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NUTRITIONAL REGULATION AND TISSUE SPECIFICITY OF GENE EXPRESSION FOR PROTEINS INVOLVED IN HEPATIC GLUCOSE METABOLISM IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary

Rainbow trout (*Oncorhynchus mykiss*) are known to use dietary carbohydrates poorly. One of the hypotheses to explain the poor utilisation of dietary glucose by these fish is a dysfunction in nutritional regulation of hepatic glucose metabolism. In this study, we obtained partial clones of rainbow trout cDNAs coding for a glucose transporter (Glut2), and for the enzymes 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2K/F-2,6BPase), fructose-1,6-bisphosphatase (FBPase) and pyruvate kinase (PK). Their deduced amino acid sequences were highly similar to those of mammals (up to 80 % similarity). In a study of nutritional regulation, the Glut2 gene was highly expressed in the liver irrespective of the nutritional status of the trout, in agreement with the role of this transporter in the input (during refeeding) and output (during fasting) of glucose from the liver. Moreover, whereas PK and FBPase gene expression was high irrespective of the nutritional status, levels of hepatic 6PF-2K/F-2,6BPase mRNA were higher in fish fed with carbohydrates than in fish deprived of food. The high levels of hepatic PK, Glut2 and 6PF-2K/F-2,6BPase gene expression observed in this study suggest a high potential for tissue carbohydrate utilisation in rainbow trout. The persistence of a high level of FBPase gene expression suggests an absence of regulation of the gluconeogenic pathway by dietary carbohydrates.

Key words: liver metabolism, glucose metabolism, glucose transporter, fructose bisphosphatase, pyruvate kinase, bifunctional enzyme, trout, *Oncorhynchus mykiss*.

Introduction

Improvement of dietary carbohydrate utilisation by fish has practical implications in aquaculture. The rainbow trout (*Oncorhynchus mykiss*) is an interesting model species recognised for its low levels of carbohydrate utilisation (Palmer and Ryman, 1972; Bergot, 1979; Cowey and Walton, 1989; Wilson, 1994; Moon and Foster, 1995). Analysis of glucose utilisation such as glucose phosphorylation (Cowey and Walton, 1989; Moon and Foster, 1995), glucose transporters (Wright et al., 1998) and insulin receptors (Mommsen and Plisetskaya, 1991; Parrizas et al., 1994), in target tissues is important in order to obtain an overall view of low dietary glucose utilisation in this carnivorous fish species. One hypothesis to explain the poor utilisation of dietary glucose by fish is a dysfunction of nutritional regulation between two major metabolic pathways in the liver: (i) a low capacity to store excess glucose in the postprandial stage (glycogen synthesis or lipogenesis) (Cowey and Walton, 1989; Wilson, 1994) and/or (ii) persistent highly active hepatic glucose production when carbohydrates are provided in the diet (Panserat et al., 2000b; Panserat et al., 2001). Indeed, we have recently shown, in rainbow trout, the induction of expression of glucokinase, the first enzyme in the glycolytic pathway, and the absence of inhibition of glucose-6-phosphatase, the last enzyme in hepatic glucose production (Panserat et al., 2000a; Panserat et al., 2000b).

The aim of the present study was to analyse two key hepatic glycolytic enzymes: (i) 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, otherwise termed the bifunctional enzyme (6PF-2K/F-2,6BPase, EC2.7.1.105/EC3.1.3.46) (Pilkis and Granner, 1992; Pilkis et al., 1995), which produces fructose 2,6-bisphosphate, a key allosteric regulator of glycolysis/gluconeogenesis in the liver (Pilkis et al., 1995), and (ii) L-type pyruvate kinase (PK, EC2.7.1.40), which catalyses the conversion of phosphoenolpyruvate to pyruvate, the last step in glycolysis (Yamada and Noguchi, 1999). We also studied the key gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase, EC3.1.3.11); this enzyme catalyses the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate (Marcus et al., 1987). Finally, we looked for a type-2 glucose transporter (Glut2) in the liver of rainbow trout. This transporter maintains the same glucose concentration between the extra and intracellular (hepatocyte) media because of its high K_m for glucose (Thorens, 1992).

In mammals, the expression of PK, 6PF-2K/F-2,6BPase and

Glut2 proteins is positively controlled by feeding, specifically by dietary carbohydrates (Thorens, 1992; Pilkis et al., 1995; Yamada and Noguchi, 1999); this induction potentiates dietary glucose utilisation in the liver. However, kinase activity of 6PF-2K/F-2,6BPase, which generates the fructose-2,6-bisphosphate, is also positively dependent on protein phosphatase 2A activity. The mechanism of induction of these proteins is mediated largely by induction of their gene expression even though, for the Glut2, the regulation of gene expression in the liver is rather complex (Thorens, 1992; Rencurel et al., 1996; Pilkis et al., 1995; Yamada and Noguchi, 1999). In contrast, feeding and dietary carbohydrates inhibit FBPase gene expression (El-Magrabi et al., 1982; El-Magrabi et al., 1988).

Nutritional regulation of the activities of PK, FBPase and 6PF-2K/F-2,6BPase has been studied previously in fish (Cowey et al., 1977; Cowey et al., 1981; Hilton and Atkinson, 1982; Fideu et al., 1983; Petersen et al., 1987; Lupianez et al., 1989; Garcia de Fructos and Baanante, 1994; Shikata et al., 1994; Shimeno et al., 1995; Meton et al., 1999a; Meton et al., 1999b; Tranulis et al., 1996; Borrebaek and Christophersen, 2000). It seems that there is an increase in the activities of PK and 6PF-2K/F-2,6BPase in response to feeding, although no clear data on the mechanism (transcriptional, post-transcriptional, post-translational) involved in this nutritional regulation are available except for the 6PF-2K/F-2,6BPase in gilthead seabream (*Sparus aurata*) (Garcia de Fructos and Baanante, 1994; Meton et al., 1999a; Meton et al., 1999b). No specific inhibition of hepatic FBPase activity in response to feeding has been observed in perch (*Perca fluviatilis*) or salmon (*Salmo salar*) (Tranulis et al., 1996; Borrebaek and Christophersen, 2000) whereas in European seabass (*Dicentrarchus labrax*) and gilthead seabream inhibition of FBPase activity in response to feeding has been demonstrated (Garcia-Rejon et al., 1996; Meton et al., 1999b). Finally, the existence of a mammalian-type glucose transporter (Glut) in fish is still a matter of debate; for example, studies using mammalian anti-Glut antibodies suggest, in tilapia (*Oreochromis niloticus*), a very limited distribution of Glut1 and the complete absence of Glut4 (Wright et al., 1998), whereas recent studies demonstrate the existence of Glut1 and Glut4 cDNAs in rainbow trout and brown trout (*Salmo trutta*), respectively (Teerijoki et al., 2000; Planas et al., 2000).

In rainbow trout, no molecular data for 6PF-2K/F-2,6BPase, PK, FBPase and Glut2 proteins are yet available. The aim of this study was the partial cloning of PK, GLUT2, FBPase and 6PF-2K/F-2,6BPase cDNAs expressed in the liver, followed by the analysis of their expression in different tissues and in fish under different nutritional conditions.

Materials and methods

Fish and diets

Juvenile immature rainbow trout (*Oncorhynchus mykiss*) were grown for 10 weeks at 18 °C at the INRA experimental fish farm (Donzacq, France) and fed with (20 %) or without (<0.5 %) carbohydrates, as described previously (Panserat et al., 2000a; Panserat et al., 2000b). On the sampling day, the fish were fed once and killed 6 h and 24 h after feeding. Food-deprived fish were deprived for 4 days. Tissues (liver, muscle, heart, kidney, brain) were quickly removed, frozen in liquid nitrogen and stored at −80 °C.

Table 1. *Origin of the cDNA sequences used in the comparative analysis*

mRNA sequence	GenBank accession number
PK sequences	
Human *Homo sapiens*	M15465
Rat *Rattus norvegicus*	M11709
Mouse *Mus musculus*	D63764
Glut2 sequences	
Human *Homo sapiens*	J03810
Chicken *Gallus gallus*	Z22932
6PF-2K/F-2,6BPase sequences	
Rat *Rattus norvegicus*	Y00702
Bull frog *Rana catesbeiana*	D25223
Gilthead seabream *Sparus aurata*	U84724
FBPase sequences	
Human *Homo sapiens*	D26055
Rat *Rattus norvegicus*	M86240
Mouse *Mus musculus*	AJ132693
Caenorhabditis elegans	AJ271466

6PF-2K/F-2,6BPase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PK, pyruvate kinase; FBPase, fructose-1,6-bisphosphatase; Glut2, glucose transporter type 2.

RNA isolation and reverse transcription

Total RNA was extracted from rainbow trout tissues as described by Chomczinski and Sacchi (Chomczinski and Sacchi, 1987). cDNA was obtained by annealing 2 µg of total RNA with 1 µg of random primers and incubating with AMV reverse transcriptase (Boehringer, Roche Molecular Biochemicals, Germany) for 1 h at 42 °C.

Cloning of partial 6PF-2K/F-2,6BPase, PK, FBPase and Glut2 cDNAs

6PF-2K/F-2,6BPase, PK, FBPase and Glut2 sequences from different species (see Table 1) were compared using the Clustal-W multiple alignment algorithm (Higgins and Sharp, 1989). The sequences of the upstream and downstream (degenerate) primers are presented in Table 2. cDNA (1 µl) was amplified by polymerase chain reaction (PCR) using 100 pmol of the degenerate primers in a reaction mixture containing 2 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl, 20 mmol l^{-1} Tris-HCl, 0.25 mmol l^{-1} dNTP and 2.5 units of Taq polymerase (Boehringer, Roche Molecular Biochemicals, Germany). 35 cycles of denaturation for 1 min at 94 °C, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s were performed. PCR products were subjected to electrophoresis in 1 % agarose gels and fragments of the expected size range were purified (Micropure System, Amicon, USA). The purified DNA fragments were inserted into the pCR™II plasmid and

Table 2. *Primers used for enzyme cDNA cloning by RT–PCR (degenerate primers) and gene expression analysis*

Enzyme	Degenerate primers for cDNA cloning	Trout-specific primers for gene expression
6PF-2K/F-2,6BPase	Forward: 5′-AAGMGKACCATYCAGACMGC-3′ Reverse: 5′-CAGCYTGRTGRCAGATSACC-3′	Forward: 5′-CCCTACGAACAGTGGAAGGC-3′ Reverse: 5′-TCTCCTGTCTCTCTAGTTCC-3′
PK	Forward: 5′-SCAGYTGTTTGAGGAGCTAC-3′ Reverse: 5′-CCTGTCACCACAATCACCAG-3′	Forward: 5′-GTCCAATGACCCTACTGAGG-3′ Reverse: 5′-CCTGTCTTGAAGAAGCCCCT-3′
Glut2	Forward: 5′-GCRGCAGGACGTGGKCCTTG-3′ Reverse: 5′-CCWGTTTATGCAACCATTGG-3′	Forward: 5′-CCGTCTTTACCATGGTGTCG-3′ Reverse: 5′-CCACAATGAACCAGGGGATG-3′
FBPase	Forward: 5′-CCMYTKGATGGMTCWTCCAA-3′ Reverse: 5′-GCMACCATSGASCCSACATA-3′	Forward: 5′-GCCCAATGAGAGGGACGCAC-3′ Reverse: 5′-CCACCATAGGGGGCACTGCC-3′

Y, C/T; M, A/C; W, A/T; K, G/T; R, A /G; S, G/C.

6PF-2K/F-2,6BPase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PK, pyruvate kinase; FBPase, fructose-1,6-bisphosphatase; Glut2, glucose transporter type 2.

RT–PCR, reverse transcription-polymerase chain reaction. See Materials and Methods for details.

used for transformation of One Shot™competent cells (Invitrogen, Carlsbad, CA, USA). Inserts were detected by *Eco*RI digestion of the extracted plasmid DNA. Two clones with inserts were sequenced (Cybergène, Evry, France).

Sequence analysis

Nucleotide sequences (excluding the primer sequences) were compared with DNA sequences from the GenBank database using the basic local alignment search tool (BLAST) algorithm (Altschul et al., 1990). Sequence alignments and percentage of amino acid conservation were assessed with the Clustal-W multiple alignment algorithm using the cloned fish sequence and sequences from other species corresponding to the amplified regions from databases.

Analysis of gene expression

Northern analysis

Extracted total RNA (20 μg) samples were electrophoresed in 1 % agarose gels containing 5 % formaldehyde and transferred by capillary onto nylon membrane (Hybond-N^+, Amersham, England). After transfer, RNA blots were stained with Methylene Blue to locate 26S and 16S rRNAs and to determine the amount of loaded RNA. Membranes were hybridized with fish ^{32}P-labelled DNA probes labelled by random priming (Stratagene, USA) and recognizing partial rainbow trout 6PF-2K/F-2,6BPase, PK, FBPase and Glut2 cDNAs. After stringent washing (2× SSC, 0.1 % SDS for 20 min; 1× SSC, 0.1 % SDS for 20 min; 0.2× SSC, 0.1 % SDS for 15 min), the membranes were exposed to X-ray film and the resulting images were quantified using Visio-Mic II software (Genomic, France).

RT–PCR analysis

cDNAs were amplified by PCR using specific primers chosen from the partial sequences for rainbow trout 6PF-2K/F-2,6BPase, PK, FBPase and Glut2 cDNAs (Table 2). PCR reactions were carried out in a final volume of 25 μl containing 1.5 mmol l^{-1} $MgCl_2$ and 4 pmol of each primer, 2 μl cDNA and 1 unit of Taq polymerase (Boehringer, Roche Molecular Biochemicals, Germany). 35 cycles of 20 s for hybridization (at 57 °C), 20 s for elongation (at 72 °C) and 20 s for denaturation (at 94 °C) were performed. The PCR products were characterized by sequencing (Cybergène, Evry, France).

Statistical analyses

The results are expressed as means ± S.D. When there were significant differences in variances (one-way analysis of variance, ANOVA), statistical differences between series of data were determined using Tuckey's post-hoc test (Systat 9 software products, SPSS Inc.). Differences were considered significant at 5 %.

Results

The available PK, Glut2, FBPase and 6PF-2K/F-2,6BPase cDNA sequences were aligned and highly conserved regions from different species (Table 1) were identified. Four sets of primers were designed (Table 2) and this made it possible to amplify partially PK, Glut2, FBPase and 6PF-2K/F-2,6BPase mRNAs. RT-PCR were performed on hepatic total RNA extracted from fish fed with or without carbohydrate (for FBPase cloning only). PCR conditions were optimized, and a major amplification product of the expected size was obtained for PK, Glut2, FBPase and 6PF-2K/F-2,6BPase transcripts (data not shown). The fragments were purified, cloned and sequenced. The cDNA sequences of 366 base pairs (bp) (PK), 293 bp (Glut2), 395 bp (FBPase) and 268 bp (6PF-2K/F-2,6BPase) (Fig. 1A, Fig. 2A, Fig. 3A, Fig. 4A) were highly similar to those of 'mammalian' genes (Blast algorithm, $p=10^{-56}$ to 10^{-46}, $p=10^{-12}$ to 10^{-8}, $p=10^{-106}$ to 10^{-11}, $p=10^{-72}$ to 10^{-62} for PK, Glut2, FBPase and 6PF-2K/F-2,6BPase, respectively). The corresponding amino acid sequences were deduced from the four cDNA sequences and showed open reading frames of 121 (PK), 97 (Glut2), 131 (FBPase) and 89 (6PF-2K/F-2,6BPase) codons highly homologous to 'mammalian' proteins (Blast algorithm, $p=10^{-56}$ to 10^{-42}, $p=10^{-47}$ to 10^{-45}, $p=10^{-47}$ to 10^{-33}, $p=10^{-54}$ to 10^{-45} for PK,

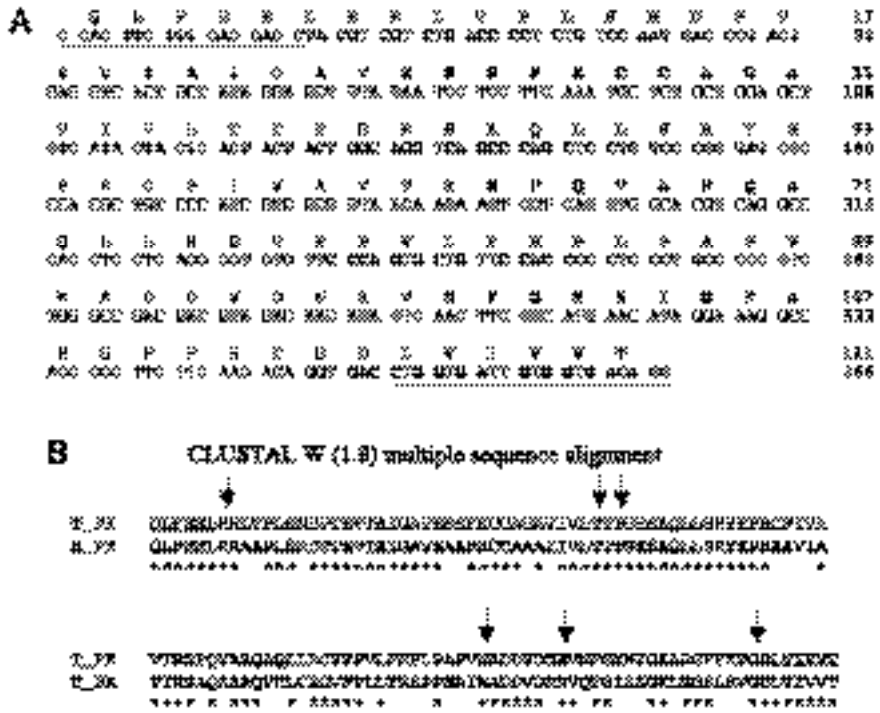

Fig. 1. Partial cloning of the pyruvate kinase (PK) gene in rainbow trout. (A) Nucleotide and deduced amino acid sequences of the rainbow trout PK clone. Underlined letters correspond to the primer sequences. (B) Alignments of the partial amino acid deduced PK cDNA of the rainbow trout (T_PK) (GenBank accession number: AF246146) with human PK (H_PK) (GenBank accession number: P30613). Underlined letters correspond to the primer sequences. Asterisks mark amino acid residues homologous between PKs. Residues involved in the interaction with the allosteric activator fructose-1,6-bisphosphate are marked by arrows (Jurica et al., 1998).

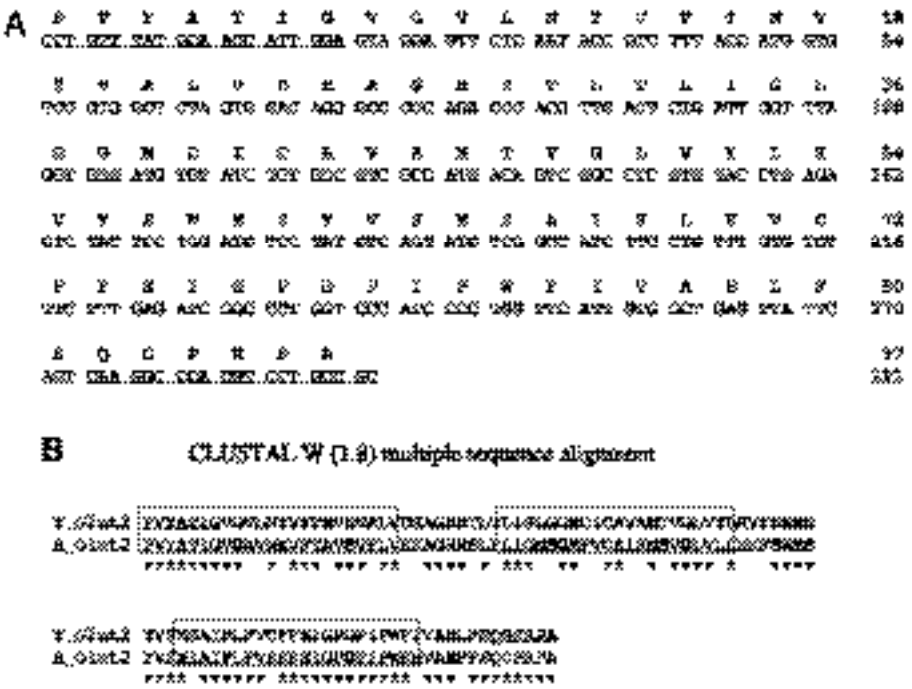

Fig. 2. Partial cloning of the glucose transporter type 2 (Glut2) gene in rainbow trout. (A) Nucleotide and deduced amino acid sequences of the rainbow trout Glut2 clone. Underlined letters correspond to the primer sequences. (B) Alignments of the partial amino acid sequence deduced from Glut2 cDNA of the rainbow trout (T_Glut2) (GenBank accession number: AF246147) with human Glut2 (H_Glut2) (GenBank accession number: P11168). Underlined letters correspond to the primer sequences. Asterisks mark amino acid residues homologous between Glut2s. Hydrophobic putative transmembrane segments are boxed (Thorens, 1992).

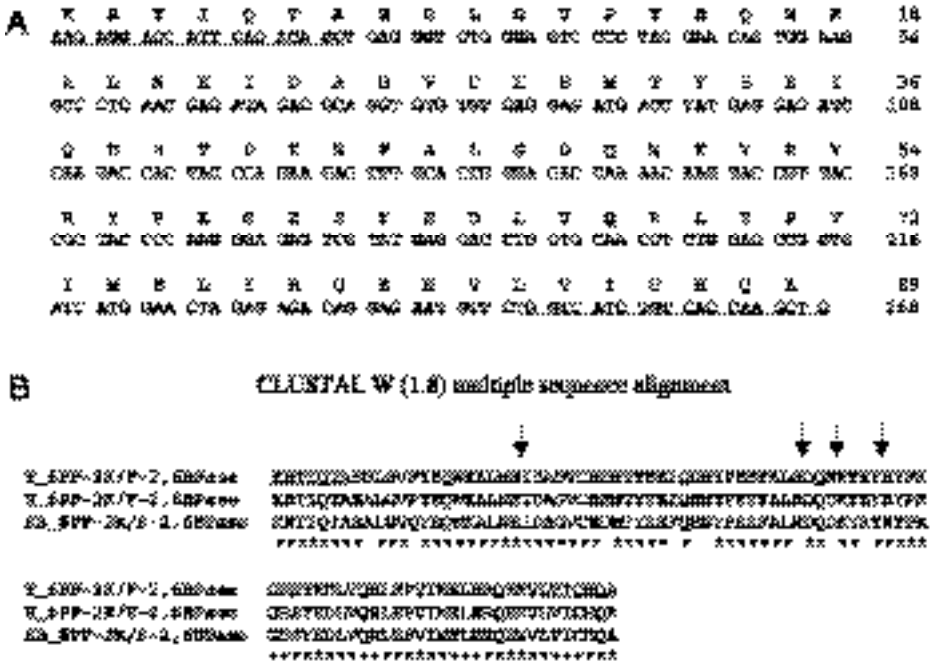

Fig. 3. Partial cloning of the 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (6PF-2K/F-2,6BPase) gene in rainbow trout. (A) nucleotide and deduced amino acid sequences of the rainbow trout 6PF-2K/F-2,6BPase clone. Underlined letters correspond to the primer sequences. (B) Alignments of the partial amino acid deduced sequence from 6PF-2K/F-2,6BPase cDNA of the rainbow trout (T_6PF-2K/F-2,6BPase) (GenBank accession number: AF246148) with human 6PF-2K/F-2,6BPase (H_6PF-2K/F-2,6BPase) (GenBank accession number: P16168) and gilthead seabream (*Sparus aurata*) 6PF-2K/F-2,6BPase (SB_6PF-2K/F-2,6BPase) (GenBank accession number: U84724). Underlined letters correspond to the primer sequences. Asterisks mark amino acid residues homologous between 6PF-2K/F-2,6BPases. Residues marked by an arrow are key residues for the 6PF-2K/F-2,6BPase (Meton et al., 1999a).

Glut2, FBPase and 6PF-2K/F-2,6BPase, respectively) (Fig. 1B, Fig. 2B, Fig. 3B, Fig. 4B).

To analyze the tissue specificity of PK, Glut2, FBPase and 6PF-2K/F-2,6BPase gene expression (Fig. 5), we performed RT–PCR using specific rainbow trout PK, Glut2, FBPase and 6PF-2K/F-2,6BPase primers (Table 2). This RT–PCR was not quantitative, and thus only the presence or absence of a specific mRNA could be determined. PK and Glut2 mRNAs were detected in liver, intestine and kidney but not in muscle, brain or heart (Fig. 5). FBPase mRNA was detected in liver, intestine and kidney but also in muscle, brain and heart of fed fish. Finally, 6PF-2K/F-2,6BPase gene expression was ubiquitous, even not much in brain of fed fish.

As growth rates of rainbow trout fed with 20 % carbohydrates or without carbohydrates were comparable (Panserat et al., 2000a), comparative analysis of the effects of dietary carbohydrates on the regulation of metabolic enzyme expression between fish groups fed different carbohydrate levels was possible. Expression of PK, Glut2, FBPase and 6PF-2K/F-2,6BPase genes was analyzed in fish livers by northern blotting (Fig. 6). Single mRNA species for PK and Glut2 cDNAs of approximately 2.2 kb and 2 kb, respectively, were found (Fig. 6A,B). In contrast, two mRNAs for 6PF-2K/F-2,6BPase and FBPase were found (Fig. 6C,D); the major forms were approximately 2 kb and 2.7 kb for 6PF-2K/F-2,6BPase and FBPase, respectively, whereas the minor mRNAs (very weak) were of approximately 1.5 kb and 2.5 kb (data not shown). As the expression of the minor fragment is not easy to analyze by northern blotting (level of gene expression is too low), only the major 6PF-2K/F-2,6FBase and FBPase mRNA species were studied in this nutritional analysis.

The effect of fasting and refeeding on gene expression was analyzed. In contrast to expression of PK, FBPase and Glut2 (in which no significant differences were observed between food-deprived and fed fish), it appeared that the level of 6PF-2K/F-2,6BPase gene expression was significantly higher in fish fed with carbohydrates 24 h after feeding than in food-deprived fish (P<0.05, Tukey's test: Fig. 7A–D). Our results indicated that dietary carbohydrates did not induce a specific effect; expression of PK, Glut2, FBPase and 6PF-2K/F-2,6BPase mRNAs were elevated at both 6 h and 24 h after feeding, similar to levels in the livers of fish fed with and without carbohydrates (Fig. 7A–D). We also analyzed the effects of the postprandial time on gene expression in fish fed with a specific diet (6 h compared with 24 h feeding): no significant effect was observed (data not shown).

Discussion

PK, Glut2, FBPase and 6PF-2K/F-2,6BPase belong to a family of enzymes, such as phosphoenolpyruvate carboxykinase (EC4.1.1.32) and fatty acid synthetase (EC2.3.1.85), whose expression in mammals has been shown to be regulated by dietary carbohydrates (Rencurel and Girard,

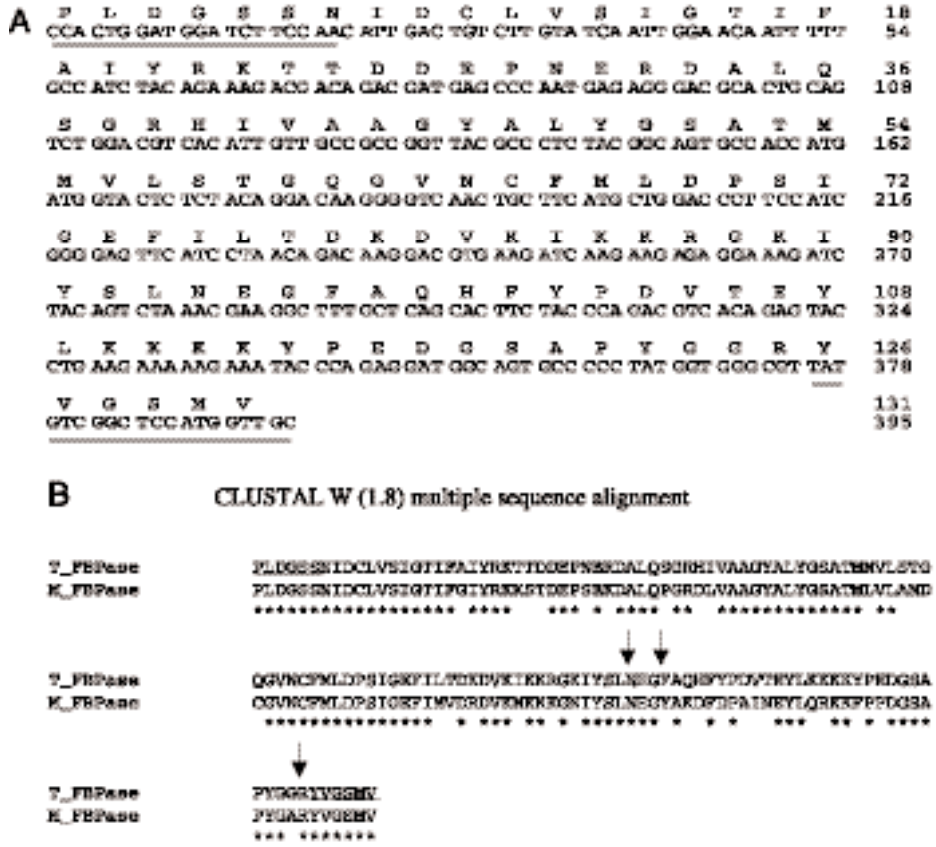

Fig. 4. Partial cloning of the FBPase gene in rainbow trout. (A) Nucleotide and deduced amino acid sequences of the rainbow trout FBPase clone. Underlined letters correspond to the primer sequences. (B) Alignments of the partial amino acid deduced sequence from FBPase cDNA of the rainbow trout (T_FBPase) (GenBank accession number: AF333188) with mouse FBPase (M_FBPase) (GenBank accession number: AJ132693). Underlined letters correspond to the primer sequences. Asterisks mark amino acid residues homologous between FBPases. Conserved residues involved in the interaction with fructose-1,6-bisphosphate are marked by arrows (Ke et al., 1989).

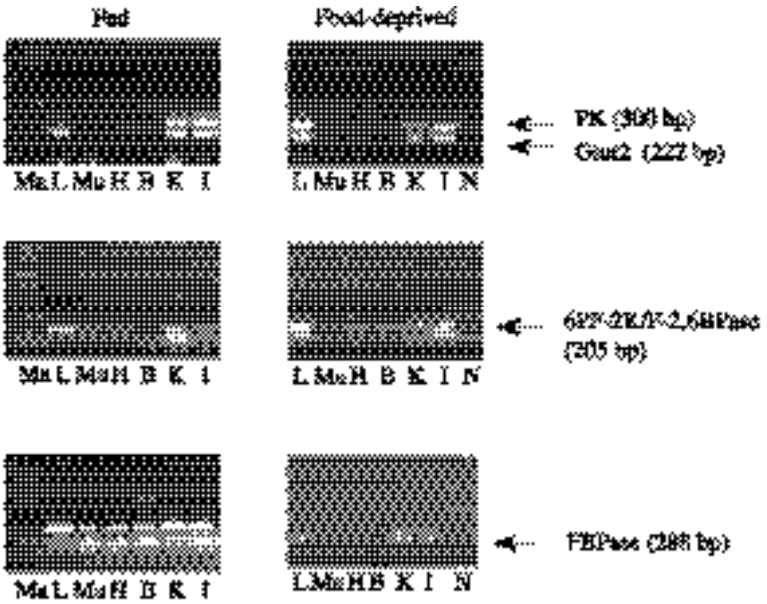

Fig. 5. Tissue specificity of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (6PF-2K/F-2,6BPase), pyruvate kinase (PK), fructose-1,6-bisphosphatase (FBPase) and glucose transporter type 2 (Glut2) gene expression in food-deprived rainbow trout and fed rainbow trout with 20 % carbohydrates (at 6 h after feeding). Analysis by non-quantitative RT–PCR (*N*=2 fish per treatment). Ma, molecular mass marker phiX174 DNA/*Hae*III (Promega, USA); N, negative control, i.e. RT–PCR reactions performed without RNA and with reverse transcriptase (other controls made with RNA and without reverse transcriptase were also performed to determine genomic DNA contamination; data not shown). L, liver; Mu, muscle; H, heart; B, brain; K, kidney; I, intestine. The exact lengths of the 6PF-2K/F-2,6BPase, PK, FBPase and Glut2 fragments (205 bp, 300 bp, 288 bp and 222 bp, respectively) were determined from known gene sequences. The quality of the first-strand cDNA used in each of the PCR assays was first confirmed by its ability to support the amplification of FBPase and 6PF-2K/F-2,6BPase cDNAs, and the failure to detect the presence of PK and Glut2 mRNA in certain tissues does not imply poor quality of RNA samples or a low-efficiency reverse transcription reaction.

1998). In fish, literature data in this area are scarce. Although a formal proof will await the cloning of the full-length cDNA sequence, the high similarity between the cDNA sequences of hepatic PK, Glut2, FBPase and 6PF-2K/F-2,6BPase in rainbow trout and of the PK, Glut2, FBPase and 6PF-2K/F-2,6BPase sequences previously characterized in other vertebrates (up to 72 %, 78 %, 76 % and 95 % similarity, respectively) strongly suggests that these sequences correspond to functional proteins (enzymes or transporter). We detected only one species of PK and Glut2 mRNA in trout liver, whereas there are two FBPase and 6PF-2K/F-2,6BPase mRNAs. In gilthead seabream, a major hepatic 6PF-2K/F-2,6BPase RNA band of approximately 4.2 kb and two minor bands of approximately 2.1 kb and 1.1 kb have been detected (Meton et al., 1999a). The existence of different mRNAs may correspond either to distinct 5′ and 3′ untranslated regions or to the existence of distinct genes. Moreover, it is worth noting that trout FBPase mRNAs (2.5 kb and 2.7 kb) are approximately twice the size of mammalian mRNAs (approximately 1.3 and 1.5 kb) (El-Magrabi et al., 1988). Furthermore, the cloning of cDNAs represented here is not exhaustive; the existence of other (different) hepatic PK, Glut2, FBPase and 6PF-2K/F-2,6BPase mRNAs is possible because (i) only two hepatic cDNA clones have been analysed and (ii) our cDNA cloning strategy based on RT–PCR with degenerate primers may amplify only one specific mRNA and not all the different mRNA species.

As in mammals (Marcus et al., 1987; Thorens, 1992; Pilkis et al., 1995; Yamada and Noguchi, 1999), PK, 6PF-2K/F-2,6BPase, FBPase and Glut2 genes are expressed in the liver, intestine and kidney. Moreover, there is no apparent nutritional effect on the regulation of PK, Glut2, FBPase and 6PF-2K/F-2,6BPase gene expression in these different tissues, except for the FBPase gene, which is expressed in non-gluconeogenic tissues such as the muscle and heart in fed fish only. To our knowledge, the biochemical consequences of FBPase gene expression in muscle (white muscle and heart) have not been studied.

PK and 6PF-2K/F-2,6BPase genes code for hepatic glucose metabolism proteins that are induced by feeding in mammals (Pilkis et al., 1995; Yamada and Noguchi, 1999). Induction of hepatic trout 6PF-2K/F-2,6BPase gene expression by feeding (and specifically by dietary carbohydrates) in rainbow trout confirms what has been already shown in mammals and gilthead seabream (Garcia de Fructos and Baanante, 1994; Meton et al., 1999a; Meton et al., 1999b; Meton et al., 2000). However, a significant change in 6PF-2K/F-2,6BPase gene expression may either increase or decrease concentrations of fructose-2,6-bisphosphate; more data on the phosphorylation/dephosphorylation status of the enzyme (induced by protein kinase A or protein phosphatase 2A activities; Pilkis et al., 1995) would be needed to determine in which direction 6PF-2K/F-2,6BPase affects fructose-2,6-bisphophate concentrations. The trout hepatic PK gene is always highly expressed, irrespective of the nutritional status. Using enzymatic methods, some authors (Cowey et al., 1977; Cowey et al., 1981; Fideu et al., 1983) found that PK activity

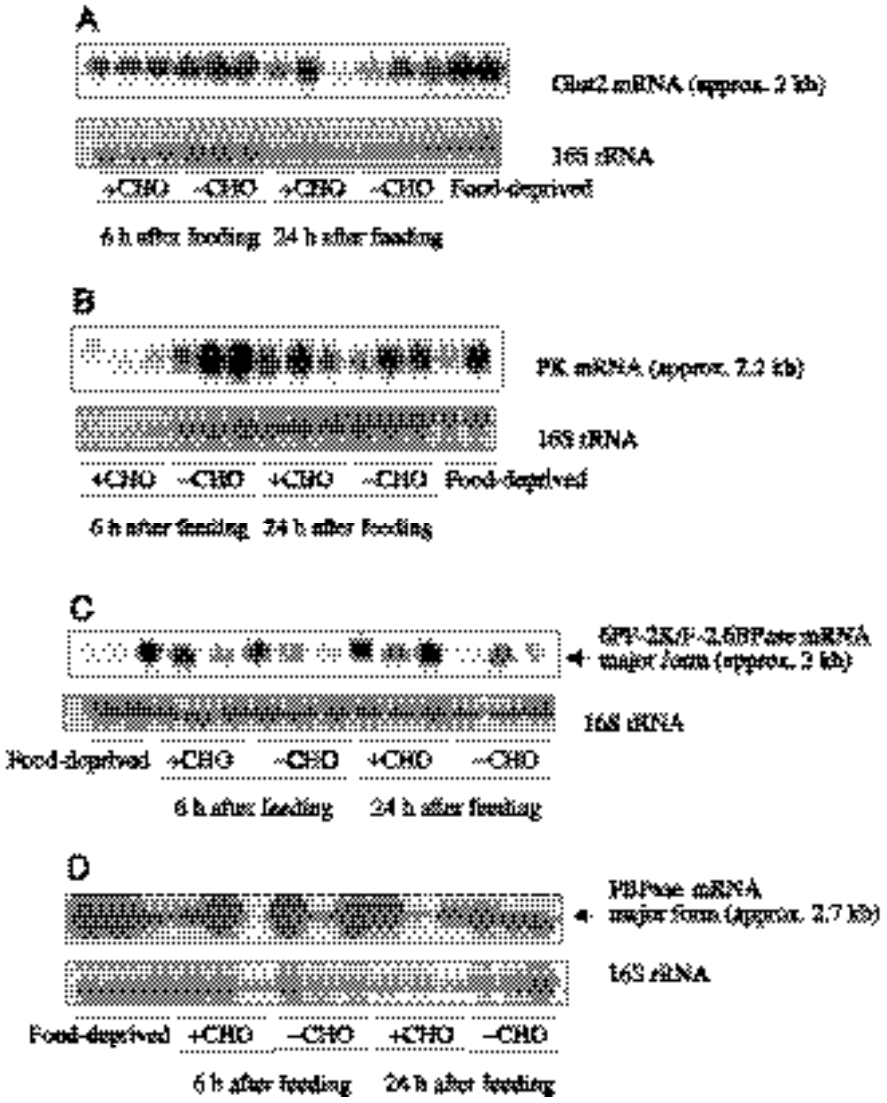

Fig. 6. Representative northern blots of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (6PF-2K/F-2,6BPase), pyruvate kinase (PK), fructose-1,6-bisphosphatase (FBPase) and glucose transporter type 2 (Glut2) gene expression in the liver of food-deprived fish or fish fed with 20 % carbohydrates (+CHO) or without carbohydrates (–CHO). Each band is from a different fish. The 16S rRNA served as an internal control of sample loading. (A) Glut2, (B) PK, (C) 6PF-2K/F-2,6BPase, (D) FBPase.

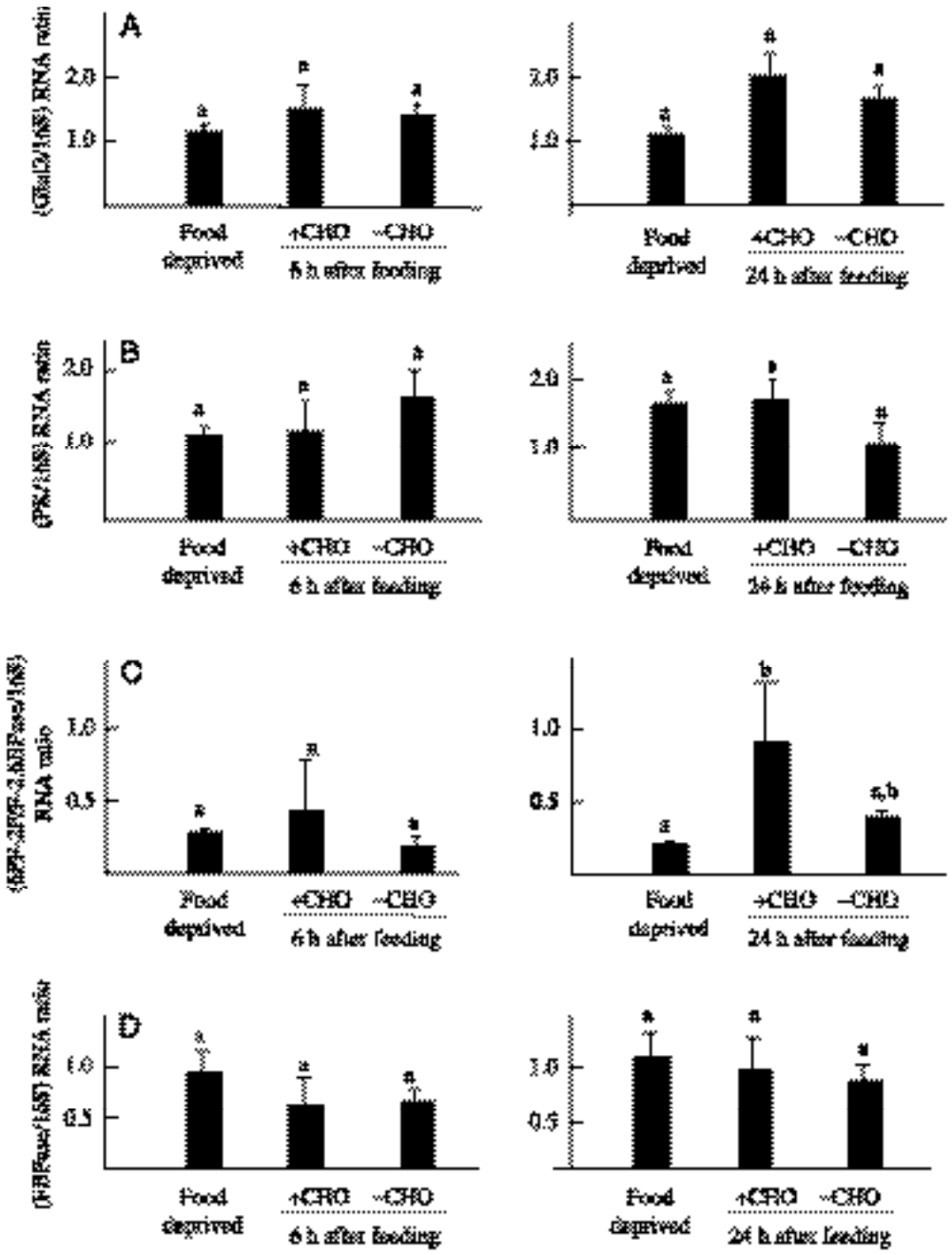

Fig. 7. Levels of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2K/F-2,6BPase), pyruvate kinase (PK), fructose-1,6-bisphosphatase (FBPase) and glucose transporter type 2 (Glut2) gene expressions in the liver of food-deprived fish and of fish fed with 20% carbohydrates (+CHO) or without carbohydrates (–CHO). (A) Glut2, (B) PK, (C) 6PF-2K/F-2,6BPase, (D) FBPase. For each gene, two different northern blots (loading with fish fed 6 h and 24 h after feeding) were done; an analysis by densitometry of mRNA levels (arbitrary units) for five fish from each treatment group weighted by 16S rRNA values was performed (Visio-Mic II software). The results are expressed as means ± S.D. (N=5). Significant differences within groups are represented by different letters (Tukey's test, P<0.05).

was induced by feeding in rainbow trout, while others (Hilton and Atkinson, 1982; Shikata et al., 1994) found no such induction. Our data suggest that this induction is not (mainly) due to a transcriptional mechanism, but is more probably linked either to post-transcriptional regulation or to qualitative alterations such as phosphorylation/dephosphorylation of PK, as in mammals (Yamada and Noguchi, 1999). Moreover, we were unable to demonstrate a significant decrease in FBPase gene expression in response to dietary carbohydrates in rainbow trout liver (at least for the major FBPase mRNA species), in accordance with the absence of inhibition of FBPase activity by dietary carbohydrates in other fish species such as perch (*Perca fluviatilis*) or Atlantic salmon (*Salmo salar*) (Tranulis et al., 1996; Borrebaek and Christophersen, 2000). It is also worth noting that some individuals (irrespective of the nutritional status) show very low levels of FBPase gene expression; the explanation for this result is unknown.

Our data demonstrate the existence of a glucose transporter very similar to the mammalian Glut2 (cloning of a full-length Glut2 cDNA is in progress in collaboration with Dr A. Krasnov and confirms the existence of a Glut2 protein in trout; our unpublished data). Glut2 seems to be expressed at high levels in rainbow trout, irrespective of nutritional status. This result is expected since Glut2 is important for the entry of glucose into hepatocytes but to allow glucose (produced by gluconeogenesis and glycogenolysis) out of the hepatocytes during fasting (Thorens, 1992).

In conclusion, our data demonstrate the existence of mammalian-type induction of 6PF-2K/F-2,6BPase gene expression by feeding, as has been shown previously for trout glucokinase (Panserat et al., 2000a; Panserat et al., 2000b) in association with high levels of PK gene expression. Taken together, these data suggest the existence of an efficient enzymatic mechanism for storing excess glucose. The existence of a type-2 glucose transporter further supports the possibility that hepatocytes store excess dietary glucose. In contrast, FBPase is not strongly inhibited by dietary carbohydrates. Such a lack of inhibition has been observed previously for two other key hepatic enzymes, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (Panserat et al., 2000a; Panserat et al., 2000b; Panserat et al., 2001), suggesting an absence of nutritional control of hepatic glucose production in trout. Further analysis of hepatic glucose production *in vivo* and *ex vivo* is necessary.

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