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Evolution of glutamine synthetase in vertebrates: multiple glutamine synthetase genes expressed in rainbow trout (*Oncorhynchus mykiss*)

Brent W. Murray[1,*], Ellen R. Busby[2], Thomas P. Mommsen[2] and Patricia A. Wright[1,†]

[1]*Department of Zoology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada* and [2]*Biochemistry and Microbiology, University of Victoria, PO Box 3055, Victoria, BC V8W 3P6, Canada*

*Present address: Biology Program, College of Science and Management, University of Northern British Columbia, Prince George, BC, Canada V2N 4Z9

†Author for correspondence (e-mail: patwrigh@uoguelph.ca)

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Summary

Glutamine synthetase (GSase) is a key enzyme in nitrogen metabolism and encoded by a single gene in mammals. Using PCR cloning techniques, including RT-PCR from total RNA and PCR from a cDNA library, we find evidence of four expressed GSase mRNAs for the tetraploid rainbow trout. For two of these mRNAs (*Onmy-GS01, -GS02*) we characterize the full-length coding regions, and for two others (*Onmy-GS03*, *-GS04*), we describe partial sequences. Northern analysis of *Onmy-GS01*, *-GS02*, *-GS03* and *-GS04* indicates that (1) *Onmy-GS02* is expressed at higher levels relative to the other transcripts in most adult tissues, with the exception of brain and gill, where *Onmy-GS01* is at the highest level, and (2) the tissue with the highest level of expression of all four transcripts is the brain, with decreasing levels in the intestine, liver, red muscle, gill/kidney, white muscle and heart. Clearly, rainbow trout possess multiple GSase genes with differing levels of tissue expression, implying manifold potential routes of regulation for this octameric enzyme. Our data also indicate that caution should be taken when interpreting mRNA expression data of a single gene, unless multiple genes have been ruled out. Consistent with a southern blot, phylogenetic and intron sequence analyses imply that the trout genes are encoded by at least four separate loci, belonging to two distinct evolutionary branches. Our data on rainbow trout, together with those from two full-length zebrafish *Danio rerio* GSase genes compiled from GenBank ESTs, support the idea that fish GSases are polyphyletic and that gene duplications have occurred at multiple points and in independent lineages throughout the evolution of bony fishes.

Key words: salmonid, L-glutamate:ammonia ligase, tetraploidization, zebrafish, *Takifugu*, brain, intestine, rainbow trout, *Oncorhynchus mykiss*, ammonia, nitrogen, mRNA.

Introduction

Glutamine synthetase [GSase; L-glutamate:ammonia ligase (ADP forming); E.C. 6.3.1.2] catalyses the ATP-dependent formation of glutamine from ammonia and glutamate. Due to its key role in nitrogen metabolism, including nucleotide, amino acid and urea biosynthesis, the enzyme has been ascribed an extraordinarily long evolutionary history (Kumada et al., 1993). Because of this, GSase has been used as a molecular clock (Pesole et al., 1991) to establish phylogenetic relationships between prokaryotes and eukaryotes (Saccone et al., 1995), and as an experimental model for molecular evolution (Kashiwagi et al., 2001) and secondary modifications of an enzyme (Berlett et al., 1996).

The functional enzyme is composed of eight identical subunits, with some microheterogeneity between the subunits, possibly due to post-translational modifications (Smirnov et al., 2000). Mammals appear to possess only a single GSase gene, although pseudogenes for GSase have been noted (Kou and Darnell, 1989; Wang et al., 1996). Elasmobranch fishes and birds also display a single GSase gene, albeit producing different transcripts for mitochondrial and cytosolic isozymes (Laud and Campbell, 1994; Pu and Young, 1989; Campbell and Smith, 1992).

In fish, GSase is a multifunctional enzyme, just as the product glutamine has many different metabolic roles. GSase is critical in the detoxification process of the highly mobile and toxic ammonia (for reviews, see Korsgaard et al., 1995; Ip et al., 2001). Neural tissues are particularly sensitive to ammonia and, not surprisingly, GSase activity is typically high in the brain (Webb and Brown, 1976; Peng et al., 1998; Wang and Walsh, 2000), although liver can also be an important site of ammonia detoxification (Jow et al., 1999; Iwata et al., 2000).

The enzyme is also key to the ‘fish type’ ornithine urea cycle, with glutamine as the N-donor substrate for the initial step catalysed by carbamoyl phosphate synthetase III (CPSase III) (for a review, see Anderson, 2001). Consequently, GSase is usually colocalised with CPSase III in the mitochondria of

fish that have a functional urea cycle (Casey and Anderson, 1982), but in some cases GSase is present in both cytosol and mitochondria (Anderson and Walsh, 1995; Felskie et al., 1998). The regulation of urea synthesis, at least in the facultatively ureogenic marine toadfish *Opsanus beta*, is upstream of the urea cycle, and present attention is focused on GSase. Stimulation of ureagenesis in *O. beta* by confinement or crowding is accompanied by a multifold induction of hepatic (cytosolic) GSase activity (Walsh et al., 1994; Julsrud et al., 1998), mRNA levels and protein concentration (Kong et al., 2000).

Fish GSase sequences have been reported in the marine toadfish *O. beta* (Walsh et al., 1999), the sleeper *Bostrichthys sinensis* (Anderson et al., 2002), and the spiny dogfish shark *Squalus acanthias* (Laud and Campbell, 1994). Additional sequence information on GSase genes from several fish species have been recorded in GenBank. A follow up study in *O. beta* has revealed a second gene, expressed primarily in the gills, which shares relatively low nucleotide and amino acid sequence similarity (approx. 73%) with the original toadfish GSase cDNA from liver (Walsh et al., 2003). GSase genes have not been isolated in any salmonid species. The common ancestor of all salmonids is believed to have undergone a tetraploidization event (duplication of the diploid set of chromosomes) between 25–100 mya (Allendorf and Thorgaard, 1984). In salmonids, therefore, one can expect two genes (i.e. up to four alleles in a single fish at two different chromosome locations or loci) instead of the single gene (i.e. up to two alleles at one locus) found in diploid fish. Studies of GSase gene expression in salmonid species may be complicated if the two genes are differentially expressed.

The objective of this study was to isolate and characterize the GSase gene(s) in the rainbow trout *Oncorhynchus mykiss*. Specifically, we analysed what effects the tetraploidization event in the evolution of salmonid fishes may have exerted on GSase genes and expression. Also, we wondered whether the negligible mitochondrial GSase activity in the trout (Korte et al., 1997) is in any way reflected in the gene structure of trout GSase.

Surprisingly, we obtained multiple, and not the expected two, GSase gene sequences. A phylogeny of bony fish genes was reconstructed, based on a nucleotide alignment of the coding regions. To study allelic relationships, we compared non-coding sequences, while the total number of GSase genes in trout was estimated through Southern blot analysis. Finally, we showed differential expression of multiple GSase genes in tissues of adult rainbow trout.

Materials and methods

Isolation of glutamine synthetase genes

Using combinations of the GSase or GSase and cloning vector (‘anchor’) primers (Table 1), the polymerase chain reaction (PCR) was used to amplify GSase genes from a primary rainbow trout *Oncorhynchus mykiss* Walbaum cDNA library constructed from mRNA isolated from the combined gill and kidney tissues of 12 trout [1×10^9 plaque forming units (p.f.i.) ml^{-1}; S. F. Perry, personal communication]. PCR reactions were conducted on a PTC-150 MiniCycler™ thermal cycler (MJ Research Inc., Incline Village NV, USA). The thermal profile consisted of an initial cycle of 95°C for 5 min, 55–60°C for 30 s and 72°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55–60°C for 30 s, 72°C for 1.5 min, and finished with an elongation of the 72°C step for 7.5 min. Reaction volumes were 25 µl, consisting of 1.5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} dNTPs, 1× Reaction buffer, 0.2 µmol l^{-1} of each primer, and 1 unit of HotStar Taq polymerase (Qiagen, Mississauga, ON, Canada). PCR products were excised from agarose gels and isolated using the QIAquick gel extraction kit (Qiagen) and ligated into the pGEM-T easy cloning vector (Promega – Fisher Sci., Nepean, ON, Canada). Plasmid DNA was isolated using the QIAprep spin miniprep kit (Qiagen).

Alternatively, we used GSase-specific primers designed from the toadfish liver GSase gene (Walsh et al., 1999) in reverse transcription-mediated PCR (RT-PCR) on total RNA isolated from a pooled sample of six rainbow trout alevin. Following total RNA isolation by standard methods, RT-PCR was performed using the Superscript II RT and 0.6 U Taq polymerase (Invitrogen/Gibco-BRL, Burlington, ON, Canada). PCR reactions were performed on a DNA Engine™ thermal cycler (MJ Research Inc.) with the following cycles. One initial incubation at 94°C for 3.5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and completed by a final elongation at 72°C for 15 min. To obtain 3′ untranslated regions (UTR) information, we used a 3′ RACE-like (rapid amplification of cDNA ends) system. Gene specific primers, oligo(dT)-containing anchor primer, and a universal amplification anchor primer (AlphaDNA, Montreal, Quebec, Canada), were used in a RT-PCR protocol, which was similar to that mentioned above. Gel bands were eluted by freeze–thaw cycles and ligated into pCR2.1 TOPO TA cloning vector (Invitrogen/Gibco-BRL). Cloned products were sequenced by Central Sequencing facilities (University of Guelph; Centre for Environmental Health, University of Victoria) and processed on ABI DNA Sequencers, model 377 (Applied Biosystems, Foster City, CA, USA).

Analysis of expressed sequence tag and pufferfish genomic databases

The GenBank expressed sequence tag (EST) (www.ncbi.nlm.nih.gov) and the DOE Joint Genome Institute – Fugu Genome (www.jgi.doe.gov/fugu) databases were searched for sequences with similarity to the deduced amino acid sequence of an identified trout GSase gene (*Onmy-GS01*, accession no. AF390021) through tBlastn searches. All EST sequences were downloaded and analysed as described below to establish sets of contiguous sequence information.

Sequence analysis

DNA sequence was edited and contiguous sequences were assembled using the Sequencher program (GeneCodes Corp.,

Table 1. *PCR primers used to amplify glutamine synthetase genes*

Primer	DNA sequence (5′–3′)	Location[1]	Orientation
GLUL*e3f1	GAR GGC TCC AAC AGY GAC ATG TA	78	F
GLUL*e4r1	TAC TCC TGY TCC ATK CCA AAC CA	132	R
GLUL*e5f1	GTG GAG GCN CAT TAY AGA GCC TG	185	F
GLUL*e6r1	TTG GTG CTR AAG TTT GTR TGG CA	254	R
GLUL-Onmy*e4f1	AAG AAG ATC ATG GAG ATG GTA	126	F
GLUL-Onmy*e4f2	GGT TTG GCA TGG AGC AAG AG	139	F
GLUL-Onmy*e5r1	TAG TGG GCT TCT ACG ATG TC	176	R
GLUL-Onmy*e5r2	CAT TGG TGC CAC AGA TCA TG	190	R
GLUL-Onmy*e6f1	GGT CCT TGT GAA GGC ATC AC	215	F
GLUL-Onmy*e6f2	CCA TAC AAA CTT CAG CAC CAA G	261	F
GLUL-Onmy*e7f1	CAC CAC GAA ACR TCC AAC AT	311	F
GLUL-Onmy*e7f2	AGG CTG GGG AGG AGG CAT A	284	F
GLUL-Onmy*e7f2	AGG CTG GGG AGG AGG CAT A	284	F
GLUL-Onmy*3UR1	TGA GAT GTA GCG CAA TAG GAC	38 bp[2]	R
GLUL-Onmy*3UR3	CAG TGG ATC TCG TTG GGT CTA	374	R
GS301	CAG CCA GCA CCG TTC CAG TT	248	R
GS501	GAG GCA TCC AAC AGC GAC ATG T	78	F
GS031I	AAC TGG AAC GGT GCT GGC TG	254	F
RTGS51	TGG TTC GGC ATG GAR CAR GAR TA	139	F
pBKCMV*r1	GCG AAT TGG GTA CAC TTA CCT G	na	na
pBKCMV*f1	CTA AAG GGA ACA AAA GCT GGA GC	na	na
M13r	TCA CAC AGG AAA CAG CTA TGA CC	na	na
T7	TAA TAC GAC TCA CTA TAG GGC	na	na

[1]Location of the 3′ end of primer relative to amino acid position (Fig. 2).
[2]Position given in base pairs (bp) from stop codon relative to 3′ end of primer.
na, not applicable.

Ann Arbor, MI, USA) (contiguous sequences or ‘contigs’ are overlapping segments of DNA). Alignments were constructed using the program MacVector 7.0 (Oxford Molecular Group). Gene phylogenies were estimated from a DNA alignment of the coding sequence using the maximum-likelihood method of the computer programs Paup 4.0b8a (Sinauer Associates Inc., Sunderland, MA, USA). 200 bootstrap trees were constructed to estimate confidence in the branch topology. Intron 4, 3′-UTR and pairwise dot-plot analyses were conducted on the computer program BioEdit (Hall, 2001).

Southern blot

10 μg of total genomic DNA was digested to completion with either the *Alu*I or *Hin*fI restriction enzymes. The resulting fragments were size separated on a 1% agarose gel and blotted onto Hybond+ membrane using a Pharmacia vacuum blotter (Amersham-Pharmacia, Baie d’Urfé, PQ, Canada). Two GSase probes were produced by amplification from cloned fragments of the *Onmy-GS01* (CA0509) and *Onmy-GS02* (CA0508) genes with the primers GLUL-Onmy*e4f1/GLUL-Onmy*3UR1 and GLUL*e6f2/GLUL-Onmy*3UR3, respectively. These products were isolated from the agarose gel as above and labelled with ^{32}P-dCTP (Amersham-Pharmacia) using a random priming kit (Invitrogen/Gibco-BRL). Southern blots were prehybridised and hybridised separately overnight at 62°C in 40 ml of Westneat solution (7% SDS, 1 mmol l^{-1} EDTA, 1% BSA, 0.25 mol l^{-1} Na_2HPO_4). Membranes were washed twice for 10 min at room temperature and once at 62°C with a 2× SSC, 0.1% SDS solution, followed by a final wash at 62°C for 10–20 min with a 0.5× SSC, 0.1% SDS solution. Hybridisation signals were detected on a Molecular Dynamics Phosphor-Imager (Amersham-Pharmacia).

Northern analysis

To study the distribution of glutamine synthetase mRNA in trout tissue, total RNA was isolated from *O. mykiss* kidney, heart, liver, gill, intestine, white muscle, red muscle and brain by the guanidine isothyocyanate method using Trizol (Invitrogen/Gibco-BRL). Total RNA (10 μg/lane) was separated in a 1.5% agarose gel in the presence of 1 mol l^{-1} formaldehyde, and transferred to nylon membranes (Hybond-N, Amersham). The membranes were initially probed using ^{32}P-labeled random-primed cDNA probes for two glutamine synthetase mRNAs: *Onmy-GS01* (198 bp) and *Onmy-GS04* (180 bp), and hybridized at 65°C. Final washing was in 40 mmol l^{-1} Na_2HPO_4, pH 7.2, 1% SDS, 1 mmol l^{-1} EDTA at 65°C. The membranes were then stripped using 0.1× Denhardt’s solution, 5 mmol l^{-1} Tris-HCl, pH 8.0, and 2 mmol l^{-1} EDTA at 65°C for 1 h and reprobed with the other two glutamine synthetase mRNAs: *Onmy-GS02* (208 bp) and *Onmy-GS03* (162 bp). The membrane was once again stripped, and finally probed with a trout β-actin gene (283bp).

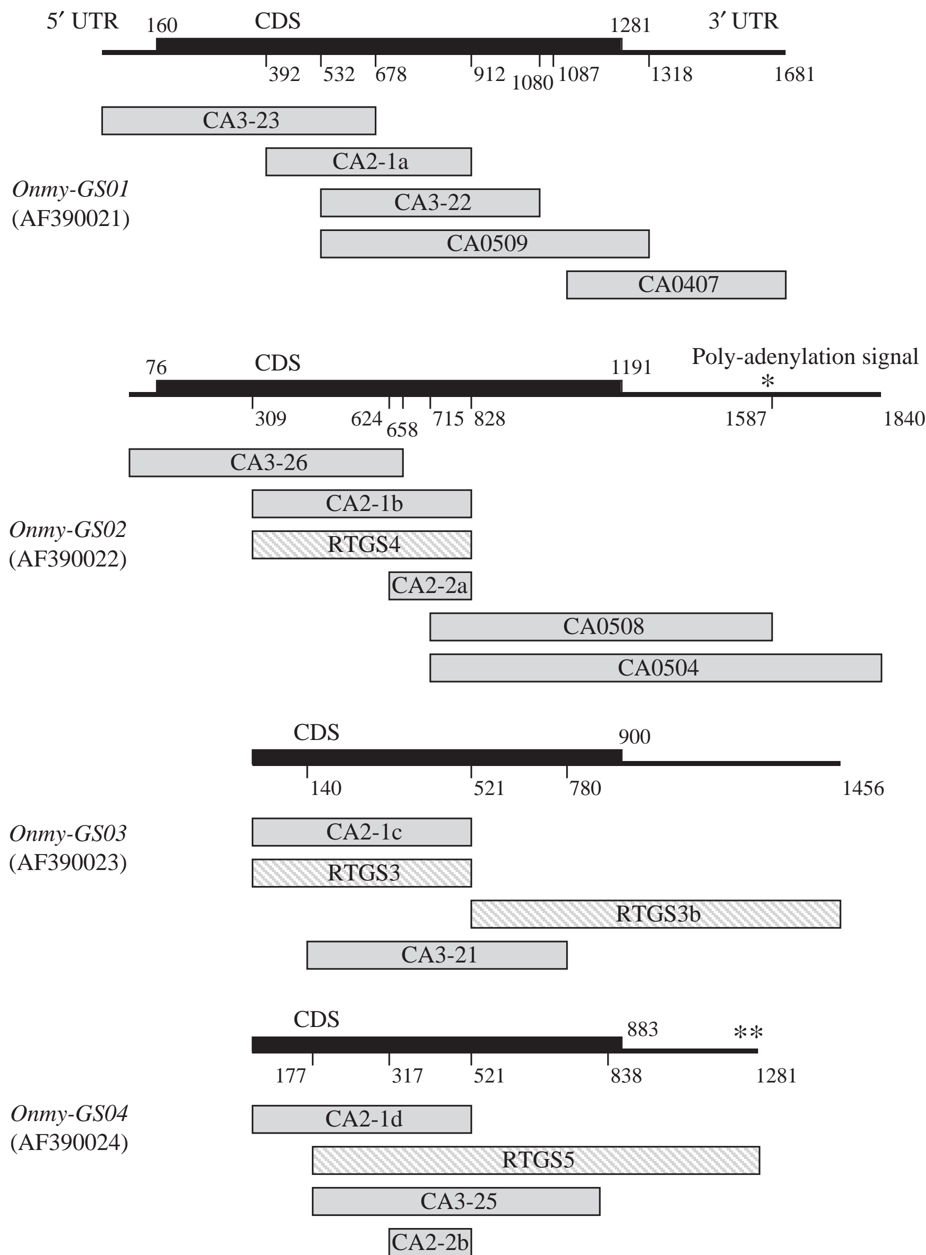

Fig. 1. Rainbow trout (*Oncorhynchus mykiss, Onmy*) glutamine synthetase cDNA sequences. A summary of the sequence information for each of the four contiguous sequences, *Onmy-GS01* – *Onmy-GS04*, is shown in black above the supporting clones. Corresponding GenBank accession numbers are shown in parentheses. Coding sequence (CDS) information is indicated by a black rectangle, while the 5′ and 3′ untranslated regions (UTR) are indicated by a thick line. Positions of the start and stop codons and the location of the supporting clones are shown in base pairs. Asterisks indicate possible polyadenylation signal. Supporting clones are shown as either grey or hatched rectangles, indicating clones isolated from the primary cDNA library (S. F. Perry, personal communication) or an alevin RNA preparation, respectively.

Results

Rainbow trout GSase sequences

We found evidence for four GSase sequences through the PCR and RT-PCR amplification of 19 sequence fragments in the analysis of a cDNA library and a total RNA preparation (Fig. 1, Table 2). Two full-length contiguous sequences were determined, *Onmy-GS01* and *Onmy-GS02* (accession nos. AF390021 and AF390022, respectively). Both contain a complete coding sequence (CDS) region and 5′ and 3′-UTRs. For *Onmy-GS03* and *GS04* we obtained sequences for the 3′-UTRs (accession nos. AF390023 and AF390024, respectively). The DNA sequences for the four 3′-UTRs were used to query the GenBank DNA database. No significant matches were found. Poly-adenylation signals were found in the *Onmy-GS02* and *Onmy-GS04* 3′-UTRs. The deduced amino acid sequences of these sequences are shown in Fig. 2.

Analysis of sequences databases

To compare a wider range of GSase cDNAs among fish species, we searched the DOE Joint Genome Institute – Fugu Genome and GenBank EST databases for entries with significant similarity to the *Onmy-GS01* amino acid sequence. Two putative genes were found in the Fugu Genomic databank, JGI Genscan Gene Model nos. 15768 and 18692. The predicted amino acid sequences are shown in Fig. 2. Of these two genes, JGI15768, lacks exon 2 and possesses its first splice site 3 codons downstream from the exon 2/3 border, predicted by comparison to the other fugu GSase-like genes (JGI-18692) and other vertebrate GSase genomic organisations (van de Zande et al., 1990; Pu and Young, 1989; Kou and Darnell, 1989).

103 eligible zebrafish (*Danio rerio, Dare*) entries were identified during a tBlastn search using the NCBI web interface (February, 2002). Two different contiguous sequences, supported by multiple entries (Fig. 2), were compiled from these EST entries. For both sequences, *Dare-GS01*, the consensus sequence of 22 entries, and *Dare-GS02*, the consensus sequence of 73 entries, a complete CDS region was determined. These sequences were reported in the Third Party Annotation GenBank database, accession numbers BK000047 and BK000048, respectively. The quality of eight remaining sequences was deemed too low to be used in the third party

Table 2. *Primers used to amplify the glutamine synthetase gene fragments*

PCR product(s)	Forward primer	Reverse primer
CA2-1 (a–d)	GLUL*e3f1	GLUL*e6r1
CA2-2 (a, b)	GLUL*e5f1	GLUL*e6r1
CA3-21	GLUL-Onmy*e4f1	T7
CA3-22	GLUL-Onmy*e4f1	M13
CA3-23	GLUL-Onmy*e5r1	T7
CA3-25	GLUL-Onmy*e4f2	T7
CA3-26	GLUL-Onmy*e5r2	M13
CA0407	GLUL-Onmy*e7f1	M13
CA0504	GLUL-Onmy*e6f1	pBKCMV*r1
CA0508	GLUL-Onmy*e6f1	pBKCMV*f1
CA0509	GLUL-Onmy*e41	GLUL-Onmy*3UR1
RTGS3, 4	GS501	GS301
RTGS3b	GS031i	3′RACE UAP
RTGS5	RTGS51	3′RACE UAP

consensus of the zebrafish genes. No evidence for additional GSase genes was noted in these entries.

A similar analysis was conducted on the portion of the EST database (March, 2001) containing entries for the tetraploid amphibian *Xenopus laevis* (*Xela*). The tBlastn search produced 107 EST entries with significant similarity to the *Onmy-GS01* peptide sequence. 44 of these entries matched the previously reported *Xenopus* GSase gene (accession no. d50062). Evidence for at least two additional sequences was found (Fig. 2). A second full-length CDS sequence (*Xela-GS03,* accession no. BK000049) was identified, based on the consensus of 44 EST entries. A partial sequence based on two entries, *Xela-GS02* (accession no. BK000050), was also noted. The relationship of *Xela-GS02* among the vertebrate GSase sequences based on this partial CDS (<1/2 of CDS) is not well supported in phylogenetic analyses (not shown) and further sequence information will be required prior to phylogenetic inference.

Sequence comparison

In the four fish cDNAs for which 5′ UTR information was determined or deduced, *Omny-GS01*, *Onmy-GS02, Dare-GS01* and *Dare-GS02*, a common initiation codon (Met) is present (position 1, Fig. 2). Upstream of this position no alternative Met codon is encountered prior to the first in-frame stop codon.

Using the *Salmonella typhimurium* GSase X-ray crystallography structure (Gill and Eisenberg, 2001) and a pairwise alignment with vertebrate GSases (not shown), we can tentatively determine the homologous active site residues in trout GSase. 15 of the 16 residues identified in *Salmonella* are identified, bolded and marked in Fig. 2, and show complete conservation within the bony fishes. Between *Salmonella* and trout, only two of 15 residues are substituted (positions 194 and 246), one of these maintaining the physicochemical nature of the residue (246: fish, N; *Salmonella*, H). An examination of vertebrate sequences at the active site positions (Fig. 2) indicates a high degree of conservation, with sequence variation found at only three positions.

Phylogenetic analysis

A maximum likelihood phenogram, based on the alignment of CDS nucleotides (positions 1–371 in Fig. 2), was constructed (Fig. 3). This phylogeny shows strong bootstrap support (>70%) for four evolutionary branches or clades, one containing all mammalian and avian genes, a second containing all teleostean genes, a third containing *Xenopus* and a single fugu sequence, JGI-15768, and a fourth containing the shark sequences. The relationship among these four clades is not as well supported (62%). Among the bony fish genes, the present phylogeny suggests two rainbow trout lineages, an *Onmy-GS01/03* and an *Onmy-GS02/04* clade. These clades are highly divergent and do not form a monophyletic clade (i.e. share a common ancestor prior to sharing an ancestor with genes from an other species), suggesting that most of the variation arose prior to speciation.

Estimating the number of GSase genes

The amino acid sequence similarities of *Onmy-GS01* to *Onmy-GS03* and *Onmy-GS02* to *Onmy-GS04* could represent allelic diversity. Alternatively, their similarities may reflect a relatively recent duplication event. To investigate these hypotheses, we conducted an analysis of the variation in intron 4. Primer pairs GLUL-Onmy*e4f1/e5r1 or GLUL-Onmy*e4f2/e5r2 were used to amplify the fourth intron of either *Onmy-GS01/GS03* or *Onmy-GS02/GS04* cDNAs, respectively. These primer pairs designed for parts of exons 4 and 5, flanking intron 4, were used to identify four unique intron sequences from a single fish. The intron sequences were 146, 256, 120 and 113 bp for *Onmy-GS01–04*, respectively. In all cases, the locations of the intron splice boundaries were identical to the locations identified in the rodent, chicken (van de Zande et al., 1990; Pu and Young, 1989; Kou and Darnell, 1989) and fugu (JGI-18692) GSase genes. Reflecting the maximum-likelihood phylogeny, a dot-plot analysis shows a slightly higher level of similarity between the *Onmy-GS01/GS03* cDNAs sequences and also between the *Onmy-GS02/GS04* sequences (Fig. 4). As expected, this similarity is most noticeable around the splice boundaries. For most of the remaining intron sequence no significant similarity exists, arguing against an allelic relationship between the sequence pairs. The analysis of the 3′-UTR sequences agrees with the intron analysis. No significant similarity is found between the 3′-UTR sequences of the two clades (i.e. *Onmy-GS01* or *GS03* compared with *Omny-GS02* or *GS04*). Separate sequence alignments could be made between the *Onmy-GS01* and *GS03* or the *Onmy-GS02* and *GS04* 3′-UTRs (not shown). In both cases, a large number of substitutions and insertions or deletions (indels) were present; *GS01/GS03*: 13 indels (involving 52 bp), 86% similar (324/377 bp, excluding indels); *GS02/GS04*: 14 indels (involving 62 bp), 84% similar (316/376 bp). Consequently, we must conclude that the similarity within these gene sequence pairs reflects a recent gene duplication event.

To estimate the number of GSase genes present in trout, a Southern blot was hybridised concurrently with two probes

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                                                             .1                  .20                 .40                 60.  , ,,
 CONSENSUS ==>                                               MATSASSHLSKGIKQMYMSLPQGEKVQAMYIWIDGTGEGLRCKTRTLDSEPKSIEELPEWNFDGSSTYQ
Onmy-GS01                                                 !K ----E-AS---TV--H--D-S--D----------------------------N-DD-------------
Onmy-GS02                                  !ILFKISRISRCFITNK --M---AE---AV--Q--E----D---V--V--------------------------------------
Dare-GS01                             !VDNPLLFYTFYFLLVSRCFIVIK -------Q---VV--Q--E----DQ-----------------------------D--------------
Dare-GS02                                           !YSTTSTT -------K---AM--Q--D--------V--------------------------V---------------
Fugu-JGL18692                                                ------AT---AV--Q--N---D-------------------------Y---V-D--------------
Fugu-JGL15768                                                MASA-L--Q-N-AVR-Q-------NTCMVT-V----------S-----YE---*****************
Opbe-af118103                       MRVRVGRLFLQLPRLSRCVTAAK  ------AS---AV-KH--G----D------------------------------D--------------
Bosi-ay071837                                                -------S---AV--Q--E----D------------------------------D--------------
Bosi-ay071838                                                -------S---AV--Q--E----D------------------------------D--------------
Xela-GS02                     AIELNPSTQQPSALPASSPPSSCCPATPV  ------AQ---A-----LE----D---------------------------T--D-----------H-
Xela-GS03                           VNNLLRDSGPVLLSLLIRSAAT   -SV-H--R-N--VREQ--K----GMA-VT-V--------V--------Q---T-D-I-------------
Xela-d50062                                                  -SV-H--R-N--VREQ--K--------VT-V--------V--------Q---T-D-I-----------H-
Rano-m91652                                                  ---------N--------N------I-L----V---------------CD--CV------------F-
Mumu-x16314                                                  ---------N----------------------V---------------C---CV------------F-
Acca-af110381                                                ---------N----------------------V---------------C---CV------------F-
Crgr-af150961                                                ---------N----------------------V---------------C---CV------------F-
Susc-z29636                                                  ---------N-----V--------------------------------C-----------------F-
Hosa-y00387                                                  -T-------N-----V--------------------------------CV----------------L-
Gaga-m29076                                                  -----------A--H---K-------------------H--------H----L-D-----------F-
Hefr-af118104                                                                  ...--DG--------------AV----K---K---N-TD-------------
Sqac-u04617                   MRICRSFLFLVKKCGNITPTIWRNQHTYK  ------AN---IV-KN--E---DG--------------AV--------N-----A--------------

                    .80                 .100                .120                .140                .,
 CONSENSUS ==>  SEGSNSDMYLIPAAMFRDPFRKDPNKLVLCEVLKYNRKPAETNLRHTCKKIMDMVSNQHPWFGMEQEYTLLGTDGHPFGWPSNGFPGPQGPYYCGVGADKAYGR
Onmy-GS01      --------------------------------------T-----LM-----E--E--V----------I-------------------------------------
Onmy-GS03          ...--------------------Q-------------RM-----E--E--V----------I-------------------------------------
Onmy-GS02      ------------S----------------------H--------L----V--L-E--I----------I-----------N-------------S------
Onmy-GS04          ...---S-------------------------------L--N-V----E--V----------I-----------N-------------S------
Dare-GS01      A-------------------------------V-----T---H--------E--GH-S----------I-------------------------------------
Dare-GS02      A---------F-Q-----------------D-----H-------QS-------Q-----------------------------------------------
Fugu-JGL18692  A-------------------------------------G--T-----I----M-E--KD----------I-------------------------------
Fugu-JGL15768  *************************************T---N-H-NS---V--K-ADS-I----------F-M-----S--K--Y-A-----------NNVF--
Opbe-af118103  A---------V---------E----------------S-S---LN-N-V-N--KD-----------I-------------------------------
Bosi-ay071837  A-D--------------------------------------I----L----AD-----------I-------------------------------
Bosi-ay071838  A----------------------------------------I----L----AD-----------I-------------------------------
Xela-GS02      ------------V--------R----------------T---------NQ-----G-E--------------M----?-L----?-R-------------N--...
Xela-GS03      A---------V-VQ------CL------M---------S-------------E--GDHR--------------IN---Y---E-----------------V---
Xela-d50062    A-----------VQ------CL------M---------S-------------E--NDHR--------------IN---Y---E-----------------V---
Rano-m91652    ----------H-V--------R------F---F-------------S--R------S-R-----------M-----------------------------
Mumu-x16314    ----------H-V--------R----------F-------------I--R-----------------------M-----------------------------
Acca-af110381  ----------S-V---------E-----F---F-------------S--R-----------------------M-----------------------------
Crgr-af150961  --S-------S-V---------E-----F---F---Q-------------R------------------------------D-------L-----------R-
Susc-z29636    ----------V-----------------F---F-----------------R-----------------------M-----------------------------
Hosa-y00387    ----------V---------------------F----R------------R-----------------------M----------------------R----
Gaga-m29076    A---------R---------------------F----QS-D-------RR--------------------------------C-------------------
Hefr-af118104  ------------S--------------I----F-----------NS-Q-V-S--AGE-----------------------------------------------
Sqac-u04617    ----------V-S--------R------------------S----S-Q---S-IA-EY------------------------C-------------------

                   .180          , ,200.  ,                  .220                .240  ,     , ,      .260
 CONSENSUS ==>  DIVEAHYRACLYAGVKICGTNAEVMPAQWEFQVGPCEGINMGDHLWVARFILHRVCEDFGVVASFDPKPIPGNWNGAGCHTNFSTKAMREEGGLKAIEEAIEKLS
Onmy-GS01      ---------------------------------------------A---------------------------------------E---D---RG--DS----G
Onmy-GS03      ---------------Q-----------------------------A---------------------------------------E---D---R----S----G
Onmy-GS02      ---------------M---------------------T------------------------------------------------E------------S--R-G

Onmy-GS04      -------K-------M---------------------S------A-----------------------------------------E------------S--R-G
Dare-GS01      ---------------M---------------------D------------------------------------------------E---D----C---C----G
Dare-GS02      -V-----------------------------------E---------L---------------------------------------E--------H---S--R-G
Fugu-JGL18692  -V-------------Q---------------------S---------L------------------S--------------------E---D---T---DS----G
Fugu-JGL15768  ----C--K----------------S---------D-A----I--YL---------II--L----ME-----------V--LQ----D---Y--Q------
Opbe-af118103  ---------------Q----------------------------F-A-----------L------------------------------E---D-------D----G
Bosi-ay071837  ---------------Q-----------------------------A----------------------------------------E------------S--R-G
Bosi-ay071838  ---------------Q-----------------------------A----------------------------------------E------------S--R-G
Xela-GS03      -------K------I---------S-----------D------M---------------TL----MT--------Y--ES--V-----H--D-----G
Xela-d50062    -V--S--K------I---------S-----------D------M---------------TL----MT--------Y--ES--V-----H--D-----G
Rano-m91652    --------------I--T-------------I------R--------------------I-T----------------------------N--RC-----D---
Mumu-x16314    -----------------T-------------I------R------I-------------I-T-----------V------------------N---C-----D---
Acca-af110381  -----------------T-------------I------R--------------------I-T------------------------------N---Y-----D---
Crgr-af150961  --M--------------T--Y---KH-----I------R-----------------K----I-T--S------------------T----N---H-K-------
Susc-z29636    --------------I--G-------------I------D--------------------I-T------------------------------N---Y---------
Hosa-y00387    -----------------A-------------I------S--------------------I-T------------------------------N---Y---------
Gaga-m29076    -----------------G--------------------E------I-------------IV--------------------------N---D----H---------
Hefr-af118104  --------------IHLS-------AS----Y--------KV-----IS-------------II-----------------------------S--------Y--DS----G
Sqac-u04617    --------------IELS-------A-----Y--------Q------IS-------------II---------------------------DD----Y--DS----G

                 .280                 ,.300 ,                ,.320,                ,340                     .360                .380
 CONSENSUS ==>  KRHQYHIRAYDPKGGLDNARRLTGFHETSNINEFSAGVANRGASIRIPRTVGQEKKGYFEDRRPSANCDPYAVTEALVRTCLLNETGDEPFQYKNGAGFSRAIGM
Onmy-GS01      R--R------------------H------H-----------------S---D--------------------------I-----S-E-E--KE-SK!
Onmy-GS03      ---R---C--------------H------H-----------------S---D-------------------------MI-----S-V-E--TE-SK!
Onmy-GS02      R--S------------------H------H------------------------------------------------I-----S-E----VD-!
Onmy-GS04      ---S------------------H------H-----------------------------------------------II-----S-E----VN-!
Dare-GS01      ---N----T-------------H------H-----------------A----M------------------------I-----D-E---TVD-!
Dare-GS02      ----------------------H-------------------------S-A---------------------------I-------E--V-EK-M!
Fugu-JGL18692  ---S------------------R---------------------------------------------G-------------E----AD-!
Fugu-JGL15768  TK-SE-------HE-N--K-----R---S-ED---------S---------Q-A--M--------------C--M-IAK----SSE-KTTD-S!
Opbe-af118103  ---H------------------H------H-----------------S---------------------G-----I-----S-E----LA-!
Bosi-ay071837  ---R------------------H------------------------S-------------------V-----G-----I-----G-E----TD-!
Bosi-ay071838  ---R------------------H------------------------S-------------------V-----G-----I-----G-E----TD-!
Xela-GS03      ---D---CV---R--K--S-----Q----S-H------------------Q----GY-----------A---------------TI-----S-TKD-!
Xela-d50062    ---D---CV---R--K--S-----Q----S-H------------------Q----GY-----------A---------------TI-----S-TKD------------
Rano-m91652    ------------------------------D--------S----------I----------------------------I-----------------!
Mumu-x16314    ----------------------A--------D------------------------------------LR-----------I-----------------!
Acca-af110381  -------------------------------D----------------------R-------------------------I----------N-------!
Crgr-af150961  ---R-----------------------K-----D-------D-S------------------A-C-------F-----I-----------Q------!
Susc-z29636    -------------------T---------------D---------------------G--------------------F-----I-----------------!
Hosa-y00387    -------------------------------D--G-----S------------------------------FS-----I-----------------!
Gaga-m29076    -----------------------------S-H-------------------N--H---------G-----------------------------E---!
Hefr-af118104  ----------------------H-------------------------S---D--------------------------D-S--K-!
Sqac-u04617    ----------------------A---H-------------------------S---D--------------------------D-S--K-IE-NKN!

                     .392
Xela-d50062    ASPRDAAVF!
```

Fig. 2. Alignment of vertebrate glutamine synthetase genes. Identity to the consensus sequence is shown with a dash, while an insertion/deletion is shown with an asterisk and a stop codon with an exclamation mark. Tentative active site residues of the glutamate binding site based on the residues identified in *Salmonella typhimurium* (Gill and Eisenberg 2001) are shown in bold, underlined and indicated with a check mark ′. The location of intron 4 is indicated by a downward arrow (⇓). Three periods (...) indicate incomplete or missing sequence information. The sequences are identified with a four-letter code based on their species name and followed by either their unique GenBank accession number, Joint Genome Institute genescan model number or, for sequences reported here, a unique gene indicator, i.e. GS01. Species included are *Oncorhynchus mykiss*, *Onmy*; *Danio rerio*, *Dare*; *Opsanus beta*, *Opbe*; *Bostrichthys sinensis*, *Bosi*; *Takifugu rubripes*, *Fugu*; *Xenopus laevis*, *Xela*; *Cricetulus griseus*, *Crgr*; *Acomys cahirinus*, *Acca*; *Rattus norvegicus*, *Rano*; *Mus musculus*, *Mumu*; *Sus scrofa*, *Susc*; *Homo sapiens*, *Hosa*; *Gallus gallus*, *Gaga*; *Heterodontus francisci*, *Hefr* and *Squalus acanthias*, *Sqac.*

made up of the *Onmy-GS01* and *Onmy-GS02* genes, respectively. In a single individual, 5–7 bands are visible in *Hin*fI and *Alu*I digests, respectively (not shown). *Hin*fI and *Alu*I restriction sites are present within the four sequence regions complementary to the *Onmy-GS01/02* probes. The observed number of fragments is consistent with the expected number of fragments that should be detectable (i.e. >500 bp) upon digestion of genomic DNA with *Hin*fi and *Alu*I, assuming all four sequences are present and an average intron size of 200 bp. Exact fragment expectations cannot be made without complete intron sequence information.

Northern analysis

The expression of the four GSase mRNAs in trout was studied by northern analysis. Within each tissue, the intensity of the GS signal was compared to that of β-actin. *Onmy-GS02* was expressed at higher levels than *Onmy-GS01, GS03* and *GS04* in most tissues (Fig. 5). In the brain and gill, however, *Onmy-GS01* was expressed at the highest level relative to the other three transcripts. When comparing between tissues, expression relative to β-actin is not appropriate because β-actin levels vary in different tissues (Foss et al., 1998). The amount of total RNA loaded appeared very consistent between lanes. For each individual transcript, the order of the highest to the lowest level of mRNA was: *Onmy-GS01*: brain, intestine, gill, liver, kidney/red muscle, white muscle, heart; *Onmy-GS02*: brain > intestine > liver > red muscle > kidney > white muscle > gill > heart; *Onmy-GS03*: brain > intestine > liver > red muscle > kidney > white muscle > gill > heart; *Onmy-GS04*: brain > intestine > gill > liver > kidney > red muscle > white muscle > heart. Overall, the highest level of expression of the total of the four transcripts was in the brain, followed by (in descending order) intestine > liver > red muscle > gill/kidney > white muscle > heart.

Discussion

Our data show direct evidence for four GSase mRNAs in rainbow trout. The high degree of DNA sequence divergence among these sequences, found in both CDS and non-coding regions (3′-UTR and intron 4), suggests that these cDNAs represent four separate loci. These loci are divided into two distinct evolutionary lineages, *Onmy-GS02/04* and *OnmyGS01/03*. The trout *Onmy-GS02/04* sequence lineage first shares a common ancestor with a toadfish/sleeper/fugu lineage and this combined assemblage next shares a common ancestor with the second trout, *Onmy-GS01/03* lineage (Fig. 3). Given this phylogeny, it is likely that ancestral genes of the two functional trout GSase lineages were present in the common ancestor of all salmonids prior to the tetraploidization event that preceded the rise to the modern salmonids. We also report the existence of two distinct GSase cDNAs

Fig. 3. Maximum-likelihood phenogram based on a DNA alignment of the coding sequence of glutamine synthetase genes in vertebrates. The nucleotide alignment used (available upon request) is identical in form to the amino acid alignment (Fig. 2). Nomenclature of genes is similar to that used in Fig. 2, except that unique GenBank accession numbers have been added to the sequences reported to assist comparison with other papers that may use slightly different nomenclature schemes (i.e. Walsh et al., 2003). Genes coding for mitochondrial isozymes are underlined. Bootstrap values above 50%, based on 200 bootstraps, are placed at the appropriate nodes.

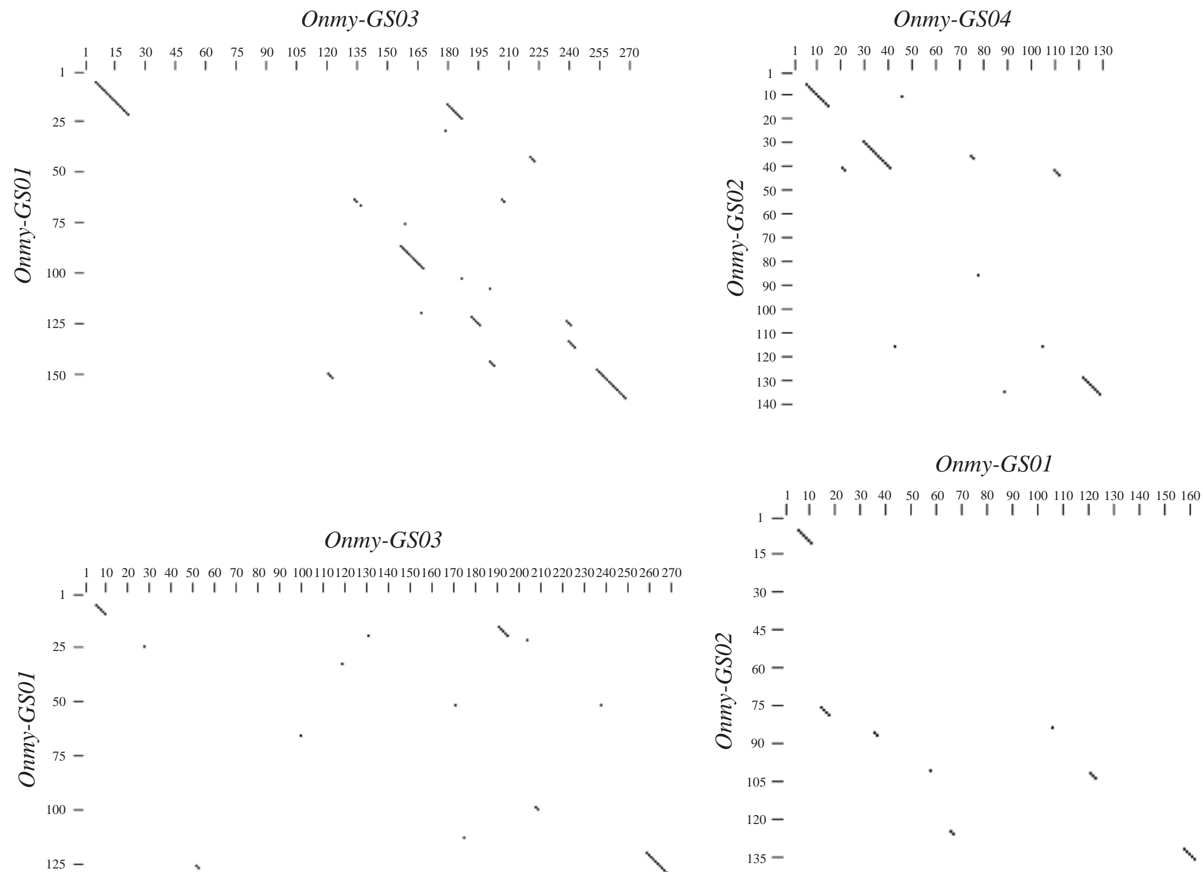

Fig. 4. Dot-plot comparisons of rainbow trout (*Oncorhynchus mykiss*) glutamine synthetase intron 4 sequences. The last and first 10 base pairs of exons 4 and 5 respectively, flanking intron 4, are included in the comparison.

in zebrafish. In zebrafish, nominally a diploid species, there have been numerous reports of duplicated loci (e.g. Postlethwait et al., 1998; Force et al., 1999; Chiang et al., 2001). The relationship of the zebrafish cDNAs to one another is not well resolved in the phylogeny, but their duplication appears to be independent of the duplication that led to the two trout lineages.

The two lineages of trout GSase cDNAs, *Onmy-GS01/03* and *Onmy-GS02/04*, are strongly supported in the phylogenetic analysis. The genetic distance estimates between the members of each pair (0.0948 and 0.0825 substitutions/site, respectively) are greater than those observed between *mus* and *rattus* (0.0798) (Fig. 3). The divergence of rat and mouse lineages is thought to have occurred between 10–35 mya (see Li, 1997). GSase genes have been shown to evolve in a clock-like manner over a wide range of evolutionary times (Pesole et al., 1991). Assuming a molecular clock for bony fish GSase genes, the *Onmy-GS01/03* and *Onmy-GS02/04* values are, therefore, consistent with the duplication of these loci (from 2 to 4) during the ancestral tetrapoidization event estimated to have occurred 25–100 mya (Allendorf and Thorgaard, 1984).

The presence of two GSase genes in zebrafish and fugu and four in trout suggests that multiple, independent gene duplication events of GSase have occurred within bony fishes. Taylor et al. (2001) examined 27 groups of orthologous genes (i.e. homologous genes, derived from a common ancestral gene, which are found in different species), each including two zebrafish genes as well as genes from *Xenopus*, chicken, mouse and man. They concluded that a single genome duplication event in the ancestor of teleostean fishes was the most parsimonious explanation for the duplicated gene copies found in zebrafish. Our data are not inconsistent with the results of Taylor et al. (2001), which did not examine orthologous loci of teleost species beyond zebrafish, but they do indicate that the ancestral genome duplication event may have been specific to the lineage leading to zebrafish and not ancestral to all teleosts. The presence of the trout GSase sequences in a sister clade to that of the two zebrafish sequences suggests the occurrence of a second ancestral lineage-specific gene duplication event. Within the lineage containing the trout sequences, two GSase genes are reported for *B. sinensis* (*Bosi*, Fig. 2) (Anderson et al., 2002). Due to their sequence similarity these genes appear to be alleles of a single locus; however, the possibility of additional GSase genes in this species was not excluded (Anderson et al., 2002). In the gulf toadfish, a single gene was reported that may be differentially spliced leading to cytosolic and mitochondrial isozymes (Walsh et al., 1999). Subsequent studies, however, have revealed the presence of a

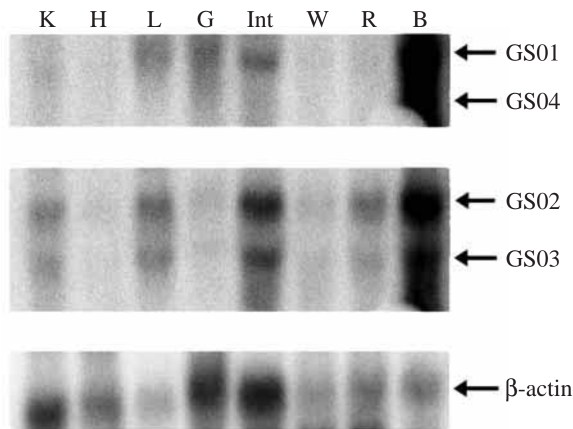

Fig. 5. Tissue distribution of rainbow trout glutamine synthetase (GS) using high-stringency northern analysis. Membranes were probed using ^{32}P-labelled random-primed cDNA probes for four GSase genes (*Onmy-GS01, Onmy-GS02, Onmy-GS03 and Onmy-GS04*) and β-actin, and hybridized at 65°C. Each lane contained 10 μg of total RNA. K, kidney; H, heart; L, liver; G, gill; Int, intestine; W, white muscle; R, red muscle; B, brain. Arrows indicate the location of the respective band.

second cytosolic *O. beta* gene (Walsh et al., 2003), supporting our proposal of multiple GSase duplication events in ray-finned fishes.

Evidence for two GSase loci is found in the draft sequence of the fugu genome. One of these sequences, JGI-18692 is clearly orthologous to the trout *Onmy-GS02/04* lineage. We predict that this is a functional GSase gene in fugu. In contrast, the second locus, JGI-15768, is divergent from all other bony fish genes. Interestingly, this gene lacks the second exon, which contains a number of conserved active site residues (Fig. 2), and may be a pseudogene. Alternatively, it may have evolved a new function. In either case, its relationship to the divergent amphibian GSase genes (Fig. 3), and to a second gill-specific GSase gene reported in *O. beta* (Walsh et al., 2003) is intriguing. Although not detected in the present study, the presence of additional trout GSase genes that belong to this divergent lineage remains to be fully tested. At least three GSase cDNAs are also present in the tetraploid African clawed frog, *Xenopus laevis*. The surprising, although weakly supported, phylogenetic position of the *Xenopus* genes (Fig. 3) suggests these genes are not the true orthologues of the other tetrapod genes or alternatively, they may have undergone substantial evolutionary change. The presence of bony fish genes within this clade suggests that the genes of this lineage are not the true orthologues of the other vertebrate genes. More work, however, in both fugu and *Xenopus,* is needed to determine if (1) both putative fugu GSase-like genes identified from genomic sequence actually code for an expressed functional protein, (2) there are additional orthologous GSase-like genes in amphibians and (3) how the complete sequence of the *Xela-GS02* gene fits into the present phylogeny.

The phylogenetic analysis of vertebrate GSase cDNAs reported here is similar to previous phylogenies (Pesole et al., 1991; Laud and Campbell, 1994; Walsh et al., 1999). Consistent with the original proposal of Pesole et al. (1991), independent origins can be postulated for each of the dogfish, chicken and Gulf toadfish mitochondrial GSase cDNAs. There is strong support for the inclusion of the toadfish *Opbe-af118103* cDNA within a monophyletic bony fish clade (Fig. 3). The convergent evolution of mitochondrial targeted GSases in several animals (see above), including toadfish, is further supported by (1) the apparent lack of mitochondrial GSase activity in the closely related *O. tau* and *Porichthys notatus* (Anderson and Walsh, 1995), (2) the lack of a mitochondrial transport leader sequence in all other bony fish GSase cDNAs fully characterised, and (3) the dissimilarity of the kinetic properties (i.e. phenotype) between the dogfish and toadfish mitochondrial isozymes (Walsh, 1996). Concerning the latter, one would predict that if both fish mitochondrial isozymes had evolved from a common mitochondrial isozyme, then they would share a similar phenotype.

The function of multiple GSase genes in some bony fishes is intriguing. The high degree of conservation of GSase genes throughout eukaryote and prokaryote evolution (Pesole et al., 1991), and the lack of variation noted at the inferred glutamate binding site residues, suggest that these genes are all functioning in the expected manner. Levels of *Onmy-GS01–04* gene expression differ among tissues and would presumably lead to functional octamers of differing subunit make-up. [We have also measured the levels of *Onmy-GS01* and *GS02* mRNAs in adult trout tissues using semi-quantitative RT-PCR and the ribonuclease protection assay, and observed a very similar pattern of mRNA expression (P. Essex-Fraser, N. Bernier, B. Murray and P. Wright, unpublished data). Given this assumption, two hypotheses may explain the retention of multiple GSase genes in bony fishes: (1) the presence of a mutant subunit in the functional octameric protein may disrupt the enzymatic function of the entire protein and be selected against, i.e. a dominant-negative mutation (Gibson and Spring, 1998) and (2) the various duplicated genes may develop specialised regulatory subfunctions, i.e. the duplication–degeneration complementation model (Force et al., 1999). The multifunctional role of GSase and the tissue-specific expression patterns reported here favour the second hypothesis. In bony fishes the regulation of the various isozymes, i.e. octameric proteins of differing subunit make-up, may be coupled to different developmental stages. GSase activity is detected early in embryonic rainbow trout (Steele et al., 2001) and the levels of activity are much higher in whole larvae relative to adult liver tissue (Wright et al., 1995). Studies are underway to determine the pattern of mRNA expression of the four genes (*Onmy-GS01–04*) during early stages of rainbow trout development. The data presented here suggest that GSase genes are differentially expressed in different tissues and therefore possibly involved in different metabolic pathways. In most teleosts, GSase activities are highest in the brain (Webb and Brown, 1976; Chamberlin et al., 1991; Peng et al., 1998) and this is also true in rainbow trout (Mommsen et al., 2003), followed, in descending order by kidney > liver > intestine >

white muscle in adults (Korte et al., 1997). While this descending order of GSase activities is not entirely consistent with the relative levels of total mRNA expression of the four trout GSase genes in the present study, it is unclear how the differing subunit make-up of the octameric protein would affect the GSase activity. Of interest, the seemingly contradictory results between enzyme assay and mRNA expression studies of GSase in other species (e.g. Kong et al., 2000), especially when the full complement of coding genes was not identified, may also be explained by the presence of multiple genes expressed at different levels in each tissue. This study clearly emphasizes the need to fully identify the number of genes coding for the protein of interest prior to the interpretation of data showing changes in the levels of mRNA expression, and suggests a complex interaction of the gene products of duplicated loci in multimeric proteins, such as GSase.

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