Molecular identification of a glucose transporter from fish muscle[1]

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Received 8 July 2000; revised 27 August 2000; accepted 29 August 2000

Edited by Giorgio Semenza

Abstract In mammals and birds, several isoforms of facilitative glucose transporters have been identified (GLUT1–4), but no information is available regarding the molecules involved in glucose transport in other vertebrates. Here we report the cloning of a GLUT molecule from fish muscle with high sequence homology to GLUT4 and containing features characteristic of a functional GLUT. Fish GLUT is expressed predominantly in skeletal muscle, kidney and gill, which are tissues with known high glucose utilization. These results indicate that fish GLUT is structurally, and perhaps functionally, similar to the other known GLUTs expressed in muscle in mammalian and avian species.

Key words: Glucose transporter; Muscle; Fish; Trout

1. Introduction

Facilitative glucose transporters (GLUTs) are key proteins that transport glucose across cell membranes and, therefore, important for the control of glucose homeostasis. In mammals, four different GLUT isoforms have been clearly identified (GLUT1–4), each being the product of a separate gene and with a distinct tissue distribution and physiological function. These different GLUT isoforms belong to a family of structurally related proteins that are characterized by the presence of 12 putative membrane-spanning regions and intracellular amino- and carboxy-termini [1]. Recently, a novel GLUT isoform (GLUTX1) has been described and it has been suggested that it may belong to a separate GLUT subfamily because of sequence and structural differences with known GLUTs [2]. In addition to mammals, three isoforms belonging to the GLUT family (GLUT1–3) have been identified in birds [3–5]. However, no information is available regarding the identity of the molecules involved in glucose transport in any other vertebrate group.

Among the lower vertebrates, fish have been extensively studied with regard to their glucose metabolism and they differ mainly from mammals in that their peripheral tissues (e.g. muscle, since it represents more than 50% of the body weight) have a much lower ability to utilize glucose [6]. Regardless of the lower glucose uptake rate in fish tissues [6,7], there is indirect evidence suggesting that glucose entry into cells could be mediated by transporters of the GLUT family. In one study, antibodies against mammalian GLUT1 immunoreacted with a protein only in heart and brain of tilapia, an omnivorous fish [8]. In another study, transgenic trout embryos overexpressing human GLUT1 showed increased glucose uptake and metabolism [9]. In addition, glucose uptake by fish erythrocytes and cardiac muscle was shown to be inhibited by cytochalasin B [10–12], a known inhibitor of facilitative glucose transporters [13]. However, the exact nature and identity of the transporter molecule(s) involved in glucose uptake in fish tissues has not yet been determined.

In order to identify and characterize the GLUT involved in glucose uptake in fish (brown trout) muscle, we have isolated and characterized, for the first time in a lower vertebrate, the cDNA that encodes a GLUT molecule belonging to the GLUT family of proteins. The brown trout GLUT, expressed primarily in skeletal muscle, gill, kidney and adipose tissue, is structurally similar to mammalian and avian GLUTs and shows the highest sequence homology with mammalian GLUT4 proteins.

2. Materials and methods

2.1. cDNA cloning

Total RNA was extracted from brown trout (*Salmo trutta*) tissues using an established RNA purification method [14]. Poly(A)$^+$-enriched RNA from trout red muscle was isolated with oligo-dT columns (Pharmacia) and reverse-transcribed with an Oligo-dT primer using a commercial kit (First Strand, Pharmacia). PCR amplification was performed for 40 cycles at 94°C for 1 min, 50°C for 2 min, and 65°C for 2 min with Taq DNA polymerase (Bioline) using degenerate primers (200 μM) designed against conserved regions of various mammalian glucose transporters (Table 1). Primers 1 and 2 amplified a 96-nucleotide product and primers 3 and 4 amplified a 386-nucleotide product. PCR products were separated on 2% agarose gels, excised, purified from agarose (Qiaex II, Qiagen) and cloned into a vector (pGEM-T Easy, Promega). Sequencing was performed on each strand of at least two independent PCR products using a dye terminator cycle sequencing kit (Thermo Sequenase II, Amersham) and specific primers were designed for rapid amplification of cDNA ends (RACE) (primers 5–8, Table 1). A commercial kit (Smart RACE, Clontech) was used to amplify by PCR the 5′- and 3′-ends of the first-strand cDNA synthesized from poly(A)$^+$-enriched RNA from trout red muscle (obtained as described above). Initially, primers 5 and 6 (10 μM) were used in the 3′- and 5′-RACE reactions, respectively, under the conditions recommended by the manufacturer. A 5′-RACE product of 486 nucleotides was obtained, isolated, cloned and sequenced as described above and, based on this sequence, another specific primer was designed (primer 9). Nested PCR was performed using one-fiftieth volume of the initial RACE reactions as template and Clontech's nested primers in conjunction with specific primers: primers 7 and 9 for 5′-RACE reactions and primer 8 for 3′-RACE reactions. Products ranging from 400 to 1100 nucleotides were obtained and products from at least two independent amplifications were sequenced on both strands. The full reading frame was subsequently amplified by PCR using gene-specific primers for the 5′- and 3′-untranslated re-

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[1] The nucleotide sequence data reported in this paper have been submitted to GenBank under accession number AF247395.

Abbreviations: RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; TM, transmembrane domain

0014-5793/00/$20.00
PII: S0014-5793(00)02020-2

gions (primers 10 and 11, respectively) and sequenced on both strands. Sequence compilations, comparisons and features were obtained using the Wisconsin Package Version 9.0 (Genetics Computer Group) and NetPhos Program Version 2.0 [15]. Phylogenetic analyses were performed with the PHYLIP Program Package Version 3.52 [16].

2.2. Northern blot analysis

Approximately 20 μg of total RNA from various tissues was loaded onto a formaldehyde-agarose gel, transferred onto a nylon membrane (Hybond-N, Amersham) and cross-linked. The membrane was hybridized overnight at 42°C with a ^{32}P-labelled 1.1-kb cDNA fragment (1.5×10^7 cpm/10 ml), amplified from red muscle poly(A)$^+$ RNA with specific primers 12 (forward) and 6 (reverse) under the conditions described below. The membrane was washed three times with 2×SSC/0.1% SDS at room temperature, once with 1×SSC/0.1% SDS at 42°C and once with 0.1×SSC/0.1% SDS at 42°C. Subsequently, the membrane was exposed to Kodak X-AR film at −80°C. After stripping, the same blot was rehybridized with a trout 18S ^{32}P-labelled cDNA fragment to control for RNA loading and transfer.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Poly(A)$^+$ RNA from different tissues (100 ng) was reverse-transcribed as described above and subjected to PCR using specific primers 12 and 6 for 40 cycles at 94°C for 1 min, 65°C for 2 min, and 72°C for 2 min. Parallel RT-PCR reactions were carried out with primers against a conserved region of β-actin (primers 13 and 14) as a control.

3. Results and discussion

3.1. Cloning of brown trout muscle GLUT

Initially, the amino acid sequences of various mammalian GLUTs were aligned to identify conserved regions as well as GLUT4-specific regions. Degenerate primers against these regions were designed to amplify a homologous region from brown trout muscle mRNA by RT-PCR. Primers 1 and 2 (Table 1) were designed to anneal to the Ser^{3}GlyPheGlnGlnIleGly^9 sequence of the amino-terminus of human GLUT4 and to the conserved Ser^{35}LeuGlnPheGlyTyrAsn^{41} sequence of GLUT4, respectively. Primers 3 and 4 (Table 1) were designed to anneal to the Ile^{303}AsnAlaValPheTyrTyr^{309} and Asn^{427}TrpThrSerAsnPhe^{432} conserved sequences (GLUT4), respectively, of the carboxy-half of known GLUTs. Primers 1 (GLUT4-specific) and 2 amplified a 96-nucleotide product that showed 84% and 59% homology to human GLUT4 and GLUT1, respectively. Primers 3 and 4 amplified a 386-nucleotide product that showed 86% and 89% homology to human GLUT4 and GLUT1, respectively. Based on these sequences, several specific primers (Table 1) were designed and used to obtain the full-length sequence by 3′- and 5′-RACE. The brown trout muscle cDNA sequence obtained is 2894 nucleotides long and contains a 271-nucleotide 5′-untranslated region, a 1509-nucleotide open reading frame and a 1114-nucleotide 3′-untranslated region, which includes the poly(A) tail. The deduced amino acid sequence corresponds to a protein containing 503 amino acids, with predicted molecular mass of 55.1 kDa and isoelectric point of 6.67, which shows a high degree of sequence homology with the family of facilitative glucose transporters (from 79.5% with rat GLUT4 to 66.3% with human GLUT3). Therefore, we designated the trout sequence the brown trout muscle glucose transporter (btGLUT).

Table 1
Sequences of oligonucleotides

Primer	DNA sequence
1	5′-C (AGCT) GG (AGCT) TT (CT) CA (AG) CA (AG) AT (ACT) - GG-3′
2	5′-C (AGCT) (AG) TT (AG) TA (AGCT) CC (AG) AA (CT) TG- (AGCT) AA-3′
3	5′-T (ACT) AA (CT) GC (AGCT) GT (AGCT) TT (CT) TA (CT) - TA-3′
4	5′- (AG) AA (AG) TT (AGCT) GA (AGCT) GTCCA (AG) TT-3′
5	5′-TCAACTCTGCCTTCACTGTGGTC-3′
6	5′-GAACACTGTCCAATAAGGCGAGC-3′
7	5′-ATAGCCGAATTGCAAGGAGC-3′
8	5′-CTCGCCTTATTGGACAGTGTTC-3′
9	5′-TCCTTCATCTCCGCCAGCATATCC-3′
10	5′-GTGCCAGGCTTATTGTCCATATTC-3′
11	5′-GCCCACTATGAGACTTACAGTAAC-3′
12	5′-GACAACTGTCACGGACTGTAATTGG-3′
13	5′-GCATCACACCTTCTACAACGAGCT-3′
14	5′-CTGCTCGAAGTCCAGGGCGACGTAG-3′

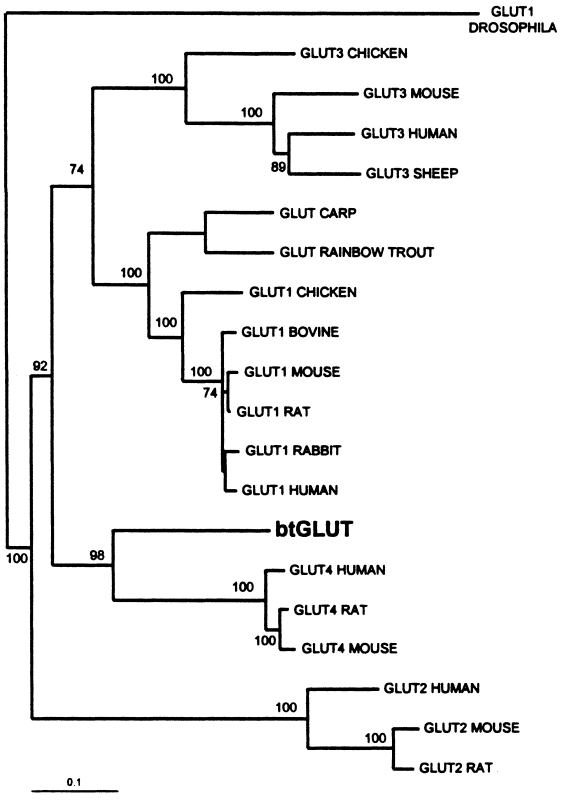

Fig. 1. Phylogenetic tree of all known vertebrate and *Drosophila* full-length glucose transporter protein sequences. *Drosophila*, rainbow trout and carp GLUT protein sequences were translated from GenBank accession numbers AF064703, AF247728 and AF247730, respectively. The tree was constructed using the neighbor-joining method (NEIGHBOR program, PHYLIP package) from a distance matrix created using the Dayhoff method (PROTDIST program, PHYLIP package). Numbers above nodes indicate bootstrap proportions (1000 replicates, SEQBOOT program, PHYLIP package). The horizontal line indicates the genetic distance.

3.2. Structural and sequence analysis of btGLUT

The deduced amino acid sequence of btGLUT shows the highest degree of sequence similarity with mammalian GLUT4 sequences (78.9–79.5%), followed by mammalian and avian GLUT1 sequences (73% and 72.6%, respectively). In addition, btGLUT shows 73% and 75% similarity with two other fish nucleotide sequences, namely carp and rainbow trout (GenBank accession numbers 247730 and 247728, respectively), which are both more similar to mammalian

GLUT1 (83–85%) than to mammalian GLUT4 sequences (71–73%). Phylogenetic analysis of GLUT isoforms shows that btGLUT is clustered together with the mammalian GLUT4 protein sequences, separate from the GLUT1, GLUT2 and GLUT3 clusters (Fig. 1). On the other hand, the above-mentioned carp and rainbow trout sequences are clearly clustered together with GLUT1 sequences and separate from btGLUT, which indicates that they most likely correspond to a GLUT isoform different from btGLUT. Together, these data suggest that btGLUT is closer in sequence to GLUT4, a protein known to be expressed mainly in muscle and adipose tissue in mammals and regulated by hormonal (e.g. insulin) and metabolic signals [17].

Alignment of the deduced amino acid sequence of btGLUT with human GLUT1 and GLUT4 evidences a high degree of structural conservation between trout and human GLUT proteins (Fig. 2). The btGLUT protein, like all other known GLUTs, contains 12 hydrophobic transmembrane domains (TMs) (I–XII), each consisting of 21 amino acids, and four major hydrophilic regions that correspond to the amino- and carboxy-termini and to the main extracellular and intracellular segments [1,18]. These structural characteristics of

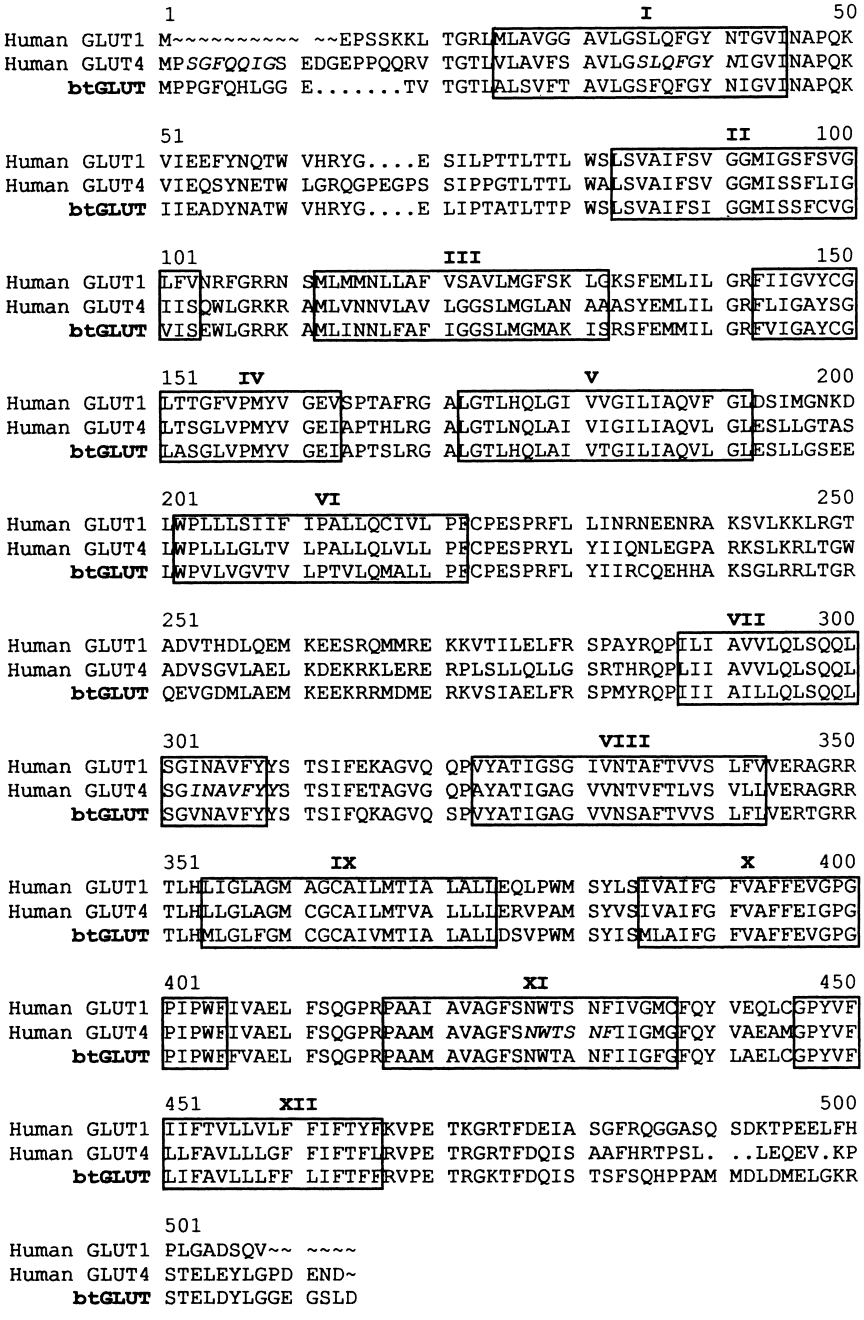

Fig. 2. Amino acid alignment of brown trout GLUT (btGLUT) with human GLUT1 and GLUT4. SwissProt accession numbers for human GLUT1 and GLUT4 are P11166 and P14672, respectively. Amino acids are represented by the single letter code and numbering corresponds to human GLUT4 residues. Boxes with roman numerals correspond to the 12 TM domains. Residues in italics correspond to residues for which degenerate primers were designed. The predicted *N*-glycosylation site is shown in bold.

btGLUT are also evidenced by the hydropathy plot of the deduced amino acid sequence of btGLUT (data not shown). A putative intracellular segment of 65 amino acids is found between TMs VI and VII and is 69.2% homologous to the corresponding segment of both human GLUT1 and GLUT4. Furthermore, the extracellular segment located between TMs I and II in btGLUT is 33 amino acids long and contains an asparagine residue at position 50, which is a predicted strong glycosylation site, common to all known mammalian and avian GLUTs. The length of the extracellular segment of btGLUT is the same as that of human GLUT1 but four amino acids shorter than that of human GLUT4 (37 residues), these differences are reflected by a higher sequence homology to the GLUT1 segment (75.7%) than to the GLUT4 segment (63.6%).

Analysis of the deduced amino acid sequence of btGLUT reveals the presence of several motifs that are characteristic of a functional GLUT. First, the ArgLeuSer sequence found in TM VII of btGLUT, also present in mammalian GLUT1, GLUT3 and GLUT4, has been shown to be important for the high-affinity recognition of the transported substrate [19]. Second, the SerThrSer sequence found in the extracellular segment between TMs VII and VIII of btGLUT, also found in mammalian GLUT1, GLUT2 and GLUT4, has been shown to be involved in the conformational change of GLUT4 during glucose transport [20]. Third, the proline residues found in TMs VI and X, which are well conserved among mammalian GLUTs with the exception of GLUT2, appear to be important for glucose transport activity [21]. Therefore, in addition to the strong sequence homology between btGLUT and mammalian GLUTs, our findings indicate that btGLUT has several structural characteristics that are known to be functionally important for mammalian GLUTs and they strongly suggest that btGLUT is indeed a member of the glucose transporter family.

Interestingly, the amino-terminus of btGLUT contains the sequence PheArg in positions 5 and 6, absent in human GLUT1, which has been shown to be important for the internalization of human GLUT4 [22]. Sequence comparison of the amino-terminus of btGLUT, human GLUT1 and GLUT4 indicates that this region of btGLUT is highly homologous to that of human GLUT4 (82.3%) and less to that of human GLUT1 (50%). The carboxy-terminus of GLUT4 also contains an additional specific motif, the SerLeuLeu sequence, which has been shown to determine its intracellular distribution [23] and is thought to be important for the regulation of GLUT4 translocation from the intracellular compartments to the cell membrane [24]. However, no such sequence is found in the carboxy-terminus of btGLUT, but rather a MetMet motif that could represent a conservative amino acid substitution. In addition, btGLUT contains a tyrosine residue in position 495, which is also found in the carboxy-terminus of human GLUT4 (Tyr^{502}) and which is also a predicted phosphorylation site for tyrosine kinases. It has been postulated recently that Tyr^{502} could be important for regulating the release of GLUT4 from its intracellular storage compartments [25], thus supporting a possible mechanism that could explain the effects of insulin in stimulating the translocation of GLUT4 to the cell membrane. Overall, the entire carboxy-terminus of btGLUT shows a higher homology to the corresponding region of human GLUT4 (67%) than to that of human GLUT1 (42%). At this point it is not possible to establish with certainty whether btGLUT could be internalized, sequestered and translocated like a GLUT4-type molecule, although the degree of similarity between the amino- and carboxy-termini of btGLUT and GLUT4 could be suggestive of this possibility. It will be important to determine if btGLUT could use the MetMet sequence as an intracellular targeting signal or if btGLUT could have other signals within its cytoplasmic carboxy-terminus that would target this fish GLUT to specific intracellular compartments.

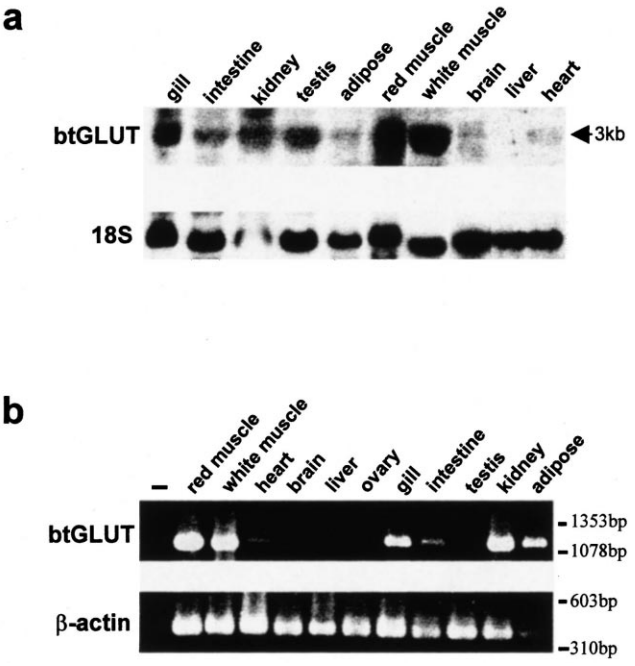

Fig. 3. Tissue distribution of brown trout (btGLUT) transcripts. a: Northern blot of total RNA from different tissues hybridized with a btGLUT cDNA probe (upper panel) or with an 18S cDNA probe (lower panel). b: RT-PCR using specific primer pairs for btGLUT (upper panel) or β-actin (lower panel). The symbol (−) indicates the negative control RT-PCR reaction performed in the absence of template cDNA. The size of the DNA markers is indicated on the right.

3.3. Tissue expression of btGLUT

Analysis of the expression of btGLUT by Northern blotting indicates that btGLUT is expressed predominantly in red and white skeletal muscle, gill and kidney (Fig. 3a). Lower levels of expression of btGLUT are detected in testis, intestine and adipose tissue, with even lower levels detected in brain, liver and heart. In all these tissues, a single mRNA species of approximately 3000 nucleotides is observed, consistent with the length of the entire nucleotide sequence of btGLUT. The expression of btGLUT, as analyzed by RT-PCR, is also shown to be high in skeletal muscle, gill and kidney, but also in adipose tissue, and very low in brain, liver and ovary (Fig. 3b). The pattern of expression of btGLUT is consistent with the reported glucose uptake rates in tissues of the brown trout: kidney and gill having high glucose uptake rates and skeletal muscle being the major site of glucose uptake [7]. Interestingly, red and white skeletal muscle are the only two tissues in brown trout that have been shown to increase the rate of glucose uptake after a glucose load [7], which suggests that glucose uptake in skeletal muscle could be regulated. In several fish species, including the brown trout, increases in

circulating glucose levels have been shown to cause increases in circulating insulin levels [26,27] and in the number of insulin receptors in muscle [28]. Therefore, it is possible that insulin, secreted in response to glucose, could stimulate glucose uptake in skeletal muscle of fish. Current efforts in our laboratory are devoted to investigating whether insulin and/or metabolic status could regulate the expression of btGLUT in skeletal muscle. Preliminary results suggest the possibility that insulin could be driving the expression of btGLUT since expression of btGLUT in red muscle appears to be lower in fasted fish than in fed fish (Planas and Capilla, unpublished).

In conclusion, we have identified a member of the GLUT family of proteins in fish muscle. The deduced amino acid sequence of btGLUT shows the highest sequence homology with mammalian GLUT4-type proteins and shares several functionally important structural characteristics with these isoforms, in addition to its expression, although not exclusive, in insulin-sensitive tissues. Nevertheless, additional information on transport properties, cellular localization and regulation will be needed to determine if btGLUT can be classified as a fish GLUT4 homologue.

Acknowledgements: We thank Dr. Antonio Zorzano and Dr. Simon MacKenzie for their critical reading of the manuscript, Dr. Antonio Celada for his technical support and Dr. Marta Riutort for her assistance with the phylogenetic analysis. This work was supported by Grants FAIR CT95-0174 and PB97-0902.

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