -1222 CATT CATAGTTAGCCTTAGTTGGAGTGACCATAGAATTCTATGGGAGTGA  
 AP-1  
 -1172 CCTTACTGAGTAAAGTATTTGCCATCATTCCCCCTGGTCGTCAGTATTA  
 Sp1  
 -1122 ACGCTGCCACAAAGTCATAATTATGGCTAAACCCTGCCCATTTCCACAAT

Fig. A. 4 (Continued)

ERE-like

-1072 TTCTCTTCTAGAAAATAGATTTTAAATCTAACCCCTAACTAATGAA**GGTCA**

Sp1

-1022 **AGTTTGATGCGTT**GGGGGGTGGTATGTGAAAAGTGCTGTAATTTCTAAAT

AP-1

-972 GGTTTACTCAAATTTCTAAACGGT**TTACTCAT**TATGGATGAAAATACCCT

-922 CAGATTAAAGCTGACAGTCTGCACTTTAAACCTCCGTCATAGCATCATTTC

AP-1

-872 AAATCAAAATTGCTGGAGTACAGAGCCAAAAACAACAACAA**TGTCTCAC**

-822 TGTCCAATTTTTTTTTTTTAAATTGACACATTAATATTTTCAGTTGAATTTA

-772 ACATCTGGATCGAACAAACAACTGTTGTAGGCCTGCATATTCAGTGTCA

AP-1

ERE

-722 **TTGACTGACAAGATGGCAGAGTCTTATCTCTAGGTCAAGCTGACC**ATTGT

-672 TGACACATGGTTGGTTCATCTGAATTACAAATACTTGTTCAGGACTTCA

ERE-like

-622 ATAGTGTG**AGGACAAGCTTGGAC**CAATGGCTGATCCATGTTCAAGTGCC

-572 AAAACTCGTTGGGTTTTGACATTACTAGTATTCTAATTTATCACATTCTC

ERE-like

-522 TAACAAATTAGAATC**GATCATACATGTACT**GATCCAAACCTAGACAATAT

-472 ATTTTATTTAGCATTGTGTTGCTTCATTAATTTAGGCACCAACTGCCTAC

-422 TTATCTCTACTGGGTTCTACTTCTAAAGTACAAGGGCCTTTCTGTACATT

-372 TGTATTTAGTGGTTAGATAAATAGAGAAAAATAACTAATGTAGTATTTAA

-322 AGAAAAATGAAGGTCGTAGTATACATAACTTGTGGATAAAACAACACGT

AP-1

ERE-like

-272 **GATTCTCCAGATAATTTTCATTAAGA**ACTGACTTAGAT**GGACATTGATC**

Sp1

-222 TGTAAGGGCTAAATGGCAGT**GGGGCAGGTT**AACCTAACCTATGGGTGTA

Fig. A. 4 (Continued)

-172 TGTGGCCACCTCAGAACTAGTGATAACCCACTCAACAGGCTTTATTATGA  
-122 GCACATACTAATCTCAACTTATGTAACGTTAGTTAATTATATATTTTGTT  
-72 ACGATTGTTTTGTCGACAGAACATTTCTTTAAAAGGCGGACTGGGACTGT  
Exon 1  
-22 TGTTCCCTCACATCACACTGGCCATGAGAGCAGTAGTACTTGCACTGACTC  
Intron 1  
29 TAGCCCTTGTTGGTAAGTACAGTTTTTCTGTCTTATTCTAGTGCAGATAT  
79 TATAACTAACTTCATGTAAATATAACTAAGTTATGTAAATATAGAAATGT  
129 ACAATTCTGTATCTATGACATTTTTTGAAAAACATTCAGTAACGTATTGT  
Exon 2  
179 GACAATAATTTTTGTTTTATCTTGTA GCGAGTCAATCTGTAACTTT

Fig. A. 4 (Continued)



-2125 ACTGGCATGCGTATTCAGTACTGAGATTTTTAACCTCTCGC **TGACC** GAGTCTG 1/2ERE  
 -2075 CAATACGTACAGGTTTAAGCAGACCACCATAGTCCCTGTGGCTAAGGAAG  
 -2025 CGAAGGTAACCTACCAAATAATTACCGCACCATAGCACTCACGTATGTA  
 -1975 GCCATGAAGTGCTTTGAAAAGCTGATCATGGATCATATCAATACCATTAT  
 -1925 CCCAGAAACCCCAGACCCAGCGTTCAACACCATAGGGCCACAAAGCTAG  
 -1875 TCACTAAGCTAAGTACCATGGAAC TAAACACCTCCCTCTGCAACTGGATC  
 -1825 CTGGATTCCTGACGGGACACCCCCAGGTGGTAAGGGTAGGCAACAACAC  
 -1775 GTCTGCCACGCTGATCCTCAACTGTGGGGACCTCAGGGGTGTGTACTTAG  
 -1725 TCCCCTCCTGTACTCCCTGATCACCCATGACTGCGCGGCCAAACACGACT  
 -1675 CAAACACCATCATTAAAGTTGGTAAACCTGATCACCAACAAT **GATGAGACA** AP-1  
 -1625 GCTTATAGGGAGGAG **GGTCAGAGACC** TGGCAGTGTGGTGCCAGGACAACAA ERE-like  
 -1575 CCTCTTCCTCAAT **GTGAGCAA** GACAAATGAGCTTATTGTGGACTACAGGA AP-1  
 -1525 AAAGGCAGGCTGAACATGCCCCATTAAACATTGACGGGGATGTAGTTTCA ERE-like  
 -1475 **AGTTCAATTGGTGTCCA** CATCTCCAACAAACGATCACAGTTGAAACACACC  
 -1425 AAGACAGTCGTAAAGAGAGACGACACCACCACCTCAGGAGACTGAAAAG  
 -1375 ATTTGACATAGGTTCCAGATCCTCAAAACCTTCTACAGCTGCAACATCG ERE-like  
 -1325 **AGAGCATCCTGACC** GGTTGCATCACTGCCTGGTATGGCAACTGCTCGGCA 1/2ERE  
 -1275 TCT **TGACC** ATAAGGCGTTACAGAGGGTAGTGCGTACGGCTCTGCTACCACA

Fig. A. 5 (Continued)

-1225 CAGCAAGTGGTACCAGAGCGCCAAGTCTTGGACCAAAGGCTCCTTAACA  
 -1175 GCTTCTACCCCAAGACTGCTTAACAATTAACAAATGGCCACTGGACTA  
           1/2ERE  
 -1125 TTTACAT**TGACC**CCCCCTCCATTTGTTTTGTACACTGCTGTTACTCTATGT  
 -1075 TTATTATCTACGCATAGTCACTTCACCCACCTACATGTATAAATTACC  
 -1025 TCGACTAACCTGTACCCCGCATATTGACTCGGTACCGGTACCCCTTGTA  
 -975 TATAGGCTCATTATTGTTATTTAATTTTGTACTTTTTATTTATTTTTTA  
 -925 CTTTAGTTTATTTGGTAAATATTTTCTTAACTCTTCTTCAACTGCACTGT  
           AP-1  
 -875 TGGTTAAGGGCTT**GTGAGTAA**GCATGTAAGGTCTACACTTGTTGTATTTG  
 -825 GTGCATGTGACAAATAGAGTTTGATTTGATTTGATTTGACTGTCAGTTG  
 -775 AGTAACTGTAGCCCGTCTGTTGCTCTGCAAAATTTGTGTCAGTCCTTTAT  
           1/2ERE  
 -725 CCTCTTTCATCAAT**TGACC**CGTTTTTCGACAACCTGGCCTGAAATTGTCTGGA  
           1/2ERE  
 -675 TGTCCCTTGGATGGT**TGACC**ATTCTTGATACAAAGAGGAAACTGTTGAGC  
                                   ERE-like  
 -625 GTGAAAAACGCTGCACCTGGTATCTACCAGCATACCCT**GTTCAAATGCA**  
 -575 **C**TTAAATATTTAGTCGTACCCATCCACCCTCTGAATTGCACACAAGCACA  
 -525 ATCCATGTCTCAATTTTATCAAGGCTTAAAAAGCCTCCTTTAAACCTGTC  
 -475 TCCTCCCCTTCATTACACTGATTGAAGTGAATTAATATCAATAAGGGAT  
 -425 CATAGCTTTCACCTGGTTAGTCTATGTCATGGAAAGCACAGGTGTTCCCTA  
 -375 ATGTTGTGTACATCCTGTGAATATATTATTTGTGCAAGCAGCACACTTTG

Fig. A. 5 (Continued)



ERE-like

-325 TGCATTCAAATAAGGTCAAAGATGTTGCATTTTAAACAATTCTTTGTAAT

-275 GTGAGTTTCACAAAGCACTGATTATTAGTGTCCATTTTAGATCACTGTTT

1/2ERE

-225 ATTTTTCCTGAAATGTCCTCCTTTTCCATGTCATACTGACCACTGTCAAC

-175 GTCACTGGTTACACTCGTCTGACATACCTTTGCCCCCTTGTTACTTTATG

ERE-like

-125 CCCCTCATTGATGCCAAAGATGTGCTGTGCAGACACTGGGTCATGTACTC

AP-1 TATA box

-75 GTTTAGTCATTGAGAACACAGCATTGCATATAAAAGGAAACGTTGAGCTG

Exon1

-25 CAGGATCCCACTCCTGGATTTCAACCATGTGGGGGTTTCCTTCTTTGTCACC

25 TTGTGGCCTTGCC

Fig. A. 5 (Continued)





-4355 ATTACATTTGTCCTTTTGGTAGCATAGTCTTGATCGGAATTTGTTATCCA  
 -4305 TTGATTGCACGTTGGCTAATAGTACTGATGGTAGAGCAATCCTTACAAGG  
 -4255 CACCCCAACCTACGTCCCCGATATCTCCGTCTCTTCTTCATG **TGAATGAC** AP-1  
 -4205 GGGGATGTGGGCCTTGTCGGGTGTCCGAAGTAAATCCTTTGCGTCGACTC  
 -4155 GTTAATGAAAAGTCTTCGTCCAGTTCGAAGTGTGTAGTCACTGTCCTGA  
 1/2ERE  
 -4105 TATCCAGAAGCTCTTTTC **GGTCA**GAGGCGGTGGCAGAACATTATGTATA  
 -4055 AAATAAGTTCAAAATAACGCGAAACACGCACAATAGTACAATTGGTTAGG  
 -4005 AGCCCGTAAAACGGCAGCCATCTCCTCCGGCGCCATCTCACAGGTGAAGC  
 -3955 TGTGCAAACCTCAGGGGTTTATTATAAGAGGCCAGCTAAATGTTGATTGT  
 -3905 TGAAAATGAAAGTGAAGTCGCATTACCAAAGATAATATGATGTTATGGCT  
 AP-1  
 -3855 GTTTTGGCAATTAATCCCAAATGTTACGATCACAGTTTCT **GTGAGTTAAT**  
 1/2ERE  
 -3805 GTCTCTGTTCTGCTCTTAGGTGTCTCTGTGGGGTAAACAGGTG **TGACCTG**  
 AP-1  
 -3755 TCCATCTCTGCTGGCATGGAGAACTCTCAGAAGGCCAGTCCCAT **TGACTC**  
 1/2ERE  
 -3705 **CCTGTC** **TGACCT**GAAAGTCTTTTATCCTGGTGGACGACTCCAACATGGTGT  
 1/2ERE  
 -3655 GGTCTGCTC **TGACCT**CTGCCCAGAGACCAGGGGAGGATGGCAAAATCACC  
 Sp1 1/2ERE  
 -3605 **CCAGGGAGGGT**GGACATCGTCCTGGACAACGCTGGCTTCGAGCT **GGTCAC**  
 -3555 AGACCTGGTCCTCGCTAACTTCTGGTGTCCGCTGGTCTGGCCAGGGAGG  
 -3505 TCCGCTTCCATGGCAAGTCCATCCCCTGGTTTGTCTCCGACGTCACCTCG

Fig. A. 6 (Continued)

-3455 CACGACTTCCAGTGGACCATCCGCCAGACCCTGGCGGCCAATCACAAGTG  
-3405 GATGTCCAAGAGCGGTGTCCAGTGGCAGAGCTACGTACGGGAGGGCGTGT  
Sp1  
-3355 GGTCTTATCACGACCACCCGTTCTGGACAATGCCTCATGAGTACTGTGAC  
1/2ERE  
-3305 ATGGCGGGCGACGCGCCTGACCTCTACGCGACACTGCAGGGAGCCGACCT  
-3255 GATTCTGTTTAAAGGAGACCTGAACTACAGGAAGCTGACTGGGGACAGGG  
-3205 AGTGGGATCACATGGTGCCGTTTGATAGGGCACTGAGGGGATTTCGGCCCA  
-3155 GCTCCCCGTGTGCAGTCTGAGGACTCTGAAGGCTAACATCCAGGTGGGGCT  
Sp1 1/2ERE  
-3105 CCAGCCGGGGCAGGGGAGAACTCAACACCCAGGACCCAGCTGGATGA  
-3055 CCAGCGGAAGTACGCTGTGGTCCAGTTCTCCAGCCCTCACAGGGAACAG  
-3005 TAGACCTGTCTGGGACAGGAAATGACGTCAATATGTTTATAATAAGTGGC  
-2955 GTCATCATAATCAGAGGAAGTAAGGGCGTGAGCAGGGCCAGTGTACTTGT  
-2905 AACCATATGCCAGGTTAGAGGTGTAGTTTGTGTTTGTGCTAGAACTACCTA  
-2855 ATGTATTTAAATGAGGAGAGTGGTTTAAAGGAGGATGATAGAGGATACTT  
-2805 TAAAAAGAGGAGGGTAGTTAAATAGGGAGTGGCTGCAGATCCGACTTCTC  
-2755 TGGACCCGTCGTCCCGACAGCTAAAGGTTTACAGCTTCTGTGAATGTGTG  
-2705 GTAATCTCTACTTTACACACCATCTCTGTTCTATTTGTTTTCAGCTGCCCAG  
-2655 AGAAGAAGCTACTCTGGCGAAAGGTGCTTGTTTAATAAAGTTAGATCAAA  
-2605 CTCGAATCATTGTAAGATTACATAAAGATAATATTCTATAGAACCAAATA

Fig. A. 6 (Continued)

-2555 AAATAGCATAGTATAACATTACTGTATGACAAAAACACCTAATTTCTTA  
-2505 TATTTTAGGATGATTGTGCTCATCGTCACATTTTTCTCTCATGCAGTCTA  
-2455 AACTCAACAGTGCACGCCACTCAGCAAAGGTATGCTACAGTTTAATACCA  
-2405 TCTGCAAAAAAATGTGCTAAGGTACATAACTAATTCAAAACACTGTAGCC  
-2355 TACTTCAAAACAACACTGTAGCCTACACTCAATAATATTTAAATGCATAT  
-2305 GCCCATGAAACTGATTGCAAATGGTTGGCGTGCAGATTCTGCATGTATGT  
-2255 TTCACCGGTAAAACCTTGAAGAATTATCATTCACGATTGACAGT**GAGAAA** AP-1  
Sp1 1/2ERE  
-2205 TGCGAAGGGAGGGT**AGACC**AGAAGTTTGGGTGTGCGTAGAGTTAAAGAAAC  
-2155 ACATGCACAGAACGTGAGTGGGATCTTCAGCTCTGGGGAAAGGGATCCAG  
Sp1 1/2ERE  
-2105 TGGGAT**GGGGCTGAGACC**TCCGGATCCCCAGCCTCTGCCATTTAAATAAT  
-2055 GATCAACTGCTGGTTAGCAATCCATCCTGGCATAACTACAAAACAATGAC  
-2005 GTTTACTTCTCCCATTTAACAAATGTGTTGACGGTTGTTTACCATCATTTC  
-1955 AGTACTAGGGTCGGGCTCTACCTCGATTCCAGTTCAAATCAGTTAATTTA  
-1905 GAAACATTGAAATTC AAGTCATGAATTGAAACATGCTTATCTGATCTTA  
-1855 AGTGACTTTTTTCCAGACATATTATGTAATTTATGTAATGTTTTTGTCTC  
-1805 CCCAACAGCGACTACACAAAATATAGATTTGAGACACATTTTCATTCATTA  
-1755 TCAGAGGCAATAAGATGCAAGGTGCAATTTGAACATTTACATTTTAAGTT  
-1705 GGCCTGTCGTAAATAGTTTAGAATTGTTATTTTAAGTTGAGCCCTTATGT

Fig. A. 6 (Continued)

-1655 AACACCCAAAGCCAGCGGCTATTTTTTAGCCATTTATTTTGCATGAGCTT  
-1605 GGCAAGACATTACAGAGTTAACTTGTGGTTGCTGGTATTTTCTGGGTCTT  
-1555 AATCAACGTCATCAGTTTGTCTTGTCAATTTATTGCATGCGTTTTTGT  
1/2ERE  
-1505 GCATACATCGGGGTTTGCACAGT**TGACC**ACAGTCCCGGAGTTTACATTTTC  
-1455 ATAGTTAAATACCTGAAGTTGAACGACAAGGTAATGTTCCCACTCTGGGA  
-1405 AACTATAGATAAATCATAGTCTTTGGGGCAGTGCAATCACACGTACAAGG  
-1355 TGTGTGTTGTTAAACACCTGTCTATGATGTATTTCTGTTTGTGGATAGT  
-1305 ACAAAATGTTATTTCCGACTCATTAGAATGCCTCTGTAACCAGTGCAGGT  
-1255 GCCGTTGTGATCCTAAATGACTGGATTGTTCTCTTTGCAGAAATCTGGAA  
-1205 CAAAAATTGTTATATTATGATGGACTTGGTTTAAATAAAGAACTTCAATG  
AP-1  
-1155 ACTGGTCTTATCTCATACTGTTGTATAAAAT**TGAATGAC**ATTAAGAAA**GAG**  
ERE-like  
-1105 **CAACCGAGGCTTGACC**TAGAAAGATGATTCCCTTTCACTCATTCTCCCA  
-1055 TACTCTTATTACGCTCTTCAATTTATCCTGGGAACATGTATAAAAAATAG  
-1005 AGTTATAATCAGACTGCTTGTTTAGCACTTGACATCACATGTCTGAATCT  
-955 GGGCTGTTTACTGAGTGAAGAGACGTTGTAGAGAATGGAGCTTCTCTAGG  
1/2ERE  
-905 CAGCA**GGTCA**AACAGGAGGCCATTTTCTCCAGTTTGCAGAGATAGAGGT  
-855 TAATGAACCATCACTTTAATAAGCAAAAAGTTAACTTTCCCCCGTAAAT  
-805 CCAGCTGTCAATTTGTCAAGAAAGTTTGGAAAAATCAGAATCCCCAGTAT

Fig. A. 6 (Continued)

-755 GATCTCACTGAATTGTTTGTGGAAATGAAGACACTCATCTTCATACTGTA  
 -705 ATATGGGGAGCTGATCTGTCCATTTTTATGTACTGAGAATTGTTGCACAC  
 -655 GTTGAGGACTGGAAATACTAGTAGTCTTAAATTAGTAATAACAGTGTTGT  
 -605 GCTAGGCACACCCTGCATTTCAACAAGTGACACCGTTATTCCTTTAGAGA  
 ERE-like  
 -555 AACAGAGTTATCTTCCTGTGTAGGTTGAAAGGTCAAGAGTTGTCCCTTGTA  
 -505 GCCCTTGAAACTTACCTAAAGGAATGGGGAAGACATTAAATGACTTTTCA  
 -455 AACATTGGTTGAATTTTCATCATGATGCAGCATGTACTTGAACCCCTTTG  
 Sp1  
 -405 TTTGCTGCTTTGGCGGGATAAATAGTTTATATTTGATCAGTGAATATCGTT  
 CAAT box  
 -355 GATAAGGTATTCCTCTTGAAAGGCCCAATGATAGCAAGACAATGTACTTT  
 1/2ERE AP-1  
 -305 AAATTAGTAGCTATTCCTTGGTCACAGTAGAGAGTGATTTAGTCAGTGTA  
 -255 GTTTGGTGATCTTACTGCAATGCTGTATTCTGTTTTCTGAATAGATTTA  
 -205 GGCTATGAAAAGGGGAACATGTAAGAACATGCATTGCCTGATGTCTGAA  
 -155 TCCTCTCATCTTTTCCCCACTGGGAGTTACATAATGTTGGCATCAGAAGG  
 1/2ERE ERE-like  
 -105 GTGTTGACCTCACATACTGTTTGCTGTGTGTCATGTTGACCTGCTCTAGAGA  
 TATA box  
 -55 TACACTATCAATATCGATCCGGCTGCGTTCATTTGTTTCTACCTTTTTTA  
 Exon1  
 -5 AATCTCTTTTTTTCTCATTGTGATGCGAAGCCAGATCTCAGAAAGAGGGA  
 +46 TGAGAGCGAGAGGACAGGGAGAAAGAGGAACCACTCAACACAACAATGCT  
 +96 CATGATTAGACCCAAAGAGCTGAATATTGATCCATAACGTCATTAAGGTA

Fig. A. 6 (Continued)



Intron1

+146 AGTACTGTATATATGACGTCTTTGTGTGTATTTTCATGTAATGGGTTCCCTA

+196 TTTTATTGCTTCCTTCTCGTGGAATTGTTTTGTATAATTTTTCCATATTT

+246 GCACATAAATCCACTGATAGACACATTTAATAATGATTTAACTTCTTAGA

+296 AGAGTGAGTCAGAAAGGAGTGATATTCTGGGCCACGGCAGTACATGTAAC

+346 TAAATGACTGATAAGCATTTCAGAGAGGAAGTCACACCAGGGTGTAACCTCT

+396 CCTTGGAGCCTAGCAGCGTCTCTAAAAGCTTTTCAGGGAAGAGAAAAAT

+446 AAGCTGCATTTTAGAAAAAATGTAAGGGGACAGGGGAGTTGTTGGAGTG

+496 GTTGCCTCTTTCTTTTCGTTCTCTCACTCTCCTTCTCTCTTGCTCTCTATC

+546 TCGAAATATCTGTCACCTGTTGTCCTTTCTCCACCCCTCTCTCCCACAG

+596 CAGTGTCGATAGTTAAGGGAAAAGAGAAAAGCTCTGCCTCTCAAAAATAA

+646 GTGGGAGGGAGGAATGTTTTTGGGCTGTCTCCAGGCGGAACCTGGGAGGG

+696 AAAGAGAGAGCAAGGAGGGACGAGAAAAGAGAGAGAGAGAACCTAGTGAA

+746 TGCCTCTTCCCTTCTCTTCCCAACAGCCAGTATTGAGTTGCTTAGCACG

+796 GGCTGTTAAGGAAGAAACAGAGCAAGAGAGGGACGAGAGAAAAGAGAGAG

+846 AGAAGACAGAACAGAGCCCTTCTCCCCTCCCACCCCTTAGTGAGCCAGTC

1/2ERE

+896 TAAACCAAGCTGCTTGTCACTGCTGTTGTTCTGTGAATGTGATGCTGGTC

+946 AGACAGTCCCATACGCAGATTTCCAAACCTCTCGGAGCTCCTCTCAGATC

Exon2

+996 CCGAACGACCCTGGAGAGCCACGTCATCTCCACCCCAAACCTCTCACCAC

Fig. A. 6 (Continued)

+1046 AGCAGCCGACCACCCCAACAGCAACATGTACCCTGAGGAGACACGCGGA

+1096 GGTGGTGGGGCGGCCGCCTTTAACTACCTGGACGGAGGGTATGACTACAC

+1146 AGCCCCTGCCCAA

Fig. A. 6 (Continued)