Molecular cloning and expression analysis of rainbow trout (*Oncorhynchus mykiss*) CCAAT/enhancer binding protein genes and their responses to induction by GH *in vitro* and *in vivo*

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**Abstract**

CCAAT/enhancer-binding proteins (C/EBPs) are transcription factors consisting of six isoforms and play diverse physiological roles in vertebrates. In rainbow trout (*Oncorhynchus mykiss*), in addition to the reported C/EBP$\beta_1$, we have isolated cDNA of four other isoforms, C/EBP$\alpha$, C/EBP$\beta_2$, C/EBP$\delta_1$, C/EBP$\delta_2$, from the liver. Comparison of the deduced amino acid sequence of rainbow trout C/EBPs with those of other vertebrates revealed that C/EBP isoforms are highly conserved. The profiles of tissue-specific expression of individual C/EBP isoform mRNA, determined by quantitative real-time (RT)-PCR showed distinct patterns. Furthermore, injection of bovine GH into yearling rainbow trout resulted in a significant increase of mRNA levels of C/EBP$\beta_1$, C/EBP$\beta_2$, and C/EBP$\delta_2$ but not C/EBP$\alpha$ and C/EBP$\delta_1$ in the liver. GH-dependent increase of mRNA levels of C/EBP$\beta_1$, C/EBP$\beta_2$, C/EBP$\delta_2$, and IGF-II were also confirmed by treating rainbow trout hepatoma cells expressing a goldfish GH receptor with bGH. Together with our previous findings, the results presented in this paper strengthen our previous hypothesis that GH may regulate the expression of the IGF-II gene via mediating the expression of C/EBP$\beta_1$, C/EBP$\beta_2$, and C/EBP$\delta_2$ mRNA.


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**Introduction**

Growth hormone (GH) is a pleiotropic hormone that mediates cellular metabolism, growth, and differentiation by regulating the expression of specific genes (Schwartz et al. 2002). In mammals, several end target genes controlled by GH have been identified, including serine protease inhibitor 2.1, insulin like growth factors (IGF-I and IGF-II), cytochrome P450 (CYP), alcohol dehydrogenase, and many others (Huo et al. 2006). GH is known to exert its effect by first binding to the GH receptor (GHR) and then triggering signal transduction cascades resulting in physiological responses (Argetsinger & Carter-Su 1996). Although several transcription factors were believed to be involved in GH signaling, only a few of them, such as signal transducer and activator of transcription (STAT) or serum response factor are known to act directly as mediators to control the expression of GH-regulated end target genes (Argetsinger & Carter-Su 1996, Schwartz et al. 2002).

CCAAT/enhancer-binding proteins (C/EBPs) are a family of leucine zipper transcription factors that consists of six members ($\alpha, \beta, \gamma, \delta, \epsilon, \zeta$), and have been isolated from several animal species since they were first identified (Landschulz et al. 1988, Ramji & Foka 2002). In teleost fish, five isoforms of C/EBPs were identified in zebrafish (Lyons et al. 2001a, b), two in Japanese flounder (Tucker et al. 2002), and one in rainbow trout (Fujiki et al. 2003). The C/EBP family proteins share highly conserved C-terminal basic DNA-binding domains and leucine zipper regions among different animal species, and the N-terminal regions have various functional domains among all of the isoforms that account for their distinct functions (Lekstrom-Himes & Xanthopoulos 1998, Takiguchi 1998, Ramji & Foka 2002, Schrem et al. 2004). C/EBP isoforms are known to play numerous biological functions including cellular differentiation, inflammatory response, liver regeneration, and energy metabolism (Lekstrom-Himes & Xanthopoulos 1998, Takiguchi 1998, Ramji & Foka 2002, Schrem et al. 2004). A well-studied gene, phospho(enol)pyruvate carboxykinase (PEPCK), involved in gluconeogenesis is activated by thyroid hormone, glucocorticoid, and cyclic AMP through the mediation of C/EBPs (Croniger et al. 1998, Roessler 2001).

We previously reported that IGF-I and IGF-II are the target genes regulated by GH, and that C/EBPs could act as mediators for the induction of IGF-II gene expression by GH in rainbow trout (Shambolt et al. 1995, 1998). To dissect this system further, we report here the isolation and characterization of the cDNA of another four C/EBP isoforms, namely C/EBP$\alpha$, C/EBP$\beta_2$, C/EBP$\delta_1$, and C/EBP$\delta_2$ from rainbow trout liver RNA.
Distinct patterns of tissue-specific expression of C/EBP isoform mRNAs were observed when compared with those of other vertebrates. Results of in vitro studies conducted in rainbow trout hepatoma (RTH) cells (Chen et al. 2004) expressing the goldfish GHR, together with results of in vivo studies conducted in yearling rainbow trout revealed that levels of C/EBPβ2, C/EBPδ2, and C/EBPδ6 mRNAs are readily induced by treatment with bovine GH (bGH). These results lend additional evidence that C/EBPs may act as mediators in regulating the expression of the IGF-II gene in rainbow trout by GH. Furthermore, the RTH cells (Chen et al. 2004) could serve as experimental models for examining the molecular mechanism of GH regulation of the IGF-II gene in teleost fish.

**Materials and Methods**

**Animals**

Rainbow trout were obtained from the Salmon Disease Laboratory of Oregon State University, Corvallis, OR, USA. The fish used in this study were 6–12 months old with uniform body weights of 60–180 g and comprise both sexes. They were maintained in the fish facility at the University of Connecticut in tanks with a flow through of fresh water (12–15 °C) under a photoperiod of 12 h light:12 h darkness for a minimum of 2 weeks before use. The fish were fed to satiety, once a day, with commercial trout feed (Melick Aqua Feeds, Catawissa, PA, USA).

**Cloning of rainbow trout C/EBP cDNA isoforms and sequence analysis**

Sequence information of zebrafish C/EBP isoforms was used to search the rainbow trout expressed sequence tag (EST) database (Rexroad et al. 2003; http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gdb=r_trout). All of the EST sequences under the assigned tentative consensus sequence number with a high degree of similarity to individual C/EBP isoforms were retrieved from Genbank and analyzed by Vector NTI (InforMax, Fredrick, MD, USA) Advance 9.0 suite software (Invitrogen) to determine the open reading frame.

To verify sequence authenticity and acquire the complete coding sequence, total RNA was isolated from rainbow trout liver tissue with TRIzol reagent (Invitrogen) following the protocol provided by the supplier. First strand cDNA was synthesized as described below. For C/EBPα2, a 810 bp fragment was amplified by PCR in a total volume of 25 μl using standard buffer conditions as described by the manufacturer (Promega), supplemented with 1.5 mM MgCl₂, 120 μM dNTP, 0.4 μM primers, 0-625 units Taq polymerase (Promega), and 1 μl diluted first strand cDNA (Table 1). The amplification cycle profile consisted of an initial denaturation step of 1 min at 94 °C, followed by 35 cycles of 10 s each at 94 °C, 10 s at the annealing temperature of 55 °C, and 1 min at 72 °C for synthesis. The PCR fragment was cleaned up by QIAquick PCR Purification Kit (Qiagen) followed by restriction enzyme digestion and cloned into pBluescriptII SK(−) (Stratagene, La Jolla, CA, USA) at HindIII and SalI sites. The nucleotide sequences were determined by using the BigDye terminator cycling sequencing method (Applied Biosystems, Foster City, CA, USA). Based on the partial sequence information, two internal gene specific primers (GSP; Table 1) were designed for 3′ rapid amplification of the cDNA ends (RACE). The first strand cDNA used for 3′ RACE was synthesized under the same conditions except using a T17 adaptor primer. GSP1 and T17 adaptor primers were used for first round PCR amplification under conditions described above. The amplification cycle of 1 min at 94 °C, followed by 35 cycles of 10 s each at 94 °C, 10 s at the annealing temperature ranging from 50–60 °C, and 1.5 min at 72 °C was used to determine optimal conditions. The PCR mixture from the first round of PCR at different annealing temperatures was used as the template for the second round of PCR using a second GSP and adaptor primer under identical conditions as described above. The amplification cycle profile consisted of 1 min at 94 °C, followed by 35 cycles of 10 s each at 94 °C, 10 s at 59 °C, and 1.5 min at 72 °C. The PCR products revealed three major bands on an agarose gel and individual fragments were cloned into pBluescriptII SK(−) at ClaI and SalI sites. The longest clone identified was 640 bp with a 190 bp overlap to the 5′ partial sequence. For the cloning of complete coding sequences of individual C/EBP isoforms, high fidelity Deep Vent DNA polymerase (New-England Biolabs, Woburn, MA, USA) was used for PCR in a total volume of 25 μl containing 1X thermal polymerase buffer (NewEngland Biolabs), 120 μM dNTP, 0.4 μM primers (Table 1), 0.25 unit of Deep Vent, and 1 μl diluted first strand cDNA. The PCR fragments were cloned into pC DNA 3.1 (+) (Invitrogen), and at least three positive clones for individual C/EBP isoforms were selected for plasmid isolation and sequenced using the BigDye terminator cycling sequencing method.

Multiple amino acid sequence alignments were carried out using ClustalW-based program, AlignX, in Vector NTI Advance 9.0 suite software. The deduced amino acid sequences were subjected to an AlignX analysis to derive sequence information, two internal gene specific primers (GSP; Table 1) were designed for 3′ rapid amplification of the cDNA ends (RACE). The first strand cDNA used for 3′ RACE was synthesized under the same conditions except using a T17 adaptor primer. GSP1 and T17 adaptor primers were used for first round PCR amplification under conditions described above. The amplification cycle of 1 min at 94 °C, followed by 35 cycles of 10 s each at 94 °C, 10 s at the annealing temperature ranging from 50–60 °C, and 1.5 min at 72 °C was used to determine optimal conditions. The PCR mixture from the first round of PCR at different annealing temperatures was used as the template for the second round of PCR using a second GSP and adaptor primer under identical conditions as described above. The amplification cycle profile consisted of 1 min at 94 °C, followed by 35 cycles of 10 s each at 94 °C, 10 s at 59 °C, and 1.5 min at 72 °C. The PCR products revealed three major bands on an agarose gel and individual fragments were cloned into pBluescriptII SK(−) at ClaI and SalI sites. The longest clone identified was 640 bp with a 190 bp overlap to the 5′ partial sequence. For the cloning of complete coding sequences of individual C/EBP isoforms, high fidelity Deep Vent DNA polymerase (New-England Biolabs, Woburn, MA, USA) was used for PCR in a total volume of 25 μl containing 1X thermal polymerase buffer (NewEngland Biolabs), 120 μM dNTP, 0.4 μM primers (Table 1), 0.25 unit of Deep Vent, and 1 μl diluted first strand cDNA. The PCR fragments were cloned into pC DNA 3.1 (+) (Invitrogen), and at least three positive clones for individual C/EBP isoforms were selected for plasmid isolation and sequenced using the BigDye terminator cycling sequencing method.

Multiple amino acid sequence alignments were carried out using ClustalW-based program, AlignX, in Vector NTI Advance 9.0 suite software. The deduced amino acid sequences were subjected to an AlignX analysis to derive sequence identity, and phylogenetic distance among isoforms in different animal species using neighbor-joining method (Saitou & Nei 1987).

In vivo GH treatment and tissue collection

Two groups of fish weighing about 60 and 200 g were deprived of food for at least 5 days prior to injection with bGH (10 μg/g body weight; USDA-bGH-B-1) dissolved in a carrier solution (10 mM NaHCO₃, pH 8.0, and 0.15 M NaCl) or with the carrier solution as controls. Each treatment and time point was done in triplicate. Liver tissues were collected from fish 3 and 6 h after GH treatment, and the samples were snap frozen at −80 °C until further analysis.
Tissues samples for a C/EBP tissue distribution study were isolated from juvenile male fish under normal physiological conditions without any treatment.

Cell culture and GH treatment

RTH-1B1A (Chen et al. 2004) was transfected with a goldfish GHR (Lee et al. 2001) expression vector by electroporation using gene pulser II (Bio-Rad) under the following conditions: 250 μl of 3×10^6 cells/ml suspension in 0.4 cm cuvette, 725 V/cm field strength, 100 μF capacitance, low Ω, 60 ms burst duration, 20% modulation, 50 KHz frequency, 6 bursts with 0.5 s burst interval. Permanent transfectants were selected by G418 and single cell cloned. Cells were cultured at 20 °C in L-15 medium (GibcoBRL Invitrogen) supplemented with 10% fetal bovine serum. Prior to hormone treatment, cells were seeded onto a 6-well plate at about 80–90% confluency, the culture medium was changed to serum free L-15 medium after cell attachment, and the cells were starved for 24 h before treatment with various concentrations (50–800 ng/ml) of bGH (USDA-bGH-B-1). Cells were harvested at 6 h after GH treatment.

Measurement of C/EBP mRNA levels

RNA extraction and reverse transcription

Tissue and cell samples were homogenized in TRizol reagent (Invitrogen) and total RNA was extracted following manufacturer’s protocol. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water following DNase (RQ1 DNase Promega) digestion. DNase treated RNA samples were extracted again by TRIzol reagent. First strand cDNA was synthesized from 4 μg of tissue total RNA and 3 μg of cell total RNA at 42 °C using Superscript III reverse transcriptase (Invitrogen) and oligo(dT)17 following conditions provided by the supplier.

Measurement of mRNA by real-time PCR analysis

The gene expressions analysis was performed by iCycler iQ real-time PCR detection system (Bio-Rad) using SYBR Green I (BMA, Rockland, ME, USA). The reaction was carried out with three repeats in each sample. Amplification efficiencies were determined by serial diluted cloned standard molecules (standard curve), and controlled above 90% for each pair of primers (Table 1) by adjusting conditions (1X reaction buffer, 0.4 μM primers, 0.2 mM dNTP, 3 mM MgCl2, 0.4× SYBR Green I, 0.01 μM fluorescence

Table 1 List of primers used in this study

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<tr>
<th>Gene</th>
<th>Sequence (5’–3’)</th>
<th>Function</th>
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<td>C/EBP</td>
<td>F: AGCTAAGCTTCCGACGCTCTCACCC</td>
<td>Initial partial cDNA sequence cloning and complete coding sequence cloning</td>
</tr>
<tr>
<td></td>
<td>Initial partial cDNA sequence cloning</td>
<td>T17 adaptor primer for 3’ RACE</td>
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<td>Gene specific primer 2 for 3’ RACE</td>
<td>Complete coding sequence cloning</td>
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<td>C/EBP1</td>
<td>T: CCCAGATCCAGGAAACGCAGGTTC</td>
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<td></td>
<td>CACCCTCAAGTACATTCCAGTG</td>
<td>Complete coding sequence cloning</td>
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F, forward primer; R, reverse primer; RACE, rapid amplification of cDNA ends; 18S rRNA, 18S ribosomal RNA GenBank number: C/EBP, DQ423469; C/EBP1, AY144611; C/EBP2, DQ423470; C/EBP3, DQ423472; C/EBP4, DQ423471; IGF-II, M95184; β-actin, AJ438158; 18S rRNA, AF308735.
(Bio-Rad) in 25 μl reaction volume) and amplification cycles (95 °C 2 min followed with 50 cycles of 95 °C 10 s; 59 °C 10 s for IGF-II, C/EBPα, C/EBPβ1, C/EBPβ1, 18S rRNA, β-actin or 54 °C for C/EBPβ2, C/EBPβ62; 72 °C 20 s). A melting curve, from 55 °C to 95 °C with 0.5 °C increments at every 10 s, was added after each amplification run to control the possible contamination and primer dimer formation (quality control). Primer specificities between C/EBPβ1 and C/EBPβ2 and between C/EBPβ1 and C/EBPβ62 were examined by including reciprocal standard molecules. No cross reaction was detected.

In an assay for the expression of C/EBP genes in different tissues, serial diluted standard molecules were used to determine the absolute cDNA copy number. To account for the differences in cDNA template amount or quality, β-actin expression level was used as control, and a similar level of β-actin mRNA was observed. In GH induction assay, threshold cycles were further analyzed by Q-Gene (Muller et al., 2002), using equation 3 (The mean normalized gene expression is calculated by averaging the three concomitant threshold cycle values of the target gene and of the reference gene respectively) and assuming 100% amplification efficiency for both the target genes and housekeeping genes.

Statistical analysis

Data were analyzed by Statistica 7.0 software (Statsoft, Tulsa, OK, USA). The tissue expression data were analyzed by one-way ANOVA followed by Fisher least significant difference test. Data of the in vivo GH induction assay were analyzed by factorial ANOVA followed by Newman–Keuls test. Results of the in vitro GH induction assay were analyzed by a nonparametric sign test. The in vitro GH responsiveness trend line was determined by polynomial regression analysis using Microsoft Excel. P values lower than 0.05 were considered to be significant.

Results

Isolation and sequence analysis of trout C/EBP isoforms

The coding regions of rainbow trout C/EBPα, β2, β1, and δ2 cDNA were isolated by RT-PCR amplification of the liver RNA and the nucleotide sequences of each C/EBP isoform were determined. The nucleotide sequences of the C/EBP isoforms were submitted to the gene bank with the GenBank numbers: DQ423469 (C/EBPα), DQ423470 (C/EBPβ2), DQ423472 (C/EBPδ1), and DQ423471 (C/EBPδ2). Since the nucleotide sequence of the C/EBPβ1 cDNA isolated in this study was identical to that reported earlier (Fujiki et al., 2003), we did not submit this sequence to the gene bank. A comparison of sequence similarity and identity of the deduced amino acid sequence revealed that all of the rainbow trout C/EBP isoforms shared high degrees of sequence identity (C/EBPα, 69.5%; C/EBPβ1, 63.1%; C/EBPβ2, 65.8%; C/EBPδ1, 57.1%; C/EBPδ2, 55.7%) with the C/EBP isoforms of zebrafish but lower degrees with those of frog, chicken, rat, and human (Table 2). In rainbow trout, the sequence identity between C/EBPβ1 and C/EBPβ2 is 86.1% and between C/EBPβ1 and C/EBPδ2 is 83.5%. Since C/EBPβ1, C/EBPβ2, C/EBPδ1, and C/EBPδ2 cDNAs were isolated from the liver RNA sample prepared from an individual fish and the differences in nucleotide sequences among C/EBPβ1 and C/EBPβ2 or C/EBPδ1 and C/EBPδ2 disperse through the entire molecule, it is unlikely that C/EBPβ and C/EBPδ isoforms are derived from single C/EBPβ and C/EBPδ genes.

Figure 1A–C depicts the alignment of the deduced amino acid sequence of C/EBP isoforms of rainbow trout, zebrafish, frog, chicken, rat, and human. Data of the alignment showed that trout C/EBP isoforms shared a high degree of sequence conservation in the basic DNA-binding regions (BR–A, BR–B) and the leucine zipper domain with C/EBPs of zebrafish, frog, chicken, rat, and human (Fig. 1A–C). While the last leucine in the leucine zipper was replaced by a threonine in rainbow trout C/EBPβ1 and C/EBPβ2, the last leucine in the leucine zipper in trout C/EBPδ1 and C/EBPδ2 remained conserved. The consensus sequence of boxA and boxB, the transactivator regions (Nerlov & Ziff, 1995), was also identified in the N-termini of the trout C/EBPα, C/EBPβ1, and C/EBPδ2. Similar to zebrafish, the boxB of trout C/EBPβ1 and C/EBPβ2 have deviated from the conserved consensus sequence. Like in many animal species studied to date, regions including the PT region (proline–threonine rich), PS region (proline–serine rich), and PR region (proline rich) are also identified in trout C/EBPα, C/EBPβ1, and C/EBPδ isoforms.

The ATG codon of the trout C/EBPβ1 and C/EBPβ2 corresponds to a shorter isoform of the mammalian homolog termed liver–enriched transcriptional activator protein (LAP). However, there is no start codon corresponding to the longer isoform of the mammalian homolog LAP* (long form LAP) in trout C/EBPβ isoforms. It is interesting to note that trout C/EBPβ1 and C/EBPβ2 isoforms also contain an internal translation initiation codon corresponding to the mammalian homolog LIP (Descombes & Schibler, 1991, Xiong et al., 2001). Two major protein products (p42 and p30) were shown to be translated from a single C/EBPα mRNA in rat (Lin et al., 1993, Ossipow et al., 1993), however, the internal translation site for p30 is not in a relevant position in rainbow trout C/EBPα (Fig. 1A). Furthermore, a synergy control (SC) motif was found in C/EBPα, and a consensus sequence of the small ubiquitin–related modifier (SUMO) was found in C/EBPα and C/EBPδ (Fig. 1A and C). Although the functions of SC and SUMO in rainbow trout remain to be determined, SUMO is known to control the interaction among transcription factors (Subramaniam et al., 2003) and SC limits the transcriptional synergy with other DNA–binding regulators in mammals (Inguez-Lluhi & Pearce, 2000). A unique histidine–glutamine variable region (HGV) was identified in trout C/EBPα (Fig. 1A). This HGV region has various lengths and compositions of histidine and glutamine in the clones that we isolated (Fig. 1A inset), and its functional significance remains to be determined. Of the serine
Levels of C/EBP isoform mRNA in different tissues

The levels of C/EBP isoform mRNA in 13 different tissues of male rainbow trout were determined by quantitative real-time PCR analysis and the results were expressed as the number of copies of mRNA per 1 μg total RNA. As shown in Fig. 3A, high levels of C/EBPα mRNA were detected in the kidney, spleen, gill, and pancreas, and low levels were detected in the liver, pyloric ceca, heart, and testis. While high levels of C/EBPβ1 and C/EBPβ2 mRNA (Fig. 3B and C) were detected in the liver, consistent with findings by other investigators (Fujiki et al., 2003), no C/EBPβ2 mRNA was detected in muscle, blood cells and the pituitary gland. High levels of C/EBPα1 and C/EBPα2 mRNA were detected in gill and pancreas, moderate levels in liver, kidney, muscle, heart, pyloric ceca, spleen, and testis, and low levels in blood cells, brain, and the pituitary gland (Fig. 3D and E).

In vivo and in vitro induction of mRNA of C/EBP isoforms in the rainbow trout liver and trout hepatoma cells (RTH-1B1A)

Previous studies conducted in our laboratory (Shamblott et al., 1998) showed that the immunoreactive materials specific to rat C/EBPα and C/EBPβ antibodies were induced in the nuclear fraction of the rainbow trout liver after treatment with bGH. These results suggest that the expression of C/EBP isoforms in the rainbow trout liver may be regulated by GH. To verify these results, the mRNA levels of C/EBP isoforms and IGF-II in the liver were determined following administration with the same dose of bGH used previously (Shamblott et al., 1995) to yearling juvenile and 2-year adult fish. Levels of IGF-II mRNA increased two- to threefold at 3 and 6 h post bGH administration (data not shown), which confirmed the results of our previous studies (Shamblott et al., 1995). While 2-year-old fish showed an obvious increase of C/EBPβ2 mRNA levels at 3 h after bGH treatment, yearling juvenile fish showed an increase of both mRNA levels at 3 and 6 h following hormone treatment (Fig. 4A and B). Although a significant increase in C/EBPβ1 mRNA levels was observed in 2-year-old fish at 3 and 6 h following GH treatment, the same increase of C/EBPβ1

Table 2 Amino acid identities (%) among CCAAT/enhancer-binding proteins (C/EBPs) of rainbow trout and other representative vertebrates

<table>
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<tr>
<th>Organism</th>
<th>C/EBPα</th>
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<td>–</td>
</tr>
<tr>
<td>Hu C/EBPβ1</td>
<td>27-7</td>
<td>37-7</td>
<td>38-1</td>
<td>27</td>
<td>26-4</td>
<td>–</td>
</tr>
<tr>
<td>Hu C/EBPβ2</td>
<td>38-7</td>
<td>38-1</td>
<td>29-1</td>
<td>28-8</td>
<td>28-8</td>
<td>–</td>
</tr>
<tr>
<td>Hu C/EBPδ1</td>
<td>24-8</td>
<td>24-5</td>
<td>21-2</td>
<td>43</td>
<td>41-2</td>
<td>–</td>
</tr>
<tr>
<td>Hu C/EBPδ2</td>
<td>26-9</td>
<td>24-5</td>
<td>21-2</td>
<td>43</td>
<td>41-2</td>
<td>–</td>
</tr>
</tbody>
</table>

RT, rainbow trout; Ze, zebrafish; JF, Japanese flounder; X, African clawed frog; Ch, chicken; Hu, human.

and threonine phosphorylation consensus sites (i.e. Ser248, Ser277, Ser299, Ser230, Thr222, and Thr226) found in rat C/EBPβ (Mahoney et al., 1992), only Ser299, Ser230, Thr222, and Thr226 were identified in trout C/EBPα. Furthermore, three phosphorylation sites in C/EBPβ (i.e. mouse Ser184 (Piwnik-Pilipuk et al., 2001), human Thr235 (Nakajima et al., 1993), and Rat Ser240 (Trautwein et al., 1994)) known to be phosphorylated by glycogen synthase kinase 3β (GSK3β), mitogen-activated protein kinase (MAPK), and protein kinase A plus protein kinase C, were identified in both isoforms of rainbow trout C/EBPβ, but mouse Thr217 and rat Ser105 sites (Buck et al., 1999; Fig. 1B) were absent in trout C/EBPβ. A calmodulin-dependent protein kinase II phosphorylation site originally identified in mammalian C/EBPβ (Wegner et al., 1992) was found to exist in rainbow trout C/EBPδ1 but not in rainbow trout C/EBPδ2 (Fig. 1C).

A small upstream open reading frame (uORF) was identified in rainbow trout C/EBPα, C/EBPβ1 (Fujiki et al., 2003), and C/EBPβ2, upstream of the initiation codon ATG of P42 and LAP (Fig. 2). While the uORF of C/EBPα, encoding a small peptide of five amino acid residues, stops at seven nucleotides preceding the translational start site, the uORF of C/EBPβ, encoding a peptide of eight amino acid residues, stops at five nucleotides preceding the translational start site.
mRNA was only observed in yearling juvenile fish at 6 h after hormone treatment. It is interesting to note that bGH may suppress the level of C/EBPα mRNA in adult fish 6 h after bGH treatment (Fig. 4A).

We have shown previously that, although RTH cells expressed GHR2 gene (Chen et al. 2004, Very et al. 2005), we failed to demonstrate the GH responsiveness in these cells. To restore the ability of the RTH 1B1A cells to GH signaling, a goldfish GHR expression vector was introduced into RTH 1B1A cells by electroporation, and permanent transformants isolated. By treating RTH-1B1A-gfGHR transformants with 50–800 ng/ml bGH for 6 h, a dose-dependent increase of the levels of IGF-II, C/EBPβ1, C/EBPβ2, and C/EBPδ2 mRNA was observed (Fig. 5). The maximal level of induction (2- to 2.5-fold) for IGF-II was at 600–800 ng/ml bGH, while maximum levels of induction (2.5- to 3-fold) for C/EBPβ2 and C/EBPδ2 were at 300–600 ng/ml bGH. However, a lower level of induction (about 1.5 -fold) of GC/EBPα mRNA was observed at 50–300 ng/ml bGH. C/EBPα and C/EBPδ1 mRNA expression was not responsive to bGH treatment in these studies (results not shown).

Discussion

The family of C/EBPs identified in many animal species consist of six members (α, β, γ, δ, ε, ζ), which contain a basic DNA-binding region and a leucine-zipper domain.
Figure 1 (continued)
(bZIP motif) in their C-termini. Both domains have high sequence similarity among the six members (Ramji & Foka 2002). In this study, we cloned C/EBPα, β1, β2, δ1, and δ2 cDNA by RT-PCR amplification of liver RNA, and phylogenetic analysis showed that these C/EBP isoforms cluster with the corresponding isoforms of other animal species (Xu & Tata 1992, Antonson & Xanthopoulos 1995, Calkhoven et al. 1997, Lyons et al. 2001a, Tucker et al. 2002), clearly indicating that we have indeed isolated rainbow trout C/EBP orthologs. Data from the amino acid sequence alignment analysis showed that trout C/EBP isoforms shared a high degree of sequence conservation on the basic DNA-binding region (BR-A, BR-B), the leucine zipper domain, and the domains of the proline–threonine rich region (PT rich region), proline–serine rich region (PS rich region), and proline rich region (PR region) with C/EBPs of zebrafish, frog, chicken, rat, and human (Lyons et al. 2001a). Although the consensus sequence of boxA and boxB, the transactivator regions (Nerlov & Ziff 1995), was identified in the N-termini of the C/EBPα, C/EBPδ1, and C/EBPδ2 of trout (in the present study) and zebrafish (Lyons et al. 2001a), boxB of the trout C/EBPδ1 and C/EBPδ2 has deviated from the conserved consensus sequence of the other animal species (Nerlov & Ziff 1995, Fujiki et al. 2003).

C/EBPs are known to have a variety of functions in immune responses, growth, and development in many organisms (Schrem et al. 2004). Although the functions of trout C/EBP isoforms await determination, identification of a C/EBP-like sequence and the conserved consensus sequence in rainbow trout indicates that the functions of these transcription factors may be highly conserved in organisms of different phylogenetic positions. The presence of highly conserved basic leucine zipper regions among C/EBPs of various animal species indicate that these regions recognize cognate DNA sequences and exert similar functions among vertebrates (Osada et al. 1996). It is known that C/EBP isoforms form homodimers within each isomer or heterodimers with other isoforms or with other families of leucine zipper proteins (Vinson et al. 1989, Williams et al. 1991, Osada et al. 1996). Since the essential functional domains are nearly identical between C/EBPδ1 and C/EBPδ2, and between C/EBPδ1 and C/EBPδ2, it is likely that these C/EBP isoforms exhibit similar functions on their targets. It is interesting to note that, although we failed to identify more than one type of C/EBPα mRNA in several rainbow trout individuals, we have identified several cDNA clones of C/EBPα with different lengths and compositions of a short stretch of peptide (12–13 amino acid residues rich in
glutamine and histidine) from each individual animal. Although this characteristic is unique in rainbow trout and not found in zebrafish C/EBPα, its functional significance requires further investigation.

C/EBPα and C/EBPβ mRNAs from many species contain a short uORF immediately upstream of the major translation start site (Morris & Geballe 2000). These uORFs function as cis-acting elements and regulate alternative translational initiation resulting in the production of various lengths of truncated isoforms (Calkhoven et al. 1994, 2000, Lincoln et al. 1998, Morris & Geballe 2000, Xiong et al. 2001). In our study, an equivalent length uORF is also found in rainbow trout C/EBPα (Fig. 2). The highly conserved structure of

Figure 3  PCR analysis. Real-time RT-PCR analysis was used to determine the absolute copy numbers for individual C/EBP isoform mRNA in the liver (Liv), head kidney (HK), posterior kidney (PK), spleen (Spl), gill (Gi), muscle (Mu), pyloric ceca (PyC), blood (Bld), brain (Brn), pituitary (Pit), heart (Hrt), pancreas (Pan), and testis (Tes). (A) C/EBPα, (B) C/EBPβ1, (C) C/EBPβ2, (D) C/EBPδ1, (E) C/EBPδ2. ND, non-detected. Each data point is presented as mean ± S.D. (n = 2). Different lowercase letters indicate significant differences among different tissues (P < 0.05).
uORF in C/EBPα across different vertebrate species very likely suggests that C/EBPα protein expression is subjected to the regulation of the uORF in rainbow trout. The uORF of C/EBPβ2 has the same structure as that of C/EBPβ1 (Fujiki et al. 2003). In both zebrafish (Lyons et al. 2001a) and Japanese flounder (Tucker et al. 2002), there is no appropriate start codon for the uORF, although a termination codon for a potential uORF can be found in the same position (5 bp upstream of the start codon for LAP) as that of rainbow trout (Fujiki et al. 2003). Since trout and zebrafish are phylogenetically closer to each other than trout to Japanese flounder, the finding of a uORF in trout that lacks LAP*, together with zebrafish and Japanese flounder that both lack the uORF, suggests that the LAP* has evolved after the formation of the uORF (Fujiki et al. 2003). The partly conserved feature of the uORF in rainbow trout C/EBPβ with that of other teleost fish and tetrapods may also imply that C/EBPβ protein expression in rainbow trout is under the regulation of the uORF, but differs from that in tetrapods since the distance between the uORF stop codon and the ORF initiating codon (five bases in teleost fish and four bases in other vertebrates) is important for translational regulation (Lincoln et al. 1998).

In mammals, chicken, frogs, and fish (Xu & Tata 1992, Antonson & Xanthopoulos 1995, Calkhoven et al. 1997, Lyons et al. 2001a, Tucker et al. 2002), C/EBPα and C/EBPβ isoform mRNAs are expressed in the liver, intestine, adipocyte, and other tissues. We have shown in this study that high levels of C/EBPα, C/EBPβ, and C/EBPδ isoform mRNAs were detected in the liver, kidney, spleen, and gills of rainbow trout. Furthermore, reasonably high levels of C/EBPδ isoform mRNA were detected in muscle, pyloric ceca, heart, pancreas, and testis. Since the expression of PEPCK and glycogen synthase genes in the liver is known to be regulated mainly by C/EBPα (Croniger et al. 1998, Roesler 2001, Desvergne et al. 2006), finding high levels of C/EBPβ

Figure 4  Expression of C/EBP isoform mRNA in rainbow trout treated with bovine growth hormone in vivo. Fishes were starved for 5 days and injected with 10 μg bGH/g body weight or carrier solution for 3 and 6 h. Livers were collected from hormone treated and control fish and immediately frozen on dry ice. The levels of C/EBP mRNA expression were determined by real-time RT-PCR and normalized to β-actin. The expression level of 3 h control was arbitrarily set to 1. (A) Adult fish (average weight 200 g); (B) Juvenile fish (average weight 60 g). Each data point is expressed as mean ± S.D. (n=3). Different letters indicate significant differences among different treatments and different time points (P<0.05).
and C/EBPβ mRNAs suggest that these isoforms may play important roles in the trout liver. It has been reported by many investigators that C/EBPβ plays an important role in the inflammatory response (Poli 1998), and in mice, the same protein is also involved in regulating acute phase protein genes such as serum amyloid A, serum amyloid P, and complement C3 (Poli 1998). One would, therefore, expect that C/EBPβ isoform plays an important role in regulating the rainbow trout orthologs of these genes. The finding that high levels of C/EBPβ and C/EBPδ isoforms mRNAs are present in immunologically important organs such as the head kidney, posterior kidney, spleen, and liver in rainbow trout supports this hypothesis.

It has been shown with conflicting results that the expression of C/EBPα mRNA in mammals is regulated by GH (Vidal et al. 1997, Rastegar et al. 2000a,b, Strand et al. 2000, Eleswarapu & Jiang 2005). In our study, we showed that, although the expressions of C/EBPα in the rainbow trout liver and in RTH cells are not responsive to induction by GH, the level of C/EBPα mRNA decreased in adult fish at 6 h post GH injection. Thus, it is possible that GH regulation on C/EBPα mRNA in the rainbow trout liver could be time dependent and under subtle control of other factors as in mammalian systems (Vidal et al. 1997, Rastegar et al. 2000a,b).

It is known that hepatocytes need to be cultured on extracellular matrix for the expression of many liver-specific genes and maintenance of a normal hepatic phenotype (Schuetz et al. 1988, DiPersio et al. 1991, Rana et al. 1994). Our initial studies failed to demonstrate that the RTH cell lines established in our laboratory responded to GH treatment, although these cells expressed the GHR2 gene (Chen et al. 2004). However, after transfecting a gene construct containing a goldfish GHR cDNA driven by a CMV promoter into RTH cells, the permanent transformants (RTH-1B1A-gfGHR cells) responded to GH treatment by expressing IGF-II, C/EBPβ1, C/EBPβ2, and C/EBPδ2 genes. To our knowledge, this is the first report of GH induction of C/EBPβ1, C/EBPβ2, and C/EBPδ2 gene expression in teleost fish. GH is known to transmit its signal to target genes by at least four pathways, namely phosphoinositide-3 kinase (PI3K), STAT, phosphatidylinositol bisphosphate (PIP2)-Ca2+, and MAPK in mammalian systems (Argetsinger & Carter-Su 1996). These pathways are implicated by other growth factors and cytokines. It has been demonstrated that GH can regulate C/EBPβ through the PI3K pathway by activating serine–threonine kinase (Akt) and inhibiting GSK-3 phosphorylation leading to the dephosphorylation of C/EBPβ and increase of DNA-binding activity (Piwien-Pilipuk et al. 2001). GH-mediated phosphorylation of C/EBPβ through MAPK is required for C/EBPβ transcriptional activity (Piwien-Pilipuk et al. 2002). Furthermore, it has been shown that GH-dependent MAPK activation plays a role in the regulation of nuclear relocalization of C/EBPβ (Pilipuk et al. 2003). Since information about GH signaling in fish is scarce, the RTH cell lines will serve as a good comparative model.

Figure 5 Expression of C/EBP isoform mRNA in trout hepatoma cells in response to GH treatment. RTH cells were starved for 24 h in serum-free medium and treated with various doses of bGH for 6 h. Each treatment was performed in triplicate and individual experiments were repeated thrice. Relative expression levels of C/EBP mRNA were normalized to 18 S rRNA. The expression level of the control sample (i.e. without bGH treatment) was arbitrarily set to 1. (A) IGF-II, (B) C/EBPβ1, (C) C/EBPβ2, (D) C/EBPδ2. Each data point is expressed as mean ± S.D. (n=3). Asterisks indicate mRNA levels at different bGH doses that are significantly different from control (P<0.05).

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Following GH treatment, Shamblott et al. (1998) detected the increase of C/EBPα and C/EBPβ proteins in the nuclei of rainbow trout liver cells using antibodies raised against rat C/EBPα and C/EBPβ as probes. However, in our study, we showed that the mRNAs of C/EBPβ1, C/EBPβ2, and C/EBPα2 were readily induced in the trout liver or RTH cells after treatment with GH, but not the mRNA of C/EBPα. Since the rat anti-CEBPα antiserum used by Shamblott et al. (1998) was raised with a fragment of rat CEBPα (14 amino acid residues) as an antigen, of which only two amino acid residues are identical to the corresponding region of the trout C/EBPα, the observed discrepancy could be the consequence of low specificity of the rat C/EBPα antiserum to the trout C/EBPα polypeptide. In the electrophoretic mobility shift assay (EMSA) assay, Shamblott et al. (1998) showed that treatment of fish with GH resulted in an increase of C/EBP proteins bound to the rainbow trout C/EBP-binding sites residing on the proximal promoter region of the IGF-II gene. This increase of binding activity could be the consequence of upregulation of C/EBP isoform mRNA induced by GH, dephosphorylation of C/EBP or the combination of both. Nevertheless, GH is known to regulate C/EBP at multiple levels including the increase of mRNA levels, and synthesis and phosphorylation of C/EBP proteins (Liao et al. 1999, Strand et al. 2000, Piwien-Pilipuk et al. 2001, 2002, Schwartz et al. 2002). It has been shown that GH regulates the expression of CYP2C12 via the mediation of C/EBPs (Tollet et al. 1995), and C/EBP trans-activates the IGF-II promoter in human and chum salmon (Rodenburg et al. 1995, Palamarchuk et al. 2001). It is conceivable that the expression of the IGF-II gene in rainbow trout could be mediated via GH induction of C/EBPs. However, the molecular mechanism which underlies the regulation of IGF-II gene expression via the mediation of C/EBP proteins remains to be investigated.

In summary, we have isolated the cDNA of C/EBPα, C/EBPβ, and C/EBPδ isoforms from rainbow trout, and showed tissue-specific expression of these mRNAs. Furthermore, studies conducted in the whole animal as well as in trout hepatoma cells (RTH) confirmed that the expression of C/EBPβ1, C/EBPβ2, and C/EBPα2 mRNAs were regulated by GH. The results presented in this paper, together with those reported by Shamblott et al. (1998) suggest that the RTH cells may serve as an ideal system for elucidating the relationships of GH regulation of C/EBP isoform and IGF-II genes at the molecular level in teleost fish.

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