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Sequence and expression of a constitutive, facilitated glucose transporter (GLUT1) in Atlantic cod *Gadus morhua*

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Summary

A putative glucose transporter, GLUT1, is reported for Atlantic cod *Gadus morhua*. A combination of RT-PCR, RLM-RACE and genome walking were used to articulate a 4560 bp cDNA (GenBank accession number AY526497). It contains a 149 bp 5′ UTR, a 1470 bp open reading frame and a 2941 bp 3′ UTR. At the nucleotide level, the cod GLUT1 ORF shares 78.2% sequence identity to human GLUT1 and the deduced amino acid sequence clusters with GLUT1s from rainbow trout and carp. GLUT1 transcript is highly expressed in brain, gill, heart and kidney and expressed to a lower level in at least six other tissues. Expression is evident immediately upon fertilization of eggs. Six hours of hypoxia at 40% DO_2 did not alter expression levels in brain, gill, heart or kidney. The level of expression is not substantially altered in heart during low temperature challenge, although there is a suggestion that colder temperature could lead to lower levels of expression, consistent with the concept that the cold-acclimated heart has a reduced dependence upon glucose as a metabolic fuel. Two months of starvation did not significantly alter the level of expression of GLUT1 in heart. This is in marked contrast to the rat heart where fasting leads to a substantial decrease in GLUT1 levels. Overall, there is a ubiquitous tissue distribution of GLUT1, consistent with other species, and the level of gene expression, especially in heart, is relatively constant over a range of physiological conditions.

Key words: Atlantic cod, glucose transporter, *Gadus morhua*, GLUT1, heart, hypoxia, larval development.

Introduction

The nature and function of facilitated glucose transporters (GLUTs) in fish is an area of active investigation given the importance of glucose uptake in the overall energetics and regulation of carbohydrate metabolism. At least 13 GLUT isoforms have been reported to occur in mammals. These fall into three general classes based upon amino acid sequences. Class I includes the sodium-independent GLUTs 1–4 that are the best understood (Joost and Thorens, 2001). GLUT 1, which is the subject of this study, occurs in most mammalian tissues, is considered to be responsible for glucose uptake under basal non-stressed conditions, is insulin insensitive and shows increased expression under hypoxia (Behrooz and Ismail-Beigi, 1999).

The structure and tissue distribution of GLUT1 in fish has been addressed but requires further resolution. Using antibodies to mammalian GLUT1, Wright et al. (1998) reported that the protein was abundant in tilapia heart, present in brain, but undetectable in skeletal muscle, liver, adipose tissue or islet cells. A putative GLUT1 cDNA was cloned from rainbow trout alevins. The deduced amino acid sequence was 77–79% identical to avian and mammalian GLUT1 proteins and transcripts were detectable shortly after fertilization of eggs. *In situ* hybridization revealed transcript distribution in a variety of cell types during embryogenesis. Micro injection of this GLUT1 mRNA into *Xenopus laevis* oocytes resulted in Na^+-independent glucose transport that was inhibited by cytochalasin B and phloretin. These features and competitive inhibition studies with a number of other sugars support the view that the transcript was indeed similar to a mammalian GLUT1. It was also stated but data were not provided, that in adult rainbow trout, abundant transcripts were detected in heart with low level of expression in skeletal muscle, liver, spleen, blood and brain (Teerijoki et al., 2000, 2001a). Capilla et al. (2002) further reported low levels of GLUT1 transcript in red and white muscle of rainbow trout. A glucose transporter has also been sequenced from the carp cell line, epithelioma papulosum cyprini, and again, based upon deduced amino acid sequence and hexose uptake characteristics, is similar to a mammalian GLUT1 (Teerijoki et al., 2001b).

In this study, we determine the sequence of GLUT1 from Atlantic cod (*Gadus morhua*). Expression during development from fertilization to larval fish, and transcript tissue distribution in adult fish, are assessed. We also address the impact of hypoxia, fasting and temperature challenge on gene expression with a particular emphasis on heart. We focus on heart, because GLUT1 is found in both tilapia and rainbow

trout hearts (Wright et al., 1998; Teerijoki et al., 2000), facilitated glucose transport is essential to the performance and glucose uptake of Atlantic cod heart under hypoxic conditions *via* an unknown glucose transporter (Clow et al., 2004), and low temperature favors fatty acids as opposed to glucose as the preferred fuel of metabolism in the fish heart (Driedzic et al., 1996). In addition, we assess the impact of food deprivation on heart GLUT1 expression since in the rat heart fasting leads to a substantial decrease in GLUT1 mRNA level (Kraegen et al., 1993).

Materials and methods

Animals

Atlantic cod *Gadus morhua* L. were cultured and raised in the Aquaculture Research and Development Facility at the Ocean Sciences Centre, Memorial University of Newfoundland, Canada. Animals were killed by a sharp blow to the head and the tissues removed quickly.

Preparation of RNA and DNA from tissues

For RNA preparation, when long-term storage was required, tissues were stored in RNA*later* (QIAGEN Inc., Mississauga, ON, Canada) according to the manufacturer's protocol. For northern blot analysis, total RNA was extracted from tissues using Trizol Reagent (Invitrogen, Burlington, ON, Canada). For expression analysis using RT-PCR, the Trizol generated RNA was treated with Amplification Grade DNaseI (Invitrogen, Burlington, ON, Canada) prior to reverse transcription. For cDNA cloning, RNA was immediately extracted from fresh tissue and concurrently treated with DNaseI using the RNeasy Mini Kit (QIAGEN Inc., Mississauga, ON, Canada). Poly(A)$^+$ RNA was isolated from total RNA using the Oligotex mRNA Mini Kit (QIAGEN Inc., Mississauga, ON, Canada). RNA was quantitated by UV absorption at 260 nm. RNA purity was determined by calculating the OD_{260}/OD_{280} ratio.

For genomic DNA preparation, liver was snap frozen in liquid nitrogen and stored at –80°C. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. DNA was quantitated as described for RNA.

cDNA cloning

Atlantic cod GLUT1 cDNA was cloned using a combination of RT-PCR, RLM-RACE and genome walking. The sequences of all primers used in cDNA cloning are presented in Table 1.

To clone the central portions, total RNA was reverse-transcribed with an oligo(dT) primer using Superscript II RNase H$^-$ Reverse Transcriptase (Invitrogen, Burlington, ON, Canada). PCR amplification was performed using Taq DNA Polymerase (Invitrogen, Burlington, ON, Canada). Touchdown PCR was used with 40 cycles of 94°C for 1 min, 65°C↓0.5°C per cycle for 1.5 min and 72°C for 1 min. To obtain the first fragment, primers were designed based upon consensus sequences from conserved areas of aligned vertebrate GLUT1s. Primers 1 and 2 generated a 664-nucleotide product. The PCR product was electrophoresed on a 1.5% agarose gel, excised and purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., Mississauga, ON, Canada). The product was then subcloned into pCRII-TOPO (Invitrogen, Burlington, ON, Canada) and sequenced on both strands at MOBIX, McMaster University, using ABI BigDye

Table 1. *Sequences of oligonucleotides used in cloning cod GLUT1*

Primer number	DNA sequence*	Direction	Position of 5′-end in cod GLUT1
1	5′-GCTACAACAC**G**GG**A**GTCATCA-3′	F	226
2	5′-**CT**CTC**CT**CCTTCATCTCCTGC-3′	R	889
3	5′-CAAGAACGAGGAGAACAAGGC-3′	F	797
4	5′-TTGAAGTA**G**GTGAAGA**Y**GA**A**GAA-3′	R	1498
5	5′-TTGTTCCCCATGAAGGACTCG-3′	R	694
6	5′-GGAGTAGAGCCCCACCACGAA-3′	R	545
7	5′-GAGAAGCTGCCGTGGATGTCGTA-3′	F	1221
8	5′-AGCATCGTGGCCATCTTCGGCTT-3′	F	1248
9	5′-TCATCATCTTCACGGTGCTGCTG-3′	F	1447
10	5′-GTACTTCAAGGTCCCGGAGACG-3′	F	1490
11	5′-TTCGCTCTCCACAGAAACGTAGTG-3′	F	2472
12	5′-TAAGGACGATACAAGATGGCCAAATT-3′	F	2571
13	5′-GCTAATCACACTCACACCTGGTG-3′	F	3166
14	5′-ACCAGCATTCGGTGGCCCGATT-3′	F	3275
15	5′-GGCTTTCATTTGGAGTTGCCTCTT-3′	F	3724
16	5′-GTTGCTGTTGTCACTGATGAGGAT-3′	F	3823
17	5′-GAGGTGTATGCAGAGTGAATTCTGT-3′	F	4061
18	5′-CCTAAATGGACGGGACAAACACAG-3′	F	4234

*Nucleotides in bold type differ from the actual cod GLUT1 sequence. F, forward; R, reverse.

terminator chemistry and ABIPRISM® 3100 Genetic Analyzer. This partial cod GLUT1 cDNA sequence was aligned with sequence from two other glucose transporters cloned from cod, to which GLUT1 had 65.3% and 63.2% sequence identity (J.R.H. and W.R.D., unpublished data). Primer 3 was designed in an area to specifically amplify GLUT1 and used in conjunction with the degenerate primer (Primer 4) to obtain a 702-nucleotide fragment that yielded, in conjunction with the first PCR product, a total of 1273-nucleotides of ORF sequence data.

The 5′ end of cod GLUT1 was cloned with a commercial kit for RLM-RACE, GeneRacer Kit (Invitrogen, Burlington, ON, Canada) using poly(A)$^+$ RNA. PCR amplification was performed at 94°C for 1 min, 68°C for 2 min for 10 cycles followed by 94°C for 1 min, 65°C for 1 min and 68°C for 1.5 min for 30 cycles using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Burlington, ON, Canada). The gene-specific Primer 5 was used with the GeneRacer 5′ primer for the primary PCR and Primer 6 with the GeneRacer 5′ nested primer for nested PCR. The 545-nucleotide product contained the 149-nucleotide 5′UTR and the remaining 76 nucleotides of 5′ coding sequence.

The 3′ end of cod GLUT1 was obtained using a combination of genome walking and 3′ RLM-RACE. Attempts to clone the remaining 3′ sequences by 3′ RACE at this point were unsuccessful. This was not surprising since northern blot analysis indicated that the mRNA for cod GLUT1 was approximately 4.6 kb, leaving about 3.7 kb of unknown 3′ sequence. Therefore, genome walking was chosen as it would break up the long 3′ UTR into more reasonable fragments based upon the presence of restriction enzyme sites within. GenomeWalker libraries for *Dra*I, *Eco*RV, *Msc*I, *Msl*I, *Pvu*II, *Ssp*I and *Stu*I were constructed using the Universal GenomeWalker Kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. The first walk was performed using Primer 7 and the GenomeWalker Adaptor Primer 1 (AP1). The reaction was diluted 1/50 and 1 μl used as the template for nested PCR using Primer 8 and the GenomeWalker Nested Adaptor Primer 2 (AP2). Both the primary and nested PCR amplifications were performed at 94°C for 10 s, 72°C for 3 min for 7 cycles, followed by 94°C for 10 s, 67°C for 30 s, 68°C for 3 min for 32 cycles, using Elongase Enzyme Mix (Invitrogen, Burlington, ON, Canada). Bands were obtained for *Msl*I, *Dra*I, *Ssp*I, *Pvu*II and *Stu*I, the largest of which was the 1.6 kb band for *Stu*I. Although this band contained a predicted intron (191 bp), the remaining 218 bp of coding sequence and 1070 bp of the 3′ UTR were resolved using GENSCAN.

These data were used to design primers for 3′ RACE using the GeneRacer Kit (Invitrogen, Burlington, ON, Canada) and poly(A)$^+$ RNA. The primary PCR was performed using Primer 9 and the GeneRacer 3′ primer. A 1 μl aliquot of the primary PCR reaction was used as the template for nested PCR using Primer 10 and the GeneRacer 3′ nested primer. PCR amplification was performed using Touchdown PCR with 40 cycles of 94°C for 30 s, 70°C↓0.3°C per cycle for 30 s and 72°C for 3.5 min using DyNAzyme EXT (MJ Research, Waltham, MA, USA). A 1 kb band was generated that matched the sequence obtained by genome walking. However, as northern blot analysis indicates that the mRNA transcript is about 4.6 kb, this is a truncated product. The sequence generated by the *Stu*I walk shows the presence of an A-rich sequence (8As), which corresponds to the position where the 3′ RACE transcript was terminated. Although this sequence is preceded by a potential poly(A) site (ATTAAA), the truncated product is most probably the result of the mispriming of the oligo(dT) to the A-rich sequence within the mRNA.

To circumvent this truncated RACE transcript and generate further 3′ UTR sequence, another genome walk was performed as previously described using Primers 11 and AP1 for the primary PCR and Primers 12 and AP2 for the nested PCR. A band was generated from the *Dra*I library that generated sequence data to position 3431.

A second 3′ RACE was then performed using primers downstream of the alternative poly(A) site as previously described. Primer 13 and the GeneRacer 3′ primer were used for the primary PCR and Primer 14 and the GeneRacer 3′ nested primer for the nested PCR. A band was generated that uses an alternative poly(A) site at position 3996.

Still being about 600 bp from the true poly(A) signal, a third genome walk was performed to circumvent the second alternative poly(A), as previously described. Primer 15 was used with AP1 for the primary PCR and Primer 16 with AP2 for the nested PCR. A band was generated from the *Ssp*I library to generate sequence to position 4639. A potential poly(A) signal was present at position 4537 that would generate a mRNA transcript of the size predicted by northern blot.

To determine if this poly(A) site is used, a third 3′ RACE was performed using Primer 17 with the GeneRacer 3′ primer for the primary PCR and Primer 18 and the GeneRacer 3′ nested primer for the nested PCR. A band was obtained which uses this poly(A) signal. Based on the transcript size predicted by northern blot, this is probably the true poly(A) signal. The poly(A) tail is located 15 bp downstream of this site, yielding a total transcript size of 4560 bp.

Sequence analysis

Sequence data was compiled and analyzed using Vector NTI v. 6.0 (Informax Inc., Bethesda, MD, USA). Alignments were performed using AlignX (Informax Inc., Bethesda, MD, USA) which uses the CLUSTAL W algorithm (Thompson et al., 1994). For phylogenetic and molecular evolutionary analyses, alignments were imported in MSF format into *MEGA* version 2.1 (Kumar et al., 2001). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) with Poisson correction. Bootstrap analysis was performed with 1000 replicates. Exon/intron boundaries from genome walking sequences were analyzed using GENSCAN (http://genes.mit.edu/GENSCAN.html). Transmembrane helices were predicted using HMMTOP (http://www.enzim.hu/hmmtop) (Tusnady and Simon, 1998, 2001).

Northern blot analysis

Total RNA (1–2 μg) was electrophoresed on a formaldehyde-agarose gel and the ribosomal RNA bands visualized by ethidium bromide staining. The RNA was then transferred to a positively charged nylon membrane (Roche, Laval, QC, Canada) using a vacuum blotter (Bio-Rad, Mississauga, ON, Canada) and UV crosslinked (UV Stratalinker, Stratagene, La Jolla, CA, USA). The membrane was pre-hybridized at 68°C for 1 h in DIG Easy Hyb (Roche, Laval, QC, Canada). Antisense RNA probes were generated from cDNA encoding the region from 259 to 923 of the open reading frame that was subcloned into pCRII-TOPO (Invitrogen, Burlington, ON, Canada). The plasmid was linearized with *Hin*dIII and the RNA labeled in an *in vitro* transcription reaction with digoxigenin-11-UTP and T7 RNA polymerase according to the manufacturer's protocol (DIG Northern Starter Kit, Roche, Laval, QC, Canada). The membrane was hybridized with denatured probe at 100 ng ml^{-1} in DIG Easy Hyb at 68°C overnight. The membrane was then processed according to the manufacturer's protocol (DIG Northern Starter Kit, Roche, Laval, QC, Canada). The bands were visualized by exposure to X-ray film for 5–30 min. X-ray films were scanned using the Alpha Imager 1220 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA, Canada). Densitometry was performed on the bands using the 1D-Multi Line Densitometry software program.

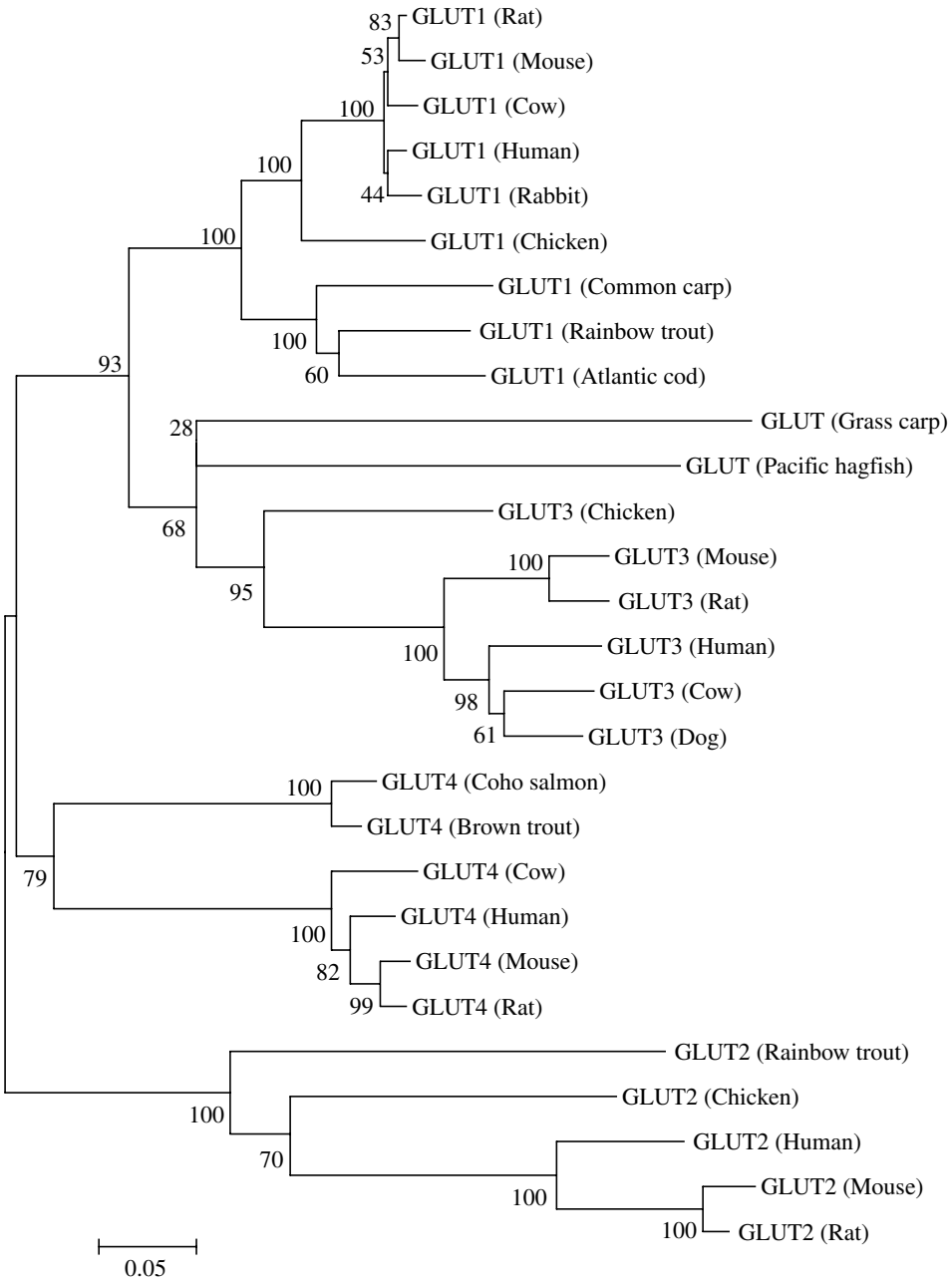

Fig. 1. Phylogenetic analysis of Atlantic cod GLUT1. A phylogenetic tree of protein sequences from cod GLUT1 and GLUTs 1-4 from other vertebrates. SwissProt accession numbers are as follows: GLUT1 common carp (AAF75683), GLUT1 rainbow trout (AAF75681), GLUT1 Atlantic cod (AAS17880), GLUT1 chicken (AAB02037), GLUT1 human (AAA52571), GLUT1 rabbit (P13355), GLUT1 cow (P27674), GLUT1 rat (P11167), GLUT1 mouse (AAA37752), GLUT2 chicken (Q90592), GLUT2 rainbow trout (AAK09377), GLUT2 human (AAA59514), GLUT2 mouse (P14246), GLUT2 rat (P12336), GLUT3 cow (AAK70222), GLUT3 dog (P47842), GLUT3 human (AAB61083), GLUT3 mouse (AAH34122), GLUT3 rat (Q07647), GLUT3 chicken (AAA48662), GLUT4 cow (Q27994), GLUT4 human (AAA59189), GLUT4 mouse (P14142), GLUT4 rat (P19357), GLUT4 coho salmon (AAM22227), GLUT4 brown trout (AAG12191), GLUT grass carp (AAP03065), GLUT pacific hagfish (AAL27090). The 28 GLUT protein sequences were aligned using the CLUSTAL W alignment mode in AlignX. The alignment was imported into *MEGA* version 2.1 and the phylogenetic tree constructed using the neighbor-joining method with Poisson correction. Bootstrap analysis was performed with 1000 replicates. Scale bar shows Tamura Nei distances.

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          1                                                                                                  80
 hGLUT1 MEPSSKKLTG RLMLAVGGAV LGSLQFGYNT GVINAPQKVI EEFYNQTWVH RYGESILPTT LTTLWSLSVA IFSVGGMIGS
ccGLUT1 .......... ..MLAVGTAV IGSLQFGYNT GVINAPQNVI EKFYNETWLD RYQEIIPKTT MTTLWSLSVA IFSVGGIVGS
rtGLUT1 MDSGGKQVTF QLMLTVGAAV IGSLQFGYNT GVINAPQKVI ERFLNETWFD RYQEPISKTS LTTLWSVSVA IFSVGGIFGS
codGLUT1 .MAEGKKVTF QLMLAVGAAV IGSLQFGYNT GVINAPQKVI ENFINETWSE RNEQPIDQTT LTTIWSVSVA IFSVGGIFGS
                    ** ** **  ********* ******* ** * * * **   *    *  *   ** ** *** ******  **

          81                                                                                                160
 hGLUT1 FSVGLFVNRF GRRNSMLMMN LLAFVSAVLM GFSKLGKSFE MLILGRFIIG VYCGLTTGFV PMYVGEVSPT ALRGALGTLH
ccGLUT1 FSVGLFVNRF GRRNSMLMAN VLAFISAALM GFSKNAASWE MLIIGRFVVG LYSGLATGFV PMYVGEVSPT ALRGALGTLH
rtGLUT1 FSVGLFVNRF GRRNSMLMAN VLAFVSAALM GFSKMGGSWE MLIIGRFVVG LYSGLSTGFV PMYVGEVAPT ALRGALGTLH
codGLUT1 FSVGLFVNRF GRKNSMLMAN ILAFASAALM GFSQMAKSYE MLIIGRFVVG LYSGLSTGFV PMYVGEVAPT ALRGALGTLH
          ********** ** ***** *  *** ** ** ***    * * *** ***  *  * ** **** ******* ** **********

          161                                                                                               240
 hGLUT1 QLGIVVGILI AQVFGLDSIM GNKDLWPLLL SIIFIPALLQ CIVLPFCPES PRFLLINRNE ENRAKSVLKK LRGTADVTHD
ccGLUT1 QLGIVVGILM AQIFGMDVIM GNDTMWPFLL CFTFIPAVLQ CCLLPFCPES PRFLLIIRNE ENKAKSVLKK LRGTSDVAAD
rtGLUT1 QLGIVTGILM AQVFGMEALM GNASLWPFLL GFTFIPALAQ CALLPFCPES PGFLLINRNE ENKAKTVLKK LRGTTDVSAD
codGLUT1 QLGIVLGILM AQVFGIESFM GNKALWPLLL GFTFFPAVVQ CAVLPFCPES PRFLLLNKNE ENKAKSVLKK LRGSADVSAD
          ***** ***  ** **    * **   ** **    * **  * *  ******* * ***   ** ** ** **** ***  **  *

          241                                                                                               320
 hGLUT1 LQEMKEESRQ MMREKKVTIL ELFRSPAYRQ PILIAVVLQL SQQLSGINAV FYYSTSIFEK AGVQQPVYAT IGSGIVNTAF
ccGLUT1 MQEMKEESRQ MMREKKVTIP ELFRSPLYRQ PMIICIMLQL SQQLSGINAV FYYSTKIFQK PGVEQPVYAT IGAGVVNTAF
rtGLUT1 MQEMKEEARQ MMREKKVTIL ELFRSPLYRQ PIFIAIMLQL SQQLSGINAV FYYSTRIFEK AGVAQPVYAT IGAGVVNTAF
codGLUT1 MQEMKSEHRQ MMREKKVTIP ELFRSPLYRQ PIFIAIVLQL SQQLSGINAV FYYSTSIFEK AGVAQPVYAT IGAGVVNTAF
          **** * ** *********  ****** *** *  *   *** ********** ***** ** *  ** ****** ** * *****

          321                                                                                               400
 hGLUT1 TVVSLFVVER AGRRTLHLIG LAGMAGCAIL MTIALALLEQ LPWMSYLSIV AIFGFVAFFE VGPGPIPWFI VAELFSQGPR
ccGLUT1 TVVSLFVVER AGRRSLHFLG LLGMAGSAVL MTIALALLEN VPWMSYISIV AIFGFVAFFE IGPGPIPWFI VAELFSQGPR
rtGLUT1 TVVSLFVVER AGRRSLHLLG LIGMAGAAVL MTIALALLDK LPWMSYVSIV AIFAFVAFFE IGPGPIPWFI VAELFSQGPP
codGLUT1 TVVSLFVVER AGRRSLHMIG LAGMAFSAVL MTIALSLLEK LPWMSYVSIV AIFGFVAFFE IGPGPIPWFI VAELFSQGPR
          ********** **** **  * * ***  * * ***** **    ***** *** *** ******  ********* *********

          401                                                                                               480
 hGLUT1 PAAIAVAGFS NWTSNFIVGM CFQYVEQLCG PYVFIIFTVL LVLFFIFTYF KVPETKGRTF DEIASGFRQG GASQSDKTPE
ccGLUT1 .LRLSVAGLC NWTANFIVGM CFQYVEELCG PYVFIIFTIF LLGFFVFTYF KVPETKGRTF DEISAGFRQA A.SAAEKHSP
rtGLUT1 PSAFAVAGFS NWTANFIVGM AFQYVEELCG PYVFVIFTIL LLSFFIFTYF KVPETKGRTF DEISAGFRQS SGTGGEKHSP
codGLUT1 PSAFAVAGFS NWTANFIVGM GFQYVEMVCG PYVFIIFTVL LLFFLVFTYF KVPETRGRTF DEISSGFRQS ..AGGEKHSP
               ***   *** ******  *****  ** **** ***   *  *  **** ***** **** ***  ****       *

          481       492
 hGLUT1 ELFHPLGADS QV
ccGLUT1 EELNSLGADS QL
rtGLUT1 EELNSLGADS QL
codGLUT1 EELAHSLGDS QL
          *       ** *
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Fig. 2. Amino acid alignment of cod GLUT1 with GLUT1s from human (hGLUT1; AAA52571), common carp (ccGLUT1; AAF75683) and rainbow trout (rtGLUT1; AAF75681). Amino acids are represented by the single letter code. Dots are inserted where gaps are present in the amino acid sequences. Numbers correspond to the human GLUT1 sequences. Asterisks indicate residues that are identical among all four sequences shown.

RT-PCR Analysis of GLUT1 expression

For RT-PCR, 1 μg of DNaseI-treated total RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen, Burlington, ON, Canada).

For analysis of GLUT1 expression, PCR amplification was performed using Taq DNA Polymerase (Invitrogen, Burlington, ON, Canada) with 40 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min. A 642 nucleotide PCR product, which encodes the region from 294 to 936 of the open reading frame, was amplified using the forward primer 5′-GTGATCGAGAACTTCATCAACGA-3′ and the reverse primer 5′-CATCATCTGCCGGTGCTCGCT-3′. These primers were designed in areas to specifically amplify cod GLUT1 when aligned with sequences from two other cod GLUT transporters (J.R.H. and W.R.D., unpublished data).

For normalization purposes, Atlantic cod actin (GenBank accession number U48856) was amplified using Taq DNA Polymerase (Invitrogen, Burlington, ON, Canada) with 40 (for developmental experiments) or 35 (for tissue distribution experiments) cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min. The 617 nucleotide product was amplified using the forward primer 5′-CCTGAACCCCAAGGCCAACAGA-3′ and the reverse primer 5′-TCTGCATGCGGTCAGCGAT-ACC-3′.

In vivo *experiments*

Developmental expression patterns

GLUT1 expression was monitored throughout the developmental period of Atlantic cod from egg to larval fish at intervals corresponding to changes in diet. The post hatch diet consisted of T-ISO enriched rotifers (to day 10), algamac 2000 enriched rotifers (to day 40), enriched artemia (to day 50) and then dry food (Shur Gain, Truro, NS, Canada). Eggs were sampled from two batches at fertilization (fertilization day 0), 7 days post fertilization (pre-hatch day 7) and upon hatching, approximately 13–14 days post fertilization (day 0). Post-hatch

```
                    I                                          50
MAEGKKVTFQ LMLAVGAAVI GSLQFGYNTG VINAPQKVIE NFINETWSER

                         II                                   100
NEQPIDQTTL TTIWSVSVAI FSVGGIFGSF SVGLFVNRFG RKNSMLMANI

       III                          IV                       150
LAFASAALMG FSQMAKSYEM LIIGRFVVGL YSGLSTGFVP MYVGEVAPTA

                  V                                VI      200
LRGALGTLHQ LGIVLGILMA QVFGIESFMG NKALWPLLLG FTFFPAVVQC

                                                           250
AVLPFCPESP RFLLLNKNEE NKAKSVLKKL RGSADVSADM QEMKSEHRQM

                                        VII                300
MREKKVTIPE LFRSPLYRQP IFIAIVLQLS QQLSGINAVF YYSTSIFEKA

               VIII                                IX      350
GVAQPVYATI GAGVVNTAFT VVSLFVVERA GRRSLHMIGL AGMAFSAVLM

                              X                            400
TIALSLLEKL PWMSYVSIVA IFGFVAFFEI GPGPIPWFIV AELSSQGPRP

            XI                                XII          450
SAFAVAGFSN WTANFIVGMG FQYVEMVCGP YVFIIFTVLL LFFLVFTYFK

                                          489
VPETRGRTFD EISSGFRQSA GGEKHSPEEL AHSLGDSQL
```

Fig. 3. Sequence analysis of Atlantic cod GLUT1. Amino acids are represented by the single letter code and numbered on the right. Boxes numbered with Roman numerals represent the putative 12 transmembrane helices. Residues that are highly conserved in all glucose transporters are highlighted in gray, while those that are specific to class I glucose transporters are highlighted in black. It should be noted that the PETKGR motif varies in Atlantic cod GLUT1 in that the K is substituted by an R.

samples were then taken at day 5 (T-ISO enriched rotifers), day 22 (algamac 2000 enriched rotifers), day 44 (enriched artemia) and day 55 (dry food). Typical body mass of day 55 larvae was about 100 mg. At each interval, for each batch approximately 100 mg of biomass was used in the RNA extraction. Samples were pooled, if necessary, to achieve the required amount of biomass.

Hypoxia challenge

Animals (body mass 396±31 g) were held at 8±0.4°C in a blackened, temperature-controlled cooler containing 35 l of water, in late spring. Aeration was achieved by gassing with an air pump supplying 100% O_2. Dissolved oxygen was monitored by pumping water from the cooler to a DO_2 electrode (VWR model 4000 DO_2 meter) housed in a Plexiglas chamber, and back to the cooler at 60 ml min^{-1}. Dissolved oxygen was reduced to selected values in less than 5 min by gassing with 100% N_2 and maintained within 4% of the desired level. When necessary, DO_2 levels were increased by gassing with 100% O_2.

For hypoxia trails, fish were placed in the chamber under normoxia and held for 1 h before DO_2 levels were reduced. After 1 h, oxygen was reduced to 60% and held for 30 min. DO_2 was then reduced to 40% and held for 6 h. Observations on temperature and DO_2 (mg l^{-1}) were collected at the end of each 30 min interval.

For normoxia trials, animals were held under identical conditions to those described above except that DO_2 in the chamber was maintained at approximately 100% saturation as described above. Animals were placed in the cooler 1 h before the experiment and observations were made on DO_2 and temperature every 60 min for a total of 7 h.

Temperature effects

Atlantic cod were divided into two groups and held in identical aerated, flow-through seawater 8100 l tanks and followed natural photoperiod. Both groups were fed a commercial diet (Shur Gain, Truro, NS, Canada). One group

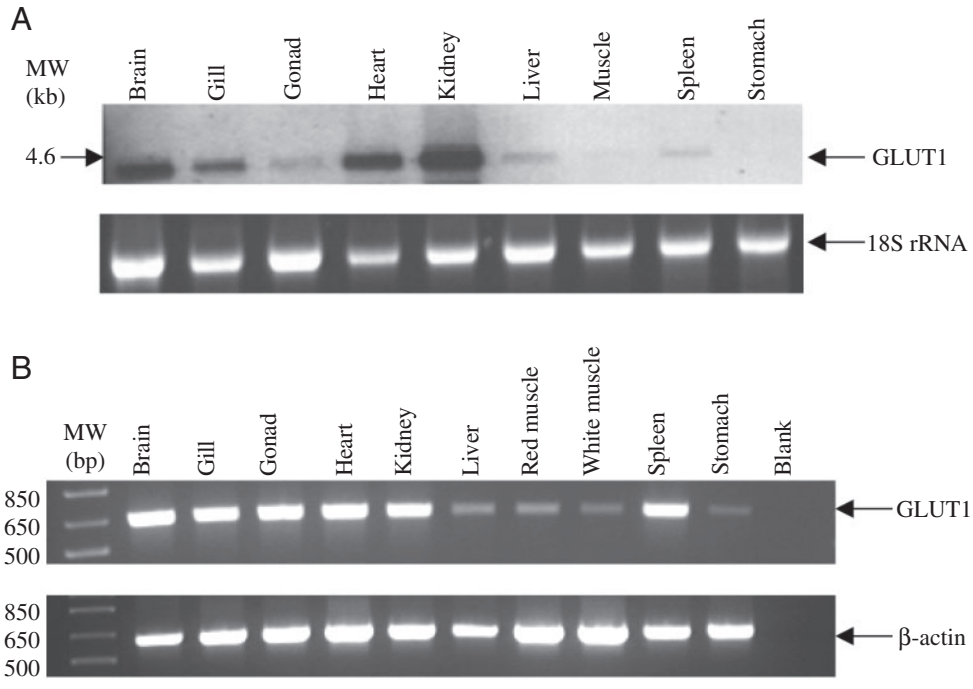

Fig. 4. Tissue distribution of Atlantic cod GLUT1. (A) Northern blot analysis of total RNA from various cod tissues hybridized with a 664 bp antisense RNA probe specific for Atlantic cod GLUT1. Top panel shows the 4560 bp GLUT1 mRNA transcript; bottom panel the 18S rRNA bands, as detected by ethidium bromide staining. MW, RNA marker (kb). (B) RT-PCR analysis. Top panel shows a 642 bp fragment amplified using primers located within the Atlantic cod GLUT1 ORF; bottom panel a 617 bp fragment amplified using cod β-actin-specific primers. MW, DNA marker (bp).

(body mass 298±29 g) was held in 8°C seawater whereas the other (body mass 195±23 g) was allowed to follow ambient seawater temperature. Cod were sampled in early June, when ambient seawater temperature had risen to 4°C from an average temperature of –0.5°C over the winter months.

Fasting

Atlantic cod (body mass 246±16 g) were divided into two groups and held in identical aerated, flow-through seawater 2000 l tanks at 8°C and natural photoperiod. One group was fed a commercial diet (Shur Gain, Truro, NS, Canada) while the other was deprived of food for 2 months, from January to March.

Statistical analysis

Expression levels are presented as mean ± S.E. A two-tailed *t*-test was used to assess if there was any significant difference in GLUT1 expression between experimental groups. A P value less than 0.05 was considered to be significant.

Results

Cloning of cod GLUT1

By combining RT-PCR, RLM-RACE and genome walking, a 4560 bp cDNA for an Atlantic cod glucose transporter was cloned (GenBank accession number AY526497). It contains a 149 bp 5′ UTR, a 1470 bp open reading frame and a 2941 bp 3′ UTR. When compared to human class I GLUTs at the nucleotide level, the cod GLUT ORF had the highest sequence identity to GLUT1 (78.2%), followed by GLUT4 (64.9%), GLUT3 (64.6%) and GLUT2 (53.7%) and is most probably a GLUT1.

Deduced amino acid sequence of cod GLUT1 protein

The 1470 nucleotide cod GLUT1 encodes a 489 aa protein that has a predicted molecular mass of 53.5 kDa and an isoelectric point of 8.97.

Phylogenetic analysis (Fig. 1) was performed to determine the relatedness of Atlantic cod GLUT1 to class I GLUTs (1–4) from other vertebrates, including all those reported in fish. A multiple alignment of 28 GLUT protein sequences was generated using the CLUSTAL W alignment mode in the program AlignX (Informax Inc., Bethesda, MD, USA). The alignment was imported into *MEGA* version 2.1 and a phylogenetic tree was constructed using the neighbor-joining method with Poisson correction. Bootstrap analysis was performed with 1000 replicates. Atlantic cod GLUT1 clusters with GLUT1s from rainbow trout and carp, as well as GLUT1s from other vertebrates. It does not cluster with other class I GLUTs from other fish including GLUT4s from brown trout and coho salmon, GLUT2 from rainbow trout nor with GLUTs from grass carp and pacific hagfish.

At the amino acid level, Atlantic cod GLUT1 had the highest degree of sequence identity to two GLUT1s from rainbow trout and common carp, with 85.2% and 79.6% identity, respectively. Atlantic cod GLUT1 shows about 78% amino acid identity with all of the other vertebrate GLUT1s, including human (Fig. 2). When compared with GLUTs 2–4 from other fish, Atlantic cod GLUT1 shows 61.4%, 60.4%, 61.2%, 55% and 53% identity to GLUT4s from brown trout and coho salmon, pacific hagfish GLUT, grass carp GLUT and rainbow trout GLUT2, respectively.

Glucose transporters have several structural motifs common to all classes, as well as class specific motifs (Joost and Thorens, 2001; Hruz and Mueckler, 2001). All glucose transporters are characterized by the presence of twelve transmembrane helices, seven glycines in the helices (1, 2, 4, 5, 7, 8, 10), two tryptophan residues in helices 6 and 11, two tyrosine residues in helices 4 and 7, as well as, several charged residues on the cytoplasmic surface. Highly conserved residues in all GLUTs include the FGY motif in helix 1, GRK/R motif in intracellular loop 2, glutamate and arginine residues in intracellular loops 4 and 10, the PESPR motif in intracellular loop 6, D/ERAGRR motif in intracellular loop 8 and the PETKG motif after helix 12. Motifs specific for class I glucose transporters include an *N*-glycosylation site in extracellular loop 1, the QL motif in helix 5, the QLS motif in helix 7, the STSIF motif in extracellular loop 7 and the PGPIPW motif in helix 10. As indicated in Fig. 3, the Atlantic cod GLUT1, identified here, is characterized by the presence of all the conserved amino acid sequences for all classes of GLUTs and most specifically the sequences unique to class I GLUTs. The only variation in amino acid sequence is in the PETKGR motif in which the K is substituted by an R. Analysis of the amino acid sequence for Atlantic cod GLUT1 using HMMTOP indicates the presence of 12 transmembrane helices. It should be noted that the boundaries predicted for helix 7 using this algorithm include the STSIF motif, which is a class I-specific feature of extracellular loop 7. It is most probable that this motif is in fact part of extracellular loop 7 and not part of helix 7 as predicted by this program.

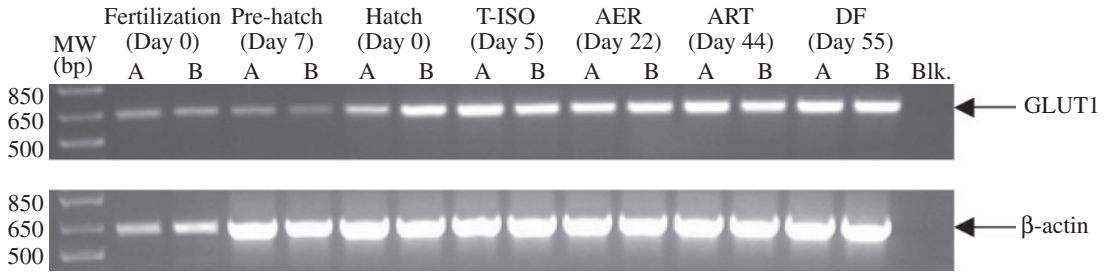

Fig. 5. Developmental expression patterns for Atlantic cod GLUT1. Two sets of Atlantic cod (A,B) were analyzed for GLUT1 expression at various stages of development as indicated, from eggs to small fish, by RT-PCR. GLUT1 is expressed at all developmental stages examined. Letters above age of post-hatch fish refer to food offered: T-ISO, T-ISO enriched rotifers; AER, algamac 2000 enriched rotifers; ART, enriched artemia; DF, dry food (see Materials and methods for details). MW, DNA marker (bp).

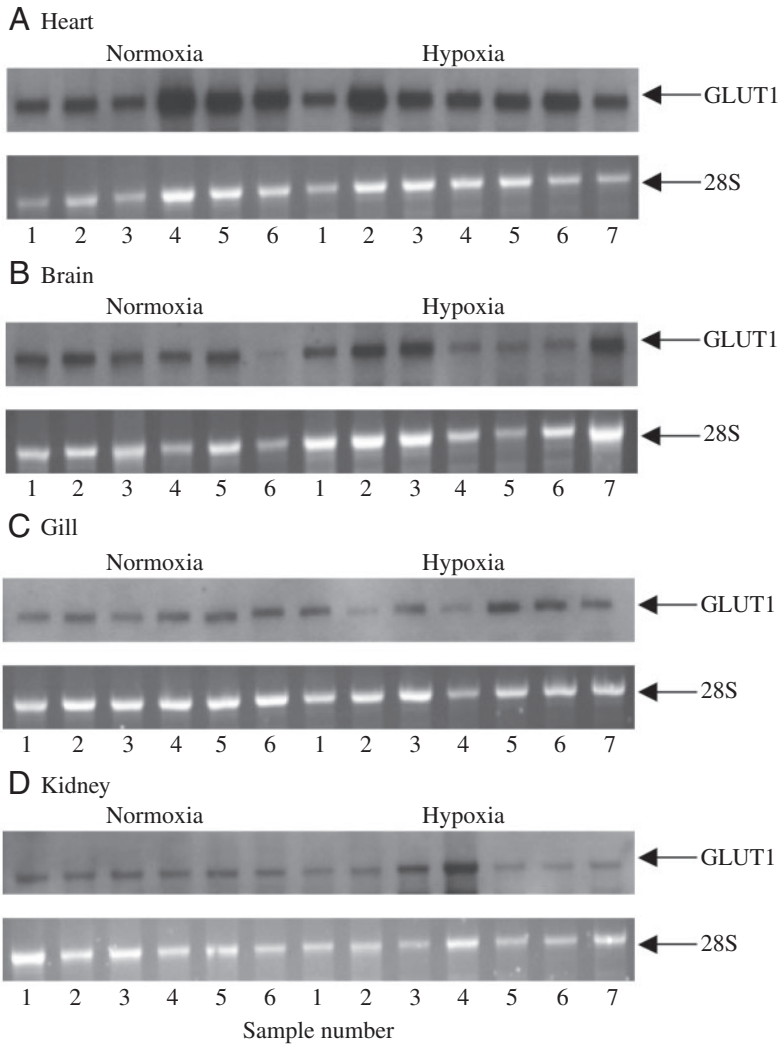

Fig. 6. The effects of hypoxia on GLUT1 expression in heart (A), brain (B), gill (C) and kidney (D). Atlantic cod were exposed to 6 h of normoxia (N=6) or hypoxia (40% DO_2; N=7). GLUT1 expression in tissues that highly express GLUT1 was analyzed by northern blot and normalized to 28S rRNA levels.

Tissue distribution of Atlantic cod GLUT1

Northern blot analysis of adult Atlantic cod tissues indicates that the 4560 bp Atlantic cod GLUT1 transcript is highly expressed in brain, gill, heart and kidney. Lower levels were detected in gonad, liver, spleen, stomach, red muscle and white muscle (Fig. 4A). The tissue expression pattern determined by RT-PCR parallels that observed by northern analysis (Fig. 4B).

Developmental expression patterns of Atlantic cod GLUT1

Two batches of Atlantic cod were monitored from eggs to larvae to determine at which point GLUT1 expression, and hence glucose utilization ability through this transporter, becomes detectable. Samples were collected at seven time points throughout development including day 0 fertilized eggs, day 7 fertilized eggs and day 0 hatched eggs. Thereafter, samples were taken while they were being fed different diets. For each batch, RNA was extracted at each time point and analyzed for GLUT1 expression by RT-PCR. As shown in Fig. 5, GLUT1 is expressed at all developmental stages examined.

GLUT1 expression under hypoxia challenge

Atlantic cod were held at either normoxic (N=6) or hypoxic (40% DO_2; N=7) conditions for about 6 h. Tissues that showed the highest levels of GLUT1 expression (heart, brain, gill and kidney) were analyzed for changes in expression under hypoxic challenge by northern analysis (Fig. 6). GLUT1 expression was measured by densitometry and normalized to 28S rRNA bands. GLUT1/28S rRNA ratios were as follows: heart (normoxic 0.97±0.09; hypoxic 1.01±0.10), brain (normoxic 0.29±0.03; hypoxic 0.25±0.03); gill (normoxic 0.31±0.01; hypoxic 0.36±0.04); kidney (normoxic 0.35±0.05; hypoxic 0.53±0.14). There was no significant difference in GLUT1 expression between normoxic and hypoxic fish in the tissues examined under these conditions.

The effects of temperature on heart GLUT1 expression

Atlantic cod were held at either 8°C (N=10) or allowed to follow ambient seawater temperature (N=10). Fish were sampled when ambient seawater temperature had risen to 4°C after an average temperature of –0.5°C over the winter months. GLUT1 mRNA levels in heart were determined by northern analysis and normalized to 28S rRNA levels (Fig. 7). GLUT1/28S rRNA ratios were 0.51±0.06 in fish held at high temperature and 0.36±0.05 in fish allowed to track lower ambient temperatures. There was a tendency for normalized GLUT1 level to be higher in fish maintained at high temperature than in fish held at low seasonal temperature (P=0.071).

The effects of fasting on heart GLUT1 expression

Atlantic cod that were deprived of food for 2 months (N=10) were compared to fish fed a commercial diet (N=10). GLUT1 expression in heart was determined by Northern analysis (Fig. 8). Level of expression was measured by densitometry and normalized to 28S rRNA bands. GLUT1/28S rRNA ratios were 0.44±0.03 and 0.58±0.08 in fed and fasted fish, respectively. There was a tendency for normalized GLUT1 level to be lower in fed than in fasted fish (P=0.099).

Discussion

On the basis of the deduced amino acid sequence the cDNA that has been cloned is considered to be a member of the class I GLUTs and more specifically a GLUT1 similar to that described for other species.

The presence of GLUT1 mRNA in at least 10 tissue types of Atlantic cod, at albeit differing levels, is similar to the ubiquitous distribution in mammalian and rainbow trout tissues (Behrooz and Ismail-Beigi, 1999; Teerijoki et al., 2000). High expression

in heart of Atlantic cod is consistent with the abundance of this transcript in rainbow trout heart (Teerijoki et al., 2000) and detection of GLUT1 protein in heart of tilapia (Wright et al., 1998). Also, the relatively low level of expression in Atlantic cod skeletal muscle, liver and spleen matches the pattern reported for rainbow trout (Teerijoki et al., 2000). These findings are in line with the inability to detect GLUT1 protein in skeletal muscle of tilapia, rainbow trout, American eel or bullhead catfish using antibodies to mammalian protein (Wright et al., 1998; Legate et al., 2001). Atlantic cod and rainbow trout differ with respect to GLUT1 expression in brain, which is relatively high in Atlantic cod but only weak in rainbow trout. In mammals, GLUT1 is expressed at highest levels in erythrocytes and brain, where it is found predominantly in endothelial and glial cells (Gould and Seatter, 1997; Devaskar et al., 1991). The reason for differences in GLUT1 expression between Atlantic cod and rainbow trout brain is unknown. Finally, we report here for the first time the presence of a high level of GLUT1 in fish gill and kidney.

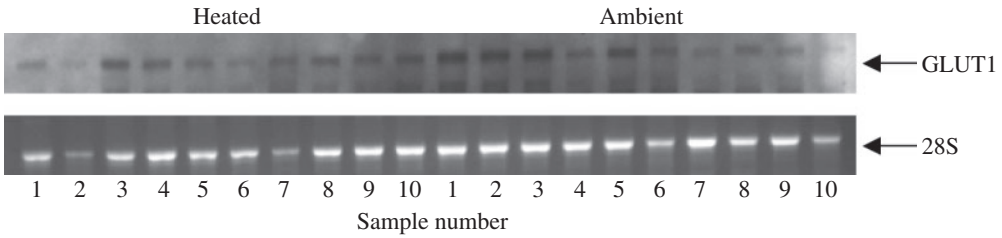

Fig. 7. The effects of temperature on GLUT1 expression in heart. Atlantic cod were either held at 8°C (*N*=10) or allowed to follow ambient seawater temperatures (*N*=10). Fish were sampled in early June when ambient seawater temperature had risen to 4°C after an average temperature of –0.5°C over the winter months. GLUT1 mRNA levels were analyzed by northern blot and normalized to 28S rRNA levels.

The tissue distribution and relative abundance of GLUT1 in Atlantic cod differs from that of other GLUTs described for fish. Planas et al. (2000) and Capilla et al. (2002) described a GLUT4 from brown trout with abundant transcript in skeletal muscle, gill and kidney but poor expression in heart, brain and liver. This GLUT4 occurs in much higher levels than GLUT1 in red and white muscle of rainbow trout and, at least in red muscle, is insulin sensitive (Capilla et al., 2002). Panserat et al. (2001) reported a mammalian-like GLUT2 in rainbow trout that is strongly expressed in kidney and liver but to a lesser extent in heart, brain and muscle. More recently, a GLUT3 was cloned and sequenced from grass carp that is most abundant in kidney with lower levels in heart, brain, gill, liver and muscle (Zhang et al., 2003). The general picture for fish tissue that is emerging is that heart, brain and gill have strong expression of GLUT1, skeletal muscle GLUT4, liver GLUT2, and kidney GLUTs 1, 2, 3 and 4.

During development of the Atlantic cod embryo and early larval stages nutrients from the yolk are mobilized to meet energetic needs. Endogenous carbohydrates constitute less than 1% of the total energetic substances with amino acids and lipids being the primary metabolic fuels (Finn et al., 1995). GLUT1 expression was monitored at various stages of development from fertilization, through hatching into larval fish, to 55 days of age when feeding on dry food with a body mass of about 100 mg. GLUT 1 transcript was detected in Atlantic cod from the very earliest stages of fertilized eggs. This finding is consistent with that of rainbow trout embryos, in which GLUT1 is evident from early gastrulation (Teerijoki et al., 2001a). In Atlantic cod, GLUT1 level became abundant at hatch and continued through to day 55. There is no indication that developmental stage and/or change in diet impact on the level of GLUT1 expression.

There was no difference in GLUT1 mRNA expression in heart, brain, kidney or gill in Atlantic cod exposed to 40% DO_2 for 6 h. This is in contrast to a wide range of mammalian tissues that show increased expression with hypoxia challenge (Behrooz and Ismail-Beigi, 1999). Moreover, Zhang et al. (2003) noted that in grass carp, a treatment of 7% DO_2 for 4 h, resulted in an increase GLUT3 expression in kidney and gill with no change in heart, brain, liver or muscle. It may be that the depth or length of the hypoxic challenge in the current study was not sufficient to induce a change in GLUT1 expression, although the treatment resulted in loss of equilibrium for about one-half of the fish. As such, the possibility that this glucose transporter isoform is not hypoxia sensitive in heart of Atlantic cod must be considered.

In hearts of a number of species there is a greater dependence on fatty acid than carbohydrate metabolism for energy production at low temperature. The corollary to this is that at high temperature, carbohydrates take on a greater relative importance in terms of ATP supply. This contention is based primarily upon performance challenges, enzyme activity levels and radioisotope studies (Driedzic et al., 1996). This is the first study that places a GLUT transporter into this framework. Hearts from Atlantic cod, held at high temperature

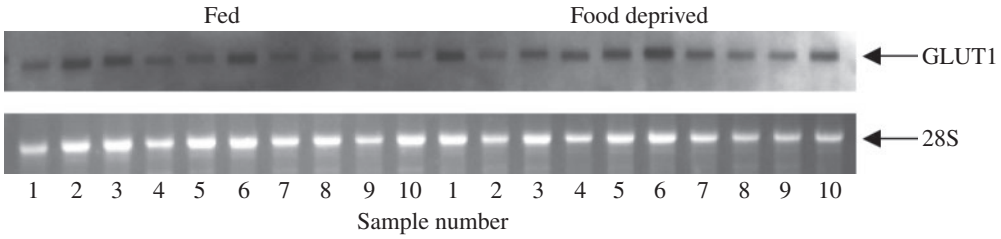

Fig. 8. The effects of food deprivation on GLUT1 expression in heart. Atlantic cod (*N*=10) were food-deprived for 2 months and GLUT1 mRNA levels compared (by northern blot analysis) with that of Atlantic cod (*N*=10) fed a commercial diet over the same time period. GLUT1 levels were normalized to 28S rRNA.

(8°C), had marginally higher levels of normalized GLUT1 transcript than animals acclimatized to winter conditions and sampled in the spring when water temperature reached 4°C (0.51±0.06 *vs* 0.36±0.05; P=0.071). It is possible that fish, at the lowest winter temperature of approximately –1°C, would have even lower levels of GLUT1 expression; however, this remains to be assessed. In relation to this point, however, the data are consistent with a decrease in the relative importance of glucose as a metabolic fuel in fish hearts at low temperature.

There was no significant difference in the level of GLUT1 expression between fed fish and those fasted for a 2-month period. If anything, there is a suggestion of a small increase in GLUT1 level in the starved fish. This finding, in Atlantic cod heart, differs from that of rat heart where GLUT1 mRNA level in 48 h fasted animals is only 14% of the level following a 5 h fast (Kraegen et al., 1993). The relatively constant level of GLUT1 mRNA in Atlantic cod heart during starvation is similar to the situation in red and white skeletal muscle of brown trout, in which a 45 day fast had no impact on GLUT1 expression (Capilla et al., 2002).

In summary, we have sequenced and cloned a putative glucose transporter from Atlantic cod that is homologous to a mammalian and trout GLUT1. This gene product is constitutive, has a wide tissue distribution, and is particularly abundant in heart, brain, gill and kidney. It is expressed upon fertilization and at all developmental stages. Levels of expression are not substantially altered under hypoxia in a number of tissues including heart, or in heart during low temperature challenge or starvation; however, the possibility that more severe hypoxia or larger temperature extremes would result in changes in gene expression cannot be ruled out. Overall, there is a ubiquitous tissue distribution of GLUT1 in Atlantic cod, consistent with other species, and the level of gene expression is relatively constant over a range of physiological conditions.

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