African Journal of Biotechnology Vol. 11(3), pp. 534-542, 10 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3174
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

cDNA sequence and tissue expression analysis of glucokinase from liver of grass carp (*Ctenopharyngodon idella*)

CHENG Han-liang*, JI Nan-jing, PENG Yong-xing, SHEN Xin, XU Jian-he, DONG Zhi-guo and WU Chen-chen

Jiangsu Key Laboratory of Marine Biotechnology, Huaihai Institute of Technology, Lianyungang 222005, People's Republic of China.

Accepted 19 December, 2011

A full-length cDNA coding glucokinase (GK) was cloned from the liver of grass carp (*Ctenopharyngodon idella*) by reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends methods. The cDNA obtained was 2066 bp exclusive of poly (A) residues with a 1431 bp open reading frame encoding 476 amino acids. The GK protein has a calculated molecular weight of 53.7 kDa and isoelectric point of 5.11. Some conserved functional sites were found including one conserved hexokinase signature sequence Leu^{156}-Phe^{181}; two N-linked glycosylation sites Asn^{176} and Asn^{214}; one cell attachment sequence Arg^{202}-Asp^{204}; one glycosaminoglycan attachment site Ser^{455}-Gly^{458}. The amino acid sequence has a high similarity to GK of other species, the percent identity compared with topmouth culter, common carp, human and rat are 98.1, 96.8, 80.3 and 79.8%, respectively. Tissue distribution of GK mRNA in brain, mesenteric adipose tissue, spleen, white muscle and liver of grass carp was analyzed by SYBR real-time fluorescence quantitative RT-PCR method using β-actin as an internal control for cDNA normalization. The result shows that the expression level of GK mRNA in liver was significantly higher than in mesenteric adipose tissue, spleen and brain ($p<0.05$). Relative expression profile of GK mRNA in liver normalized with β-actin level was 31, 454 and 649-fold compared with the levels in mesenteric adipose tissue, spleen and brain, respectively. Meanwhile, GK mRNA was not detected in white muscle.

Key words: *Ctenopharyngodon idella*, glucokinase, full-length cDNA, tissue distribution, real-time PCR.

INTRODUCTION

Glucokinase (GK, EC 2.7.1.1) is a member of the hexokinase gene family, which consists of several evolutionary related isoenzymes, hexokinase I, II, III and IV, and a conserved fifth vertebrate hexokinase was identified recently (Irwin and Tan, 2008). All of them catalyze the ATP-dependent phosphorylation of glucose to glucose-6-phosphate as the first step, and the first rate-limiting step, in the glycolytic pathway (Kawai et al., 2005). Hexokinase IV, which is often called glucokinase, has a molecular weight of 50 kDa, and is characterized by a high S0.5 for glucose of 5 of 8 mM compared with Km values of 20 to 130 μM for human hexokinases I to III, and by a lack of product inhibition by glucose-6-phosphate compared with the hexokinases (Mahalingam et al., 1999). Glucokinase is expressed in liver, pancreas, gut, and brain of humans and most other vertebrates. In each of these tissues, it plays crucial roles in the response to changes in blood glucose levels (Irwin and Tan, 2008; Panserat et al., 2001a), and converts excess glucose to lipid and glycogen in liver (Panserat et al., 2001b). Glucokinase also has an important role as glucose sensor and metabolic signal generator in pancreatic β-cells and hepatocytes (Egea et al., 2008). Moreover, mutations in the glucokinase gene

*Corresponding author. E-mail: chl3139@163.com. Tel: +86-518-85895429. Fax: 86-518-85895429.

can lead to development of an autosomal dominant form of type 2 diabetes, and nearly 100 of these mutations have been identified (Stoffel et al., 1992).

In contrast to mammals, the control of blood glucose is highly variable in fishes, carbohydrate utilization being poorly effective in many species, intermediate or good in others (Moon, 2001; Panserat et al., 2000a). In carnivorous species, the utilization of digestible dietary carbohydrates for energy purposes appears limited (Hemre et al., 2002; Stone, 2003), and a prolonged postprandial hyperglycemia has been observed after a carbohydrate-rich diet in rainbow trout (Caseras et al., 2000; Moon, 2001). But omnivorous common carp (*Cyprinus carpio*) easily use high levels of dietary carbohydrate (Panserat et al., 2000b). In addition, high-carbohydrate diets may be the main reason behind the fatty liver syndrome in cultured fish (Cheng et al., 2006). There are numerous reported studies on GK gene expression and regulation in carnivorous and omnivorous fish. The cDNA encoding GK has been cloned and sequenced in several fish, including common carp (Blin et al., 2000; Panserat et al., 2000a), rainbow trout (*Oncorhynchus mykiss*) (Panserat et al., 2000a), gilthead seabream (*Sparus aurata*) (Panserat et al., 2000a), zebrafish (*Danio rerio*) (Gonzalez-Alvarez et al., 2009) and topmouth culter (*Erythroculter ilishaeformis*) (Ge et al., 2006). The effects of feeding condition and nutritional state on GK gene expression in the liver were investigated. The expressions of GK and HK-I genes were analyzed during early ontogenesis of common carp. As far as HK-I gene is concerned, it is expressed during all developmental stages, while GK is only induced by the first feeding with high levels of dextrin as a source of carbohydrate (Panserat et al., 2001b). Hepatic glucokinase expression can be induced by dietary carbohydrates in carnivorous rainbow trout and gilthead seabream (Panserat et al., 2000b). The expression of GK in the liver of gilt head seabream is strictly regulated at the transcriptional level by hormonal and nutritional status. High-carbohydrate low-protein diets increase glucokinase expression. Starvation decreases GK mRNA in the liver, whereas GK expression increases post-prandially when feeding high carbohydrate diets (Caseras et al., 2000; Egea et al., 2007, 2008; Fernandez et al., 2007; Meton et al., 2004; Soengas et al., 2006). All of these studies lay a foundation for the regulation of carbohydrate metabolism in fish. On the other hand, there is still lack of knowledge about herbivorous fishes liver glucokinase.

Grass carp (*Ctenopharyngodon idella*) is an economically important aquaculture species in China, with a production of more than 4 million tons per year. Some diseases associated with carbohydrate metabolism, such as fatty liver syndrome, have been found to increase year after year in cultured grass carp. The aim of the present work was to clone the full-length cDNA of GK gene from liver of adult grass carp and analyze its tissue distribution. The results will be beneficial in understanding the relation between carbohydrates and lipid metabolism in herbivorous fish at the molecular level, and to control fatty liver syndrome of grass carp in aquaculture by transcriptional regulation.

MATERIALS AND METHODS

Experimental fish

Adult grass carps, with body weight ranging from 1500 to 2500 g, were collected from a pond in Lianyungang, Jiangsu province in May, 2008, and transported to the indoor tanks in the Huaihai Institute of Technology, Jiangsu province, China. The fish were maintained in 500 L tanks under natural conditions of photoperiod and fed with standard commercial diet for 2 weeks. The water temperature ranged from 23 to 27°C. The fishes were sampled 6 h after the last meal.

Total RNA extraction from liver

The fish were killed by a blow to the head and liver were rapidly excised and ground under liquid nitrogen to a fine power using a mortar. Total RNA was extracted from approximately 50 mg of liver using E.Z.N.A.™Total RNA Kit I (OMEGA, USA), according to instructions from the manufacturer. The total RNA was treated using an on-membrane DNase I digestion Kit (OMEGA) to eliminate the remaining genomic DNA. The integrity of isolated RNA was assessed by 1% formaldehyde denaturing agarose gel electrophoresis, and its quantity and purity were determined by absorbance measures at 260 and 280 nm with an UV-Visible spectrophotometer GeneQuant pro (GE). Samples were diluted to 0.5 μg μL^{-1} with DEPC-water, and then stored at -80°C for future use.

Synthesis of the first-strand cDNA and isolation of core cDNA fragments of GK

Reverse transcription was performed with total RNA as template and oligo$(dT)_{18}$ as primer using an AMV First Strand cDNA Synthesis Kit (BBI), according to the manufacturer's instructions. The total of 20.0 μL reaction mixture composed of 5.0 μL total RNA (0.5 μg μL^{-1}), 1.0 μL oligo$(dT)_{18}$ primer (0.5 μg μL^{-1}), 5.0 μL RNase-free ddH_2O, 4.0 μL 5×reaction buffer, 1 μL RNase inhibitor (20 U μL^{-1}), 2.0 μL dNTP Mix (10 mM each) and 2.0 μL AMV reverse transcriptase (10U μL^{-1}). The resulting first strand cDNA was then diluted to 1:10 with ddH_2O, and used as template for PCR. An 855-bp core cDNA fragment of GK was amplified using GK-F and GK-R as specific primers (Table 1). Approximate locations of primers are shown in Figure 1. A pair of primer was designed based on sequence information of zebrafish and common carp GK cDNA obtained from GenBank (BC122359, AF053332), using Primer Premier 5.0 software. PCR was set up in a 50.0 μL reaction mixture composed of 2.0 μL diluted first strand cDNA product, 5.0 μL 10×buffer (with 20 mM Mg^{2+}), 1.0 μL dNTP (10 mM each), 1.0 μL each primer (25 μM), 0.4 μL AmpliTag® DNA polymerase (5 U μL^{-1}, BBI), and 39.6 μL ddH_2O. Amplification was carried out in a BIO-RAD thermal cycler (iCycler). The amplification conditions were set as follows: initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturing for 40 s at 94°C, annealing for 40 s at 52°C, extension for 60 s at 72°C, and a final extension for 7 min at 72°C. Five PCR products were selected by 1% w/v agarose gel electrophoresis and delivered to Shanghai Sangon Biological Engineering and Technology and Service Co. Ltd. (Shanghai, China) for sequencing. The sequence was conducted in both forward and reverse directions using the amplification primers. The forward and reverse sequences were assembled using SeqMan II software in DNAStar Package version 5.01, and the core fragment of GK was

Table 1. Primers and expected products used for GK gene cDNA RT-PCR, RACE and real-time PCR.

Primers name	Sequence 5'→3'	Use	Expected product (bp)
GK-F	ATGCTGGTGAAAGTGGGT	Used with GK-R for RT-PCR of core fragment	855
GK-R	ACATGCCAGACGCACAAT	Used with GK-F	
Oligo(dT)$_{16}$AP	CTGATCTAGAGGTACCGGATCC(T)$_{16}$	Synthesis of the first-strand cDNA for 3' RACE	
GK$_3$-F$_1$	ATGGAGGAGATGCGTAAGGT	Used with AP for first PCR of 3' RACE	
AP	CTGATCTAGAGGTACCGGATCC	Used with GK$_3$-F$_1$ or GK$_5$-R$_2$	
GK$_3$-F$_2$	GTCTCCCAGATTGAGAGTGA	Used with Race$_3$-R for nested PCR of 3' RACE	978
Race$_3$-R	CTAGAGGTACCGGATCCTT	Used with GK$_3$-F$_2$	
GK$_5$-R	TGTTTGTCCAGGAAGTCAG	Synthesis of the first-strand cDNA for 5' RACE	
GK$_5$-R$_1$	CTTCCAGCCKCGCTCTTC	Used with Oligo(dT)$_{16}$AP for first PCR of 5'RACE	
GK$_5$-R$_2$	ACCCACTTTCACCAGCAT	Used with AP for nested PCR of 5' RACE	383
qGK-F	GCTGAAATGTTGTTTGACTA	Used with qGK-R for real-time PCR of GK	140
qGK-R	AGCAGAATGCCCTTATCC	Used with qGK-F	
qActin-F	TTCGCTGGAGATGATGCT	Used with qActin-R for PCR of housekeeping gene	152
qActin-R	ATGGGGTACTTCAGGGTC	Used with qActin-F	

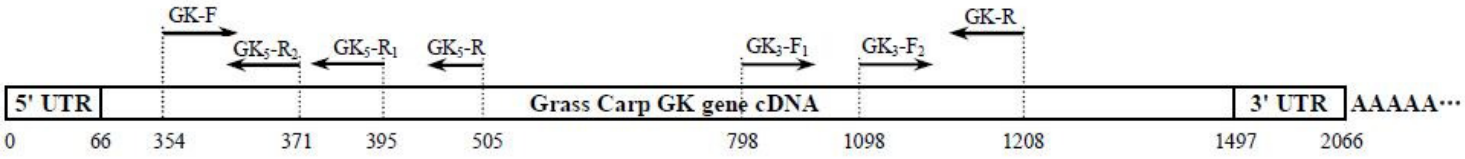

Figure 1. Primers and their approximate locations in GK gene cDNA used for RT-PCR and RACE.

obtained. According to the sequence information of this fragment, gene-specific primers were designed for 3' RACE and 5' RACE.

Rapid amplification of the 3' end (3' RACE)

Rapid amplification of the 3' end was performed using 3'-Full RACE Core Set (TaKaRa), according to the manufacturer's instructions. The primers used for 3' RACE are shown in Table 1. First, reverse transcription of 2 µg of total RNA was performed with oligo(dT)$_{16}$AP as primer, which contained an anchor sequence. The resulting first strand cDNA was diluted to 1:10 with ddH_2O and used as a template for PCR. Amplification was done with a gene-specific forward primer GK$_3$-F$_1$ and a reverse primer AP that corresponds to the anchor sequence. After the first PCR, the product was diluted to 1:10 with ddH_2O and used as template for the nested PCR, which was done with a specific forward primer GK$_3$-F$_2$ and a reverse primer Race$_3$-R. The first and nested PCRs were carried out under the same condition as above. The nested PCR product was separated by agarose gel electrophoresis and then the band of expected size was excised, and purified using the EZ-10 Spin Column DNA Gel Extraction Kit (BBI). The purified PCR product was subcloned into a PUCm-T vector (BBI) and five independent clones were sequenced in both forward and reverse directions using the universal M$_{13}$ primers.

Rapid amplification of the 5' end (5' RACE)

Rapid amplification of the 5' end was made following a protocol described by Dieffenbach and Dveksler (1995), and the primers used for 5' RACE are shown in Table 1. Briefly, reverse transcription of 2 µg total RNA was performed with a specific reverse primer GK$_5$-R to obtain the first strand cDNA. After RNase H (MBI) treatment, an oligo (dA) tail at the 5' end was added using terminal deoxynucleotidyl transferase (MBI). The resulting product was diluted to 1:10 with TE buffer, and used as a template for the first PCR. Amplification was done with a universal forward primer oligo (dT)$_{16}$AP that contained an anchor sequence, and the specific reverse primer GK$_5$-R$_1$. The first PCR product was diluted to 1:10 with ddH_2O and used as a template for the nested PCR. This was done with an anchor forward primer AP and a specific reverse primer GK$_5$-R$_2$. The nested PCR product was eluted from 1.5% agarose gel. The purified PCR product was subcloned into a PUCm-T vector (BBI) and five independent clones were sequenced in both forward and reverse directions using the universal M$_{13}$ primers.

Sequence analysis

The core fragment, 3' and 5' end sequences, were assembled using

SeqMan II software in DNAStar Package to obtain full-length cDNA of GK. The sequence was edited and analyzed using the program EditSeq of DNAStar package to search for open reading frame (ORF) and then translated into amino acid sequence using standard genetic codes. The amino acid sequence of GK was tested for the presence of signal peptide with SignalP v3.0 at http://www.cbs.dtu.dk/services/signalP/ (Bendtsen et al., 2004). Putative transmembrane regions were predicted with HMMTOP v2.0 at http://www.enzim.hu/hmmtop/ (Tusnady and Simon, 2001). The secondary structure of GK protein was predicted at http://www.predictprotein.org/ and http://www.EMBL-Heidelberg.de/. The protein sequence alignment was made by program MegAlign of DNAStar package using the Clustal W method. The phylogenetic tree was produced by the Neighbor-Joining (NJ) method (Kimura 2-parameter model, 10 000 replicates, bootstrap phylogeny test) based on GK amino acid sequences using the MEGA software Version 3.1 (Kumar et al., 2004).

Tissue expression of GK mRNA by real time PCR

Tissue expression of GK mRNA was determined by SYBR real-time fluorescence quantitative RT-PCR method using β-actin as an internal control for cDNA normalization. A total of 3 fishes were sampled 6 h after the last meal, and total RNAs were extracted from the brain, mesenteric adipose tissue, spleen, white muscle and liver of grass carp, and were treated using an on-membrane DNase I digestion Kit (OMEGA) to avoid samples DNA contamination. The integrity of isolated RNAs were assessed by 1% denaturing agarose gel electrophoresis, whose quantity and purity were determined by absorbance measures at 260 and 280 nm, and diluted to 0.5 μg μL^{-1} with DEPC-water. Reverse transcriptions were performed with equal quantities of each total RNA (2μg) as templates using Quantitect® Reverse Transcription Kit for real-time PCR (Qiagen, USA). The resulting first strand cDNA from each tissue was diluted to 1:10 with ddH_2O and used as template for SYBR real-time PCRs.

Real-time PCR (qPCR) was performed using the SYBR® Premix Ex Taq™ II Kit (TaKaRa) in iQ™5 real-time PCR detector (Bio-Rad). Amplification was done with primers qGK-F and qGK-R for GK cDNA, and qActin-F and qActin-R (Table 1), which were designed based on grass carp β-actin sequence information (GenBank accession no. M25013) and spanned a 106 bp intron in genomic DNA, for β-actin cDNA as an internal control for cDNA normalization in different tubes. Real-time PCRs were set up in a 25 μL volume reaction mixture composed of 12.5 μL SYBR® Premix Ex Taq™ II (2×), 0.5 μL each primer (25 μM), 2 μL diluted first strand cDNA product, and 10 μL ddH_2O. The amplification conditions setting were as follows: 30 s at 95°C, 40 cycles of 5 s at 95°C and 30 s at 60°C. To ensure data validity, the negative PCR control reactions were performed with ddH_2O as templates and 3 repeated well were set for each sample. The baseline was automatically set to maintain consistency. An average C_t value was determined for each sample (N = 3).

Statistical analysis

The GK expression was determined by comparative Delta-delta C_t method normalized with β-actin level. The formula $X_{01}/X_{02}=2^{-\Delta\Delta Ct}$ was used to calculate the relative expression levels of GK mRNA in each sample by comparing the tissues with liver expression level. The results from three independent experiments are presented as means ± standard deviation (SD). Differences between tissues were calculated using a function TTEST in Excel 2003 software, and considered statistically significant at a $P<0.05$ level. To confirm amplification specificity, the PCR products from each sample were examined by melting curve analysis and subsequent agarose gel electrophoresis.

RESULTS

Molecular characterization of GK

The full-length cDNA coding GK was completed from liver of adult grass carp by assembling the core fragment, 3' and 5' end sequences, and submitted to GenBank (accession number GU065314). The complete nucleotide sequence covered 2066 bp plus additional 3'-poly(A) residues with an open reading frame (ORF) of 1431 bp encoding 476 amino acid residues (Figure 2). The 5'-untranslated region (UTR) covered 65 bases. An ATG initiation codon was found 66 bases downstream of the 5'-start, and a TGA stop codon was present at 589 bases upstream of the 3'-end. The 3'- untranslated region (UTR) covered 586 bases containing one AATAAA motifs, which represent a putative polyadenylation signal.

The GK protein has a calculated molecular weight of 53.7 kDa and isoelectric point (pI) of 5.11. Some conserved functional sites were found, including one conserved hexokinase signature sequence Leu^{156}-Phe^{181}; two N-linked glycosylation sites Asn^{176} and Asn^{214}; one cell attachment sequence Arg^{202}-Asp^{204} and one glycosaminoglycan attachment site Ser^{455}-Gly^{458}. The amino acid sequence had a high similarity, and showed similar structural features to GK of other species. Complete amino acid sequence alignment of GK showed the percent identities compared with topmouth culter (*E. ilishaeformis*), common carp (*C. carpio*), human (*Homo sapiens*) and rat (*Rattus norvegicus*) were 98.1, 96.8, 80.3 and 79.8%, respectively.

Phylogenetic analysis based on GK amino acid sequences

Phylogenetic analysis among eight fish species, eleven endothermic species and one amphibian species based on glucokinase amino acid sequences is shown in Figure 3. Tree topology showed that all the fish species were in the same group forming a clade, which was distinguished from endotherms and amphibian. In the clade of fishes, grass carp (*C. idella*) was first grouped with common carp (*C. carpio*), crucian carp (*Carassius auratus*), zebrafish (*D. rerio*) and topmouth culter (*E. ilishaeformis*), which belong to the same family Cyprinidae with a high bootstrap value (100%). Gilthead seabream (*S. aurata*) and spotted green pufferfish (*Tetraodon nigroviridis*), which belong to the same superorder Percomorpha, formed another clade with a bootstrap value of 81%. In endotherms, human (*H. sapiens*), chimpanzee (*Pan troglodytes*), and rhesus monkey (*Macaca mulatta*), which belong to primates, formed a clade with a bootstrap value of 99%. Then all species of endotherms were in the

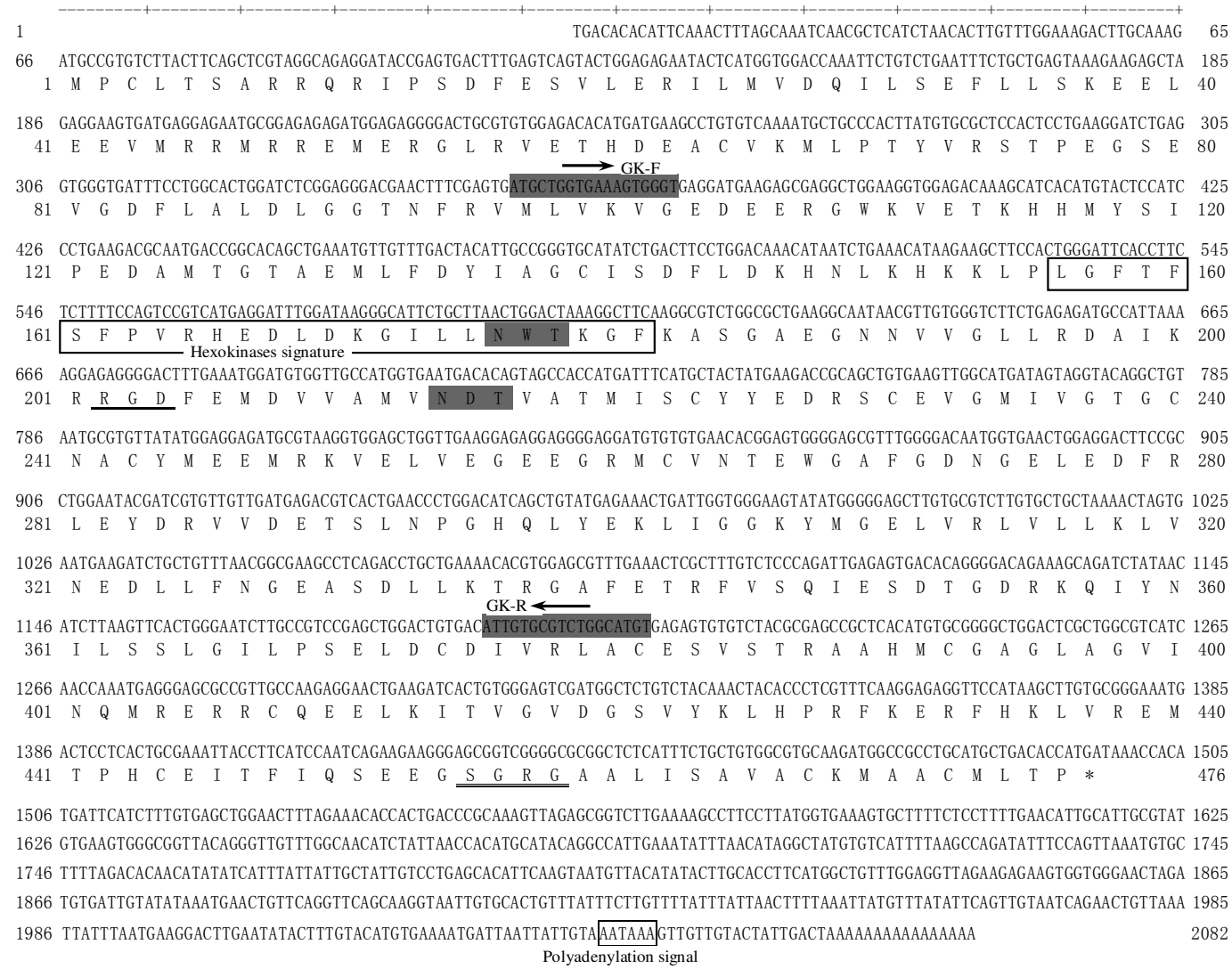

Figure 2. The nucleotide sequence of GK gene full-length cDNA and the deduced amino acid sequences of grass carp. An asterisk represents a termination codon. The Boxed base AATAAA is a polyadenylation signal. The grey bases indicate the primers used for RT-PCR; boxed amino acid sequence indicates the hexokinases signature; the grey amino acids indicate the N-linked glycosylation sites. The underlined amino acids RGD indicate the cell attachment sequence. The glycosaminoglycan attachment site SGRG is double underlined. The nucleotide sequence was submitted to GenBank with accession No. GU065314.

same group forming a clade with a bootstrap value of 99% distinguished from African clawed frog (*Xenopus laevis*), which belong to amphibian. The phylogenetic relationship based on GK amino acid sequences agreed with traditional classification.

Tissue distribution of GK mRNA

Tissue distribution of GK mRNA in the brain, mesenteric adipose tissue, spleen, white muscle and liver of adult grass carp was determined by RT-qPCR method using β-actin as an internal control for cDNA normalization. The results show that the expressions of GK mRNA were detected in liver, spleen, mesenteric adipose tissue and brain, but not detected in white muscle (Figure 4). Meanwhile the negative control exhibited no products (data not shown). The expression level of GK mRNA in liver was higher than that in the spleen, brain and mesenteric adipose tissue significantly ($p<0.05$). Relative expression profile of GK mRNA in liver normalized with β-actin level was 31, 454 and 649-fold compared with the levels in mesenteric adipose tissue, spleen and brain, respectively (Figure 4).

DISCUSSION

In this study, we reported the isolation and characterization of a full-length cDNA coding for GK from the liver

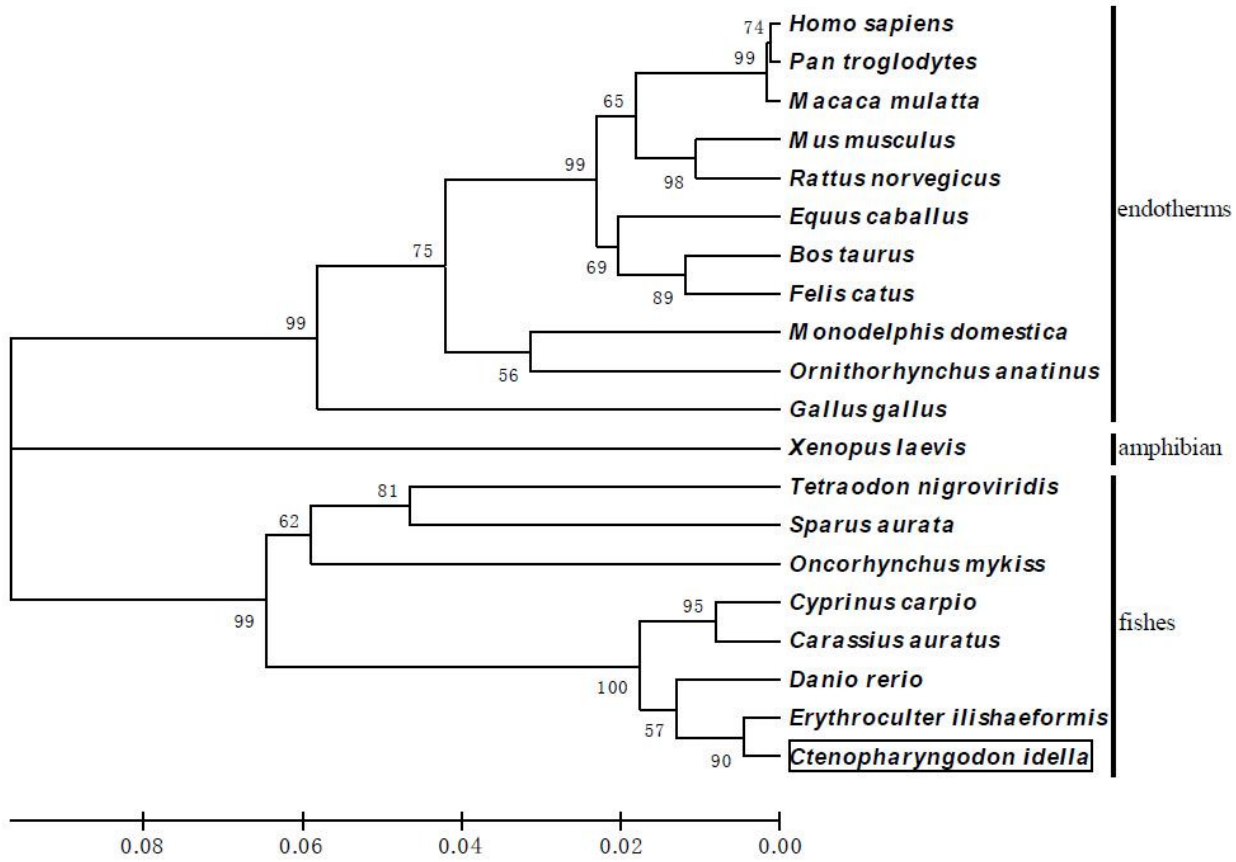

Figure 3. Phylogenetic tree based on glucokinase amino acid sequences made with MEGA 3.1 software using Neighbor-Joining (NJ) method. The distance matrix was calculated using the Amino: p-distance model, numbers represents bootstrap percentages. GenBank accession numbers: *Homo sapiens*, P35557; *Pan troglodytes*, XP_001143590; *Macaca mulatta*, XP_001092919; *Mus musculus*, AAC42074; *Rattus norvegicus*, AAA41229; *Equus caballus*, XP_001495888; *Bos taurus*, NP_001095772; *Felis catus*, NP_001077423; *Monodelphis domestica*, XP_001379711; *Ornithorhynchus anatinus*, XP_001520120; *Gallus gallus*, AAM83106; *Xenopus laevis*, CAA63761; *Tetraodon nigroviridis*, CAG08582; *S. aurata*, AAC33585; *O. mykiss*, NP_001117721; *C. carpio*, ACD37722; *C. auratus*, ADD52461; *E. ilishaeformis*, ABA41457; *D. rerio*, NP_001038850; and *C. idella* ADD52460, which was boxed to show present study.

of adult grass carp (*C. idella*), and detected the tissue distribution of GK mRNA in the brain, mesenteric adipose tissue, spleen, white muscle and liver. Amino acid sequence derived from this cDNA has a high similarity, and shows similar structural features to GK of other vertebrates. The amino acid sequence of grass carp GK was compared with the corresponding sequences form human (*H. sapiens*), rat (*R. norvegicus*), African clawed frog (*X. laevis*), common carp (*C. carpio*), topmouth culter (*E. ilishaeformis*), rainbow trout (*O. mykiss*), and gilthead seabream (*S. aurata*) (Figure 5).

Grass carp GK amino acid sequence compared with other vertebrate

Mammalian glucokinase is composed of a single polypeptide chain of approximately 50 kDa, which is identical or similar to the carboxyl-terminal portions of brain hexokinase I (Andreone et al., 1989), and not subject to allosteric regulation by glucose 6-phosphate. In contrast, hexokinases I to III consist of a single polypeptide chain of molecular mass ~100 kDa, which contain two hexokinase domains, and are allosterically inhibited by the reaction product, glucose 6-phosphate (Andreone et al., 1989). The GK amino acid sequence contains several functional sites, including the ATP-binding domain, the glucose binding sites and the regulatory protein binding sites.

In rat, the core sequence Asp78-Lys90 and an essential lysine102 (rat numbering) located 11 residues away from the core sequence is thought to be putative ATP-binding domain (Andreone et al., 1989). All of these residues are conserved in all species including grass carp (Asp88-Lys100 and lysine112, grass carp numbering). In human, the glucose binding site is formed by glucokinase residues Ser151-Pro153, Asn166-Lys169, Asn204-Thr206, Ile225-Asn231, Asn254-Gly258, Gln287, and Glu290. All

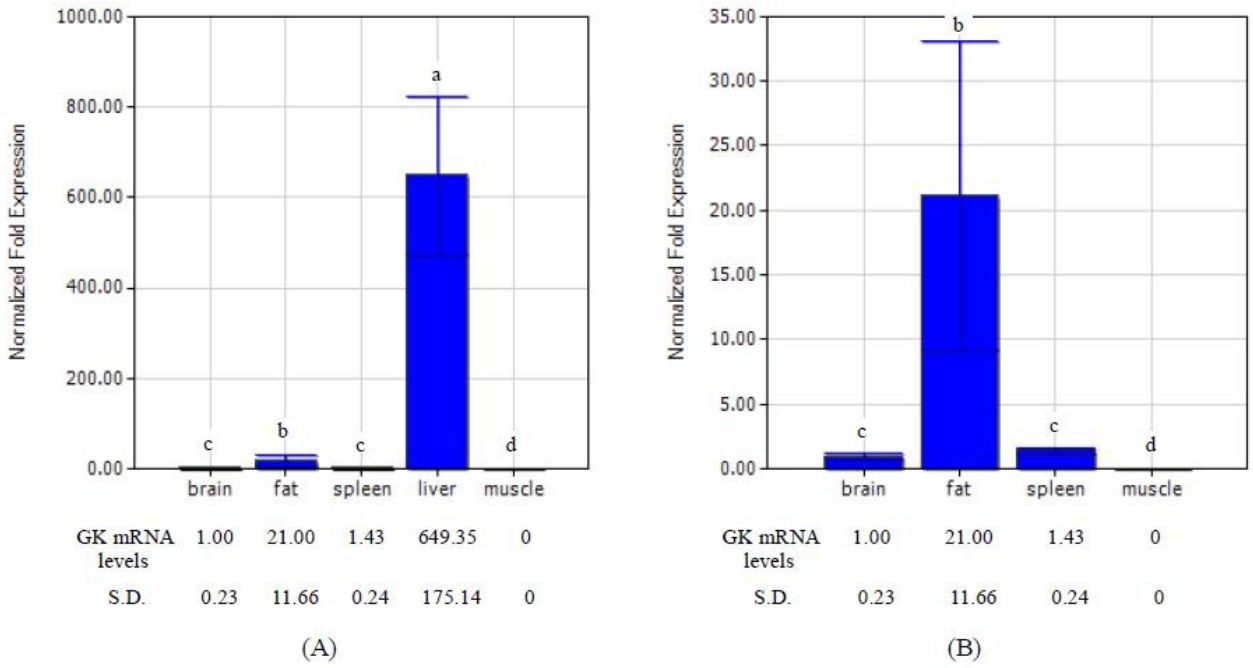

Figure 4. Expression analysis of GK mRNA in different tissues of grass carp performed by RT-qPCR, with β-actin as an internal control for cDNA normalization. (A) Relative expression profile of GK mRNA normalized with β-actin level. Data points represent the mean values ± SD of the three fish replicates; different letters indicate significant difference ($p<0.05$). Fat = mesenteric adipose tissue; muscle = white muscle. (B) Relative expression levels of GK mRNA except liver.

the oxygen atoms of glucose form hydrogen bond interactions with the side chains of glucokinase residues Thr168, Lys169, Asn204, Asp205, Asn231, Glu256, and Glu290 (human numbering) (Mahalingam et al., 1999; Veiga-da-Cunha et al., 1996). All of these residues appear to be conserved and in similar locations in all species including grass carp (Figure 5). Furthermore, to delineate the regions of liver glucokinase that are involved in the binding of its regulatory protein, five mutants of X. laevis glucokinase were constructed by replacing sets of 2-5 glucokinase-specific residues with their counterparts in the C-terminal half of rat hexokinase I. The result concluded that all of these residues; His141 to Leu144, which are located close to the tip of the small domain, as well as Glu51 and Glu52 (human numbering), which are present in the large domain of the enzyme close to the hinge region, or nearby residues participate in the binding of the regulatory protein (Veiga-da-Cunha et al., 1996), except Glu51, are conserved in all species including grass carp.

GK contains a conserved hexokinase signature sequence Leu^{146}-Phe^{161} (human numbering). Except for Ile^{158} that was replaced by Leu in Cyprinidae family fish, all other residues are conserved in all species. Beside, two potential N-linked glycosylation sites (Asn^{166} and Asn^{204}, human numbering), a cell attachment sequence (Arg^{192}-Asp^{194}, human numbering), and a glycosaminoglycan attachment site (Ser^{445}-Gly^{448}, human numbering) are also much conserved in all vertebrates including grass carp.

Tissue distribution of GK mRMA

In the present study, tissue distribution of GK mRNA in brain, mesenteric adipose tissue, spleen, white muscle and liver of adult grass carp was determined by RT-qPCR method using β-actin as an internal control for cDNA normalization. The result shows that the expressions of GK mRNA were detected in all tissues, except for white muscle (Figure 4). The expression level of GK mRNA in liver was higher than in spleen, brain and mesenteric adipose tissue significantly ($p<0.05$). The organ distribution of the GK mRNA is similar in grass carp as in mammals. In humans, glucokinase has been discovered in specific cells in four types of tissue: liver, pancreas, small intestine, and brain, but it is mainly expressed in glucose sensitive tissues, such as liver and pancreatic islet beta cells under the control of insulin (Tanizawa, 1991). In common carp, GK was only induced by the first feeding with high levels of carbohydrate (Panserat et al., 2001b). In rainbow trout, the expression of GK was assessed in different tissues under different feeding conditions: GK mRNA was identified only in liver and the four brain regions; GK expression was altered by feeding conditions, especially in liver and hypothalamus where food deprivation decreased and re-feeding increased

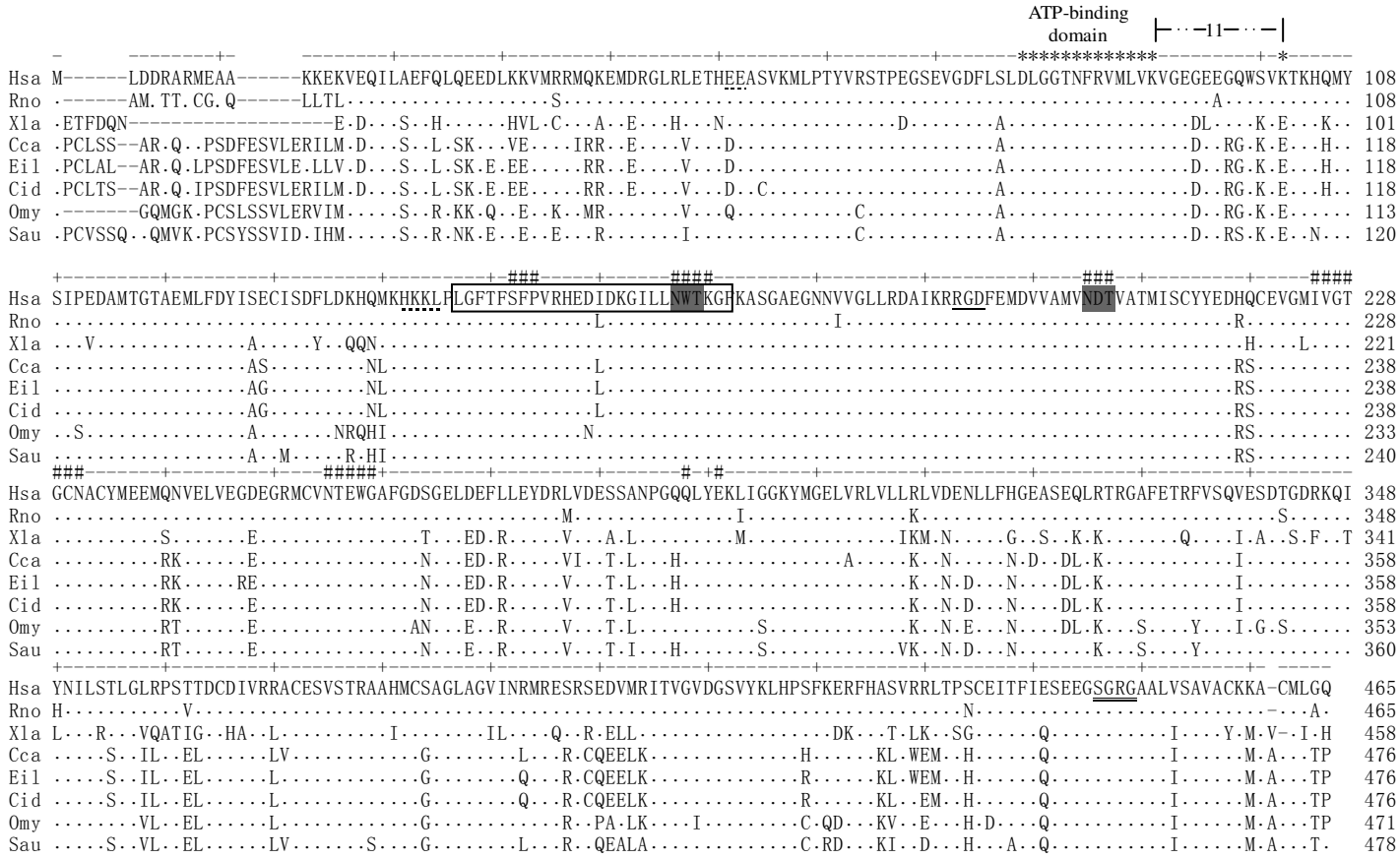

Figure 5. Multiple alignment of the GK deduced amino acid sequence from grass carp (Cid, ADD52460) with the corresponding sequences from human (Hsa, P35557), rat (Rno, NP_036697), African clawed frog (Xla, CAA63761), common carp (Cca, AAC33578), topmouth culter (Eil, ABA41457), rainbow trout (Omy, NP_001117721), and gilthead sea bream (Sau, AAC33585). Sites with an amino acid identical to that on the top line (human) indicated with a dot. Alignment gaps are indicated by dashes. Amino acid substitutions are indicated by the respective amino acid. The boxed amino acid sequence indicates the hexokinase signature. The grey amino acids indicate two potential conserved N-linked glycosylation sites. The ATP-binding amino acids are indicated by asterisks (*). The glucose-binding amino acids are indicated by pound (#). The regulatory protein binding sites are indicated by dotted lines. The conserved cell attachment sequence is underlined. A conserved glycosaminoglycan attachment site is double underlined.

expression. These studies strongly support that low dietary carbohydrate utilization in rainbow trout is not due to the absence of inducible hepatic GK as previously suggested (Capilla et al., 2003; Panserat et al., 2001a, 2000b). Carnivorous gilthead seabream can tolerate partial replacement of dietary protein by carbohydrates through the regulation of GK expression (Fernandez et al., 2007; Meton et al., 2004). Fish fed with high carbohydrate/low protein diets markedly increased hepatic GK expression, while hardly detectable levels were observed in the fish fed low carbohydrate/high protein diets (Caseras et al., 2000; Egea et al., 2007). An induction of GK gene expression by dietary carbohydrate was observed in all fish species.

In summary, we have cloned and characterized the cDNA encoding GK from the liver of the herbivorous grass carp. The main domains (ATP-binding domain, glucose binding site and regulatory protein binding sites) are basically conserved between the teleost fish and other vertebrates. Our results demonstrated that the liver is one of the main GK producing tissues in fish. Relative expression profile of grass carp GK mRNA in liver normalized with β-actin level was 31, 454 and 649-fold compared with the levels in mesenteric adipose tissue, spleen and brain, respectively. These findings, including a detailed phylogenetic analysis, revealed that GK gene of grass carp was highly homologous to mammalian with similar tissue specific expression (mainly in the liver).

ACKNOWLEDGEMENTS

This work was supported by Jiangsu Provincial Natural Science Foundation of China (Grant no. BK2008191), the major project of Natural Science Foundation of Jiangsu Educational Commission (Grant no. 10KJA240002), the Open Foundation of Zhejiang Key Laboratory for Exploitation and Conservation of Nearshore Waters Biological

Resources, Zhejiang Mariculture Research Institute (Grant no. J2010010) and the Open Foundation of Jiangsu Key Laboratory of Marine Biotechnology, Huaihai Institute of Technology (Grant no. 2009HS15).

REFERENCES

Andreone TL, Printz RL, Pilkis SJ, Magnuson MA, Granner DK (1989). The amino acid sequence of rat liver glucokinase deduced from cloned cDNA. J. Biol. Chem. 264: 363-369.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004). Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340: 783-795.

Blin C, Panserat S, Kaushik S, Krishnamoorthy R (2000). Partial molecular cloning and tissue distribution of hexokinase I cDNA in common carp. J. Fish Biol. 56: 1558-1561.

Capilla E, Medale F, Navarro I, Panserat S, Vachot C, Kaushik S, Gutierrez J (2003). Muscle insulin binding and plasma levels in relation to liver glucokinase activity, glucose metabolism and dietary carbohydrates in rainbow trout. Regul. Peptides, 110: 123-132.

Caseras A, Meton I, Fernandez F, Baanante IV (2000). Glucokinase gene expression is nutritionally regulated in liver of gilthead sea bream (*Sparus aurata*). Biochim. Biophys. Acta, 1493: 135-141.

Cheng HL, Xia DQ, Wu TT (2006). Fatty liver and regulation of lipids metabolism in fish. Chin. J. Anim. Nutr. 18: 294-298.

Dieffenbach CW, Dveksler GS (1995). PCR primer: a laboratory manual. Plainview, Cold Spring Harbor Laboratory Press, N.Y., USA.

Egea M, Meton I, Baanante IV (2007). Sp1 and Sp3 regulate glucokinase gene transcription in the liver of gilthead sea bream (*Sparus aurata*). J. Mol. Endocrinol. 38: 481-492.

Egea M, Meton I, Cordoba M, Fernandez F, Baanante IV (2008). Role of Sp1 and SREBP-1a in the insulin- mediated regulation of glucokinase transcription in the liver of gilthead sea bream (*Sparus aurata*). Gen. Comp. Endocrinol. 155: 359-367.

Fernandez F, Miquel AG, Cordoba M, Varas M, Meton I, Caseras A, Baanante IV (2007). Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (*Sparus aurata*, L.) fingerlings. J. Exp. Mar. Biol. Ecol. 343: 1.

Ge XP, Yu JH, WU TT (2006). Molecular cloning and sequence analysis of the cDNA of glucokinase in *Erythroculter ilishaeformis*. J. Fish. China, 30: 410-415.

Gonzalez-Alvarez R, Ortega-Cuellar D, Hernandez-Mendoza A, Moreno-Arriola E, Villasenor-Mendoza K, Galvez-Mariscal A (2009). The hexokinase gene family in the zebrafish: structure, expression, functional and phylogenetic analysis. Comp. Biochem. Physiol. B, 152: 189-195.

Hemre GI, Mommsen TP, Krogdahl U (2002). Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. Aquacult. Nutr. 8: 175-194.

Irwin DM, Tan H (2008). Molecular evolution of the vertebrate hexokinase gene family: Identification of a conserved fifth vertebrate hexokinase gene. Comp. Biochem. Physiol. D, 3: 96-107.

Kawai S, Mukai T, Mori S, Mikami B, Murata K (2005). Hypothesis: structures, evolution, and ancestor of glucokinases in the hexokinase family. J. Biosci. Bioeng. 99: 320-330.

Kumar S, Tamura K, Nei M (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief. Bioinform. 5: 150-163.

Mahalingam B, Cuesta-Munoz A, Davis EA, Matschinsky FM, Harrison RW, Weber IT (1999). Structural model of human glucokinase in complex with glucose and ATP: implications for the mutants that cause hypo- and hyperglycemia. Diabetes, 48: 1698-1705.

Meton I, Caseras A, Fernandez F, Baanante IV (2004). Molecular cloning of hepatic glucose-6-phosphatase catalytic subunit from gilthead sea bream (*Sparus aurata*): response of its mRNA levels and glucokinase expression to refeeding and diet composition. Comp. Biochem. Physiol. B, 138: 145-153.

Moon TW (2001). Glucose intolerance in teleost fish: fact or fiction? Comp. Biochem. Physiol. B, 129: 2-3.

Panserat S, Blin C, Medale F, Plagnes-Juan E, Breque J, Krishnamoorthy J, Kaushik S (2000a). Molecular cloning, tissue distribution and sequence analysis of complete glucokinase cDNAs from gilthead seabream (*Sparus aurata*), rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). Biochim. Biophys. Acta, 1474: 61-69.

Panserat S, Capilla E, Gutierrez J, Frappart PO, Vachot C, Plagnes-Juan E, Aguirre P, Breque J, Kaushik S (2001a). Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (*Oncorhynchus mykiss*) by a single meal with glucose. Comp. Biochem. Physiol. B, 128: 275-283.

Panserat S, Fontagne S, Bergot P, Kaushik S (2001b). Ontogenesis of hexokinase I and hexokinase IV (glucokinase) gene expressions in common carp (*Cyprinus carpio*) related to diet. Br. J. Nutr. 85: 649-651.

Panserat S, Medale F, Blin C, Breque J, Vachot C, Plagnes-Juan E, Gomes E, Krishnamoorthy R, Kaushik S (2000b). Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. Am. J. Physiol. Integr. Comp. Physiol. 278: R1164-1170.

Soengas JL, Polakof S, Chen X, Sangiao-Alvarellos S, Moon TW (2006). Glucokinase and hexokinase expression and activities in rainbow trout tissues: changes with food deprivation and refeeding. Am. J. Physiol. Regul. Integr. Comp. Physiol. 291: 810-821.

Stoffel M, Froguel P, Takeda J, Zouali H, Vionnet N, Nishi S, Weber IT, Harrison RW, Pilkis SJ, Lesage S (1992). Human glucokinase gene: isolation, characterization, and identification of two missense mutations linked to early-onset non-insulin-dependent (type 2) diabetes mellitus. Proc. Natl. Acad. Sci. USA. 89: 7698-7702.

Stone DAJ (2003). Dietary Carbohydrate Utilization by Fish. Rev. Fish. Sci. 11: 337-370.

Tanizawa YK, Welling CM, Permutt ML (1991). Human liver glucokinase gene: cloning and sequence determination of two alternatively spliced cDNAs. Proc. Natl. Acad. Sci. USA. 88: 7294-7397.

Tusnady GE, Simon I (2001). The HMMTOP transmembrane topology prediction server. Bioinformatics (Oxford, England), 17: 849-850.

Veiga-da-Cunha M, Courtois S, Michel A, Gosselain E, Van Schaftingen E (1996). Amino acid conservation in animal glucokinases. Identification of residues implicated in the interaction with the regulatory protein. J. Biol. Chem. 271: 6292-6297.