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Developmental Expression Pattern

Spatiotemporal expression of the creatine metabolism related genes *agat, gamt* and *ct1* during zebrafish embryogenesis

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ABSTRACT Glycine amidinotransferase (AGAT or GATM), guanidinoacetate methyltransferase (GAMT) and creatine transporter (CT1) are three proteins involved in the synthesis and uptake of creatine. The expression patterns of these three genes were examined in zebrafish embryos by whole mount *in situ* hybridization followed by histological sectioning. Expression of *agat* first appeared in the yolk syncytial layer (YSL) at the gastrula stage and was progressively up regulated during gastrulation. As development proceeds, *agat* was expressed in the mature somites during the segmentation stage and in the liver at 48 hpf. *gamt* showed a similar expression pattern to that of *agat* during embryogenesis. It was first detected in the center of the yolk from the cleavage to the gastrula stage. At the bud stage, its expression shifted to the YSL. *gamt* was also transiently expressed in the mature somites from 16 hpf to 24 hpf and became strongly expressed in the liver and in epithelial cells of the gut at 48 hpf. *ct1* was initially uniformly expressed from the cleavage to the early segmentation stage; it was then strongly expressed in all the somites till 30 hpf and in the gut of 48 hpf embryos. However, *ct1* transcripts also appeared in the central nervous system during the segmentation stage, but not in the YSL, the yolk or the liver. Our data reveal for the first time distinct and unique patterns of expression of the creatine metabolism genes *agat, gamt* and *ct1* during zebrafish embryogenesis.

KEY WORDS: *agat, gamt, ct1, zebrafish, developmental expression*

The creatine metabolism plays a crucial role for keeping the normal life of vertebrates. Endogenous creatine is synthesized by a two-step mechanism involving two enzymes: glycine amidinotransferase (AGAT or GATM) and guanidinoacetate methyltransferase (GAMT). Creatine is taken up by cells through CT1, a specific creatine transporter (Wyss and Kaddurah-Daouk, 2000). Defects of AGAT, GAMT and CT1 result in three kinds of creatine deficiency syndromes (CDS) occurring mostly in children (Schulze, 2003). The common clinical feature of three CDS is developmental delay/regression, mental retardation and severe disturbance of their expressive and cognitive speech (van der Knaap *et al.*, 2000). The biochemical characteristics of CDS include severe depletion of creatine/phosphocreatine in the brain, as well as changes in creatine and creatinine concentrations in body fluids (Verhoeven *et al.*, 2000, Cecil *et al.*, 2001, Schulze, 2003, Stromberger *et al.*, 2003, Almeida *et al.*, 2004, Sykut-Cegielska *et al.*, 2004). GAMT deficiency is characterized by accumulation of guanidinoacetic acid in brain and body fluids and shows intractable seizures and the movement disorder (Sykut-Cegielska *et al.*, 2004). GAMT and AGAT deficiency have autosomal-recessive traits, whereas the CT1 defect is an X-linked disorder (Mancini *et al.*, 2005, Leuzzi *et al.*, 2006). Treatment with oral creatine supplementation is in part successful in GAMT and AGAT deficiency (Battini *et al.*, 2006, Schulze *et al.*, 2006), whereas it does not work on CT1 deficiency. Recently, it has been demonstrated that AGAT, GAMT and CT1 are expressed in many embryonic tissues during rat embryogenesis (Braissant *et al.*, 2005). Here we carried out whole mount *in situ* hybridization followed by histological sectioning to determine the spatiotemporal expression patterns of *agat*, *gamt* and *ct1* during zebrafish embryogenesis.

Sequences and phylogenetic analyses of zebrafish AGAT, GAMT and CT1

Three cDNAs (GenBank accession numbers AAH56747,

Abbreviations used in this paper: AGAT (or GATM), glycine amidinotransferase; GAMT, guanidinoacetate methyltransferase; CDS, creatine deficiency syndromes; CT1, creatine transporter 1; YSL, yolk syncytial layer.

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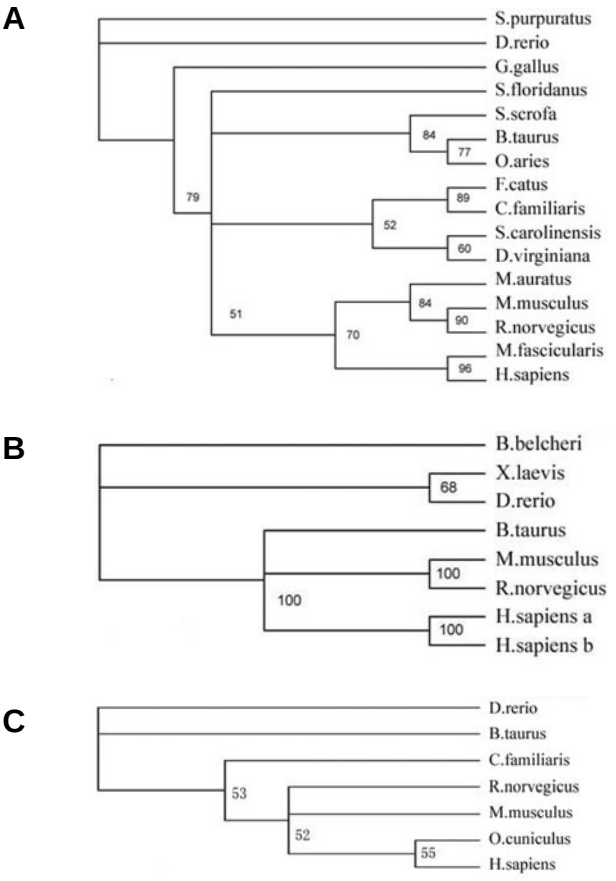

Fig. 1. Phylogenetic tree constructed using *Treepuzzle 5.0* software *according to the alignment of the protein sequences of AGAT* **(A)**, *GAMT* **(B)** *and CT1* **(C)** *in zebrafish and other species. The numbers after the internal branches indicated the bootstrap value (value/1000) for each group. Zebrafish AGAT and CT1 fell outside the other vertebrate groups and formed an independent clade in the trees respectively, while zebrafish GAMT and frog GAMT were in the same clade.*

pancreas, CNS, skeletal muscles and intestine of rat embryos. In *Xenopus, agat* (*xat*) transcripts were detected around the yolk plug of early gastrula and strong expression was found in the notochord and the midline of the neural plate of early neurula. At tailbud stage, the expression was found both in the notochord and the trunk region (Zhao *et al.*, 2001).

Spatiotemporal expression of gamt

Transcripts of *gamt* were first detected in the central area of the yolk from cleavage (Fig. 3A) to gastrula stage (not shown). They were progressively expanded in the YSL at bud and early segmentation stages, while no transcript was detected in other

NM_205741 and XM_690840) encoding zebrafish AGAT, GAMT and CT1 were identified in the database. The deduced proteins were 422, 234 and 652 amino acids respectively, showing high levels of conservation to known AGAT, GAMT and CT1 proteins in human and other vertebrates, ranging from 65% to 86% overall identities (Appendix 1). Phylogenetic analyses were performed by the neighbor-joining analysis. Zebrafish AGAT and CT1 fell outside the other vertebrate group and formed an independent clade in the tree respectively, while zebrafish GAMT and frog GAMT were in the same clade (Fig. 1).

Three cDNA fragments for *agat* (355bp), *gamt* (417bp) and *ct1* (625bp) were isolated by RT-PCR using the primers designed according to these sequences obtained from GenBank. After subcloning and sequencing, RNA probes were synthesized for whole mount *in situ* hybridization.

Spatiotemporal expression of agat

The temporal and spatial expression pattern of *agat* was determined from fertilization to 48 hpf (Fig. 2). *agat* transcripts first appeared in the yolk syncytial layer (YSL) at gastrula stage (Fig. 2A,B). At early segmentation stage, strong *agat* expression persisted in the YSL and a weak expression also appeared in the mature somites (Fig. 2 C,D). As development proceeds, *agat* still expressed strongly in the YSL and weakly in the somites (Fig. 2 E,F). By 48 hpf, the expression of *agat* disappeared in the somites; however, strong expression was detected in the liver (Fig. 2 G,H).

agat expression pattern has been described in several other vertebrate species. It was expressed in the placenta and the yolk sac of mouse embryos. However, no expression was found in the embryonic tissues of mouse (Sandell *et al.*, 2003). Similarly, human *agat* was also expressed in the placentas (Monk *et al.*, 2006). Braissant *et al.* (2005) have reported *agat* expression in the hepatic primordium of rat embryos at E12.5. High levels of *agat* expression were also detected in kidney,

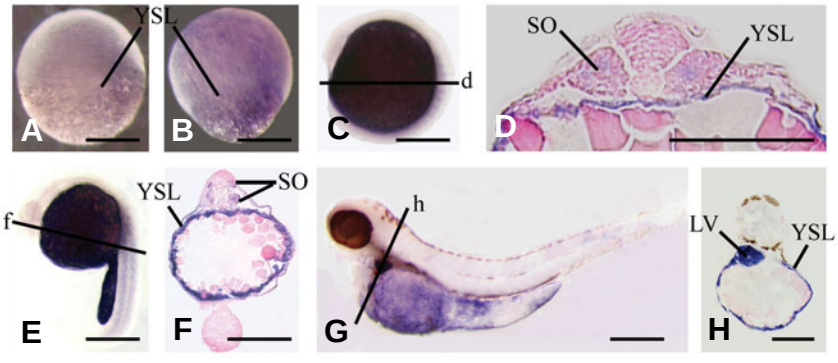

Fig. 2. Expression of *agat* in zebrafish embryos. *Left side views for the whole mount, except where noted, with anterior up and dorsal to the right.* **(A)** *50%-epiboly stage, showing that* agat *mRNA was first detected in the YSL.* **(B)** *70%-epiboly stage, showing increased expression in the YSL.* **(C)** *8-somite stage (13 h), showing agat expression in mature somites and the YSL.* **(D)** *Cross section of (C) through the line d.* **(E)** *25-somite stage (21.5 h), showing strong expression in the YSL and weak expression in the mature somites.* **(F)** *Cross section of (E) through the line f.* **(G)** *Long-pec stage (48 h), left side view, with dorsal side up and anterior to the left. Expression of agat disappeared in the somites, but persisted in the YSL and appeared in the liver.* **(H)** *Cross section of (G) through the line h, showing strong expression in the liver and the YSL. LV, liver; SO, somite; YSL, yolk syncytial layer. Scale bar: 250 μm.*

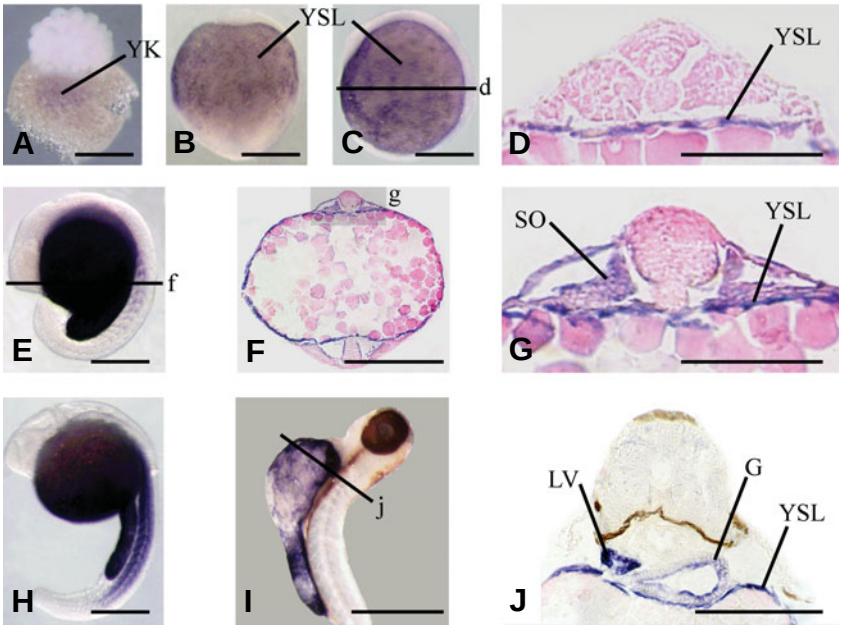

Fig. 3. Expression of *gamt* in zebrafish embryos. *Left side views for the whole mount, except where noted, with anterior up and dorsal to the right.* **(A)** *Early cleavage stage, side view. Weak expression was found in the center of the yolk.* **(B)** *Bud stage (10 h), ventral view. Expression of gamt appeared in the YSL.* **(C)** *8-somite stage (13 h), showing gamt expression in the YSL.* **(D)** *Cross section of (C) through the line d.* **(E)** *15-somite stage (16.5 h); gamt is expressed in the YSL and the mature somites.* **(F)** *Cross section of (E) through the line f.* **(G)** *Enlargement of the gray shadow area g in (F), showing gamt expression in the YSL and the somites.* **(H)** *25-somite stage (21.5 h), showing increased expression in the somites.* **(I)** *Long-pec stage (48 h).* **(J)** *Cross section of (I) through the line j. Staining of gamt disappeared in the somites, but persisted in the YSL and appeared in the liver and the gut. G, gut; LV, liver; SO, somite; YK, yolk; YSL, yolk syncytial layer. Scale bar: 80 μm for (G) and 250μm for others.*

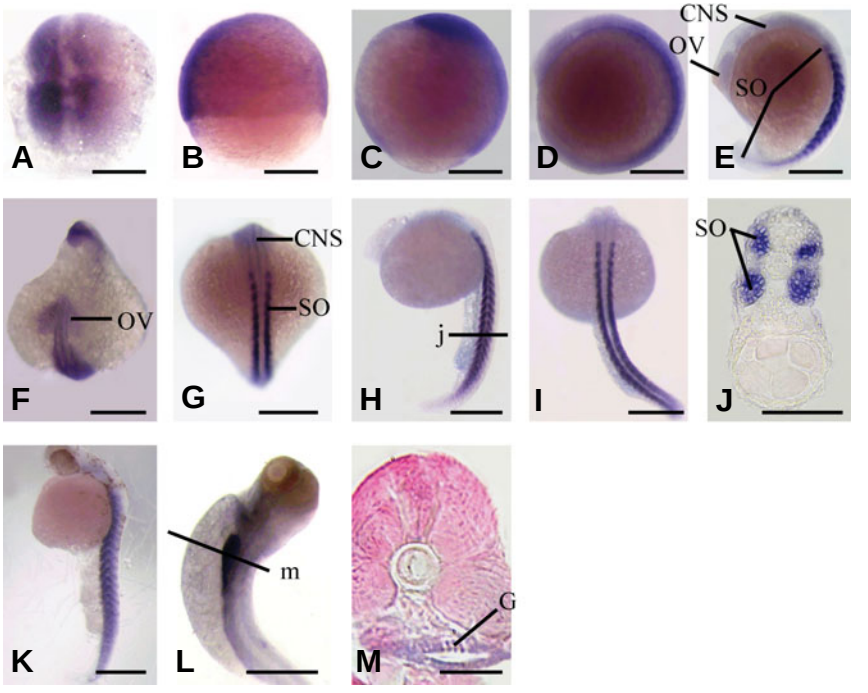

Fig. 4. Expression of *ct1* in zebrafish embryos. *Left side views for the whole mount, except where noted, with anterior up and dorsal to the right.* **(A)** *4-cell stage, animal pole view.* **(B)** *70%-epiboly stage.* **(C)** *90%-epiboly stage.* **(D)** *8-somite stage (13 h).* ct1 *mRNA was distributed ubiquitously in the embryonic tissues in (A-D) while no signal was found in the YSL.* **(E)** *15-somite stage (16.5 h).* **(F)** *Ventral view of (E).* **(G)** *Dorsal view of (E). The expression was restricted in the somites, the optic vesicles and the CNS.* **(H)** *Prim-5 stage (24 h).* **(I)** *Dorsal view of (H). The expression of* ct1 *mRNA persisted in both the newly formed and the matrue somites.* **(J)** *Cross section of (H) through the line j, showing the expression in the somites.* **(K)** *Prim-15 stage (30 h), showing* ct1 *mRNA in the somites.* **(L)** *Long-pec stage (48 h), showing the expression of* ct1 *in the foregut.* **(M)** *Cross section of (L) through the line m, showing* ct1 *expression in the epithelial cells of the foregut. CNS, central nervous system; G, gut; OV, optic vesicle; SO, somite. Scale bar: 80 μm for (J) and (M), 250μm for others.*

tissues (Fig. 3 B-D). At about 16 hpf, *gamt* expression could be detected in the mature somites besides the YSL (Fig. 3 E-G). The expression in the somites was gradually up regulated from 17 hpf to 22 hpf stage (Fig. 3H). Like *agat*, *gamt* expression in the somites disappeared at 48 hpf, but persisted in the YSL and appeared in the liver (Fig. 3 I,J). However, *gamt* mRNA was also detected in the epithelial cells of the gut where no *agat* mRNA was found (Fig. 3J).

Similar pattern of expression could be noted between zebrafish *gamt* and rat *gamt* (Braissant *et al.*, 2005). Both of them were detected in liver, muscles and gut. But the expression of rat *gamt* was also detected prominently in CNS and pancreas while zebrafish *gamt* was not.

Spatiotemporal expression of ct1

ct1 exhibited overlapping and distinct expression pattern compared with *agat* and *gamt*. *ct1* was initially expressed ubiquitously in the embryo from cleavage to early segmentation stage (Fig. 4 A-D). Then it was gradually restricted in the somites, the optic vesicles and the central nervous system (CNS) (Fig. 4 E-G). By prim-5 and prim-15 stage, *ct1* expression was strongly expressed in both the newly formed and mature somites (Fig. 4 H-K). By 48 hpf, the *ct1* transcripts decreased to a much weaker extent in the somites, but strongly

appeared in the foregut (Fig. 4 L,M). *ct1* expression has been reported in CNS, choroid plexus, dorsal aorta, hepatic primordium, skeletal muscles, epidermis and dermis, kidney, lung, stomach and intestine epithelial cells of rat embryos (Braissant *et al.*, 2005). However, *ct1* expression in zebrafish embryos was restricted to a fewer tissues, including somites, CNS, optic vesicle and gut.

Several interesting differences were noticed between the expression patterns of *agat*, *gamt* and *ct1* in zebrafish embryos. First, unlike *agat* and/or *gamt*, no transcripts of *ct1* were detected in the yolk, the YSL or the liver. Second, *ct1* expression appeared in the CNS while *agat* and *gamt* did not. Finally, high level of *ct1* expression was detected in all the somites, while weak *agat* and *gamt* expressions were detected only in the mature somites. Thus, our results show that *agat*, *gamt* and *ct1* genes exhibit unique expression pattern during zebrafish embryogenesis.

The expressions of *agat* and *gamt* in zebrafish are detected in YSL before 48 hpf and in the liver after 48 hpf. This may suggest that zebrafish YSL and liver at different developmental stages are important tissues related to creatine synthesis. In addition, the somites during segmentation stage may be also involved in this process. In zebrafish, the expression of *ct1* is detected in CNS while *agat* and/or *gamt* are not. It implies that fish CNS may not function in creatine synthesis but just transport creatine to supply itself for energy.

Compared with their homologues during rat embryonic development (Braissant *et al.*, 2005), there is a similar expression pattern of *agat, gamt* and *ct1* between zebrafish and rat embryos. They are all expressed in skeletal muscle. In addition, *agat* and *gamt* are expressed in liver. On the other side, there are differences of these gene expression patterns between two species. For example, three genes are expressed in the CNS of rat embryo while only *ct1* is expressed in the CNS of zebrafish embryo. *agat* gene is strongly expressed in rat kidney and pancreas, while no expression is found in the same organs of zebrafish embryo. Among these three genes related to creatine metabolism, the spatiotemporal expression of *ct1* shows a remarkable conservation between fish and mammal. It implies that the similar mechanism of creatine transport may occur in fish and mammal.

The expressions of muscle-specific creatine kinase and brain subtype creatine kinase have been reported during zebrafish development (Xu *et al.*, 2000, Dickmeis *et al.*, 2001). Our data show that three primary elements related to creatine metabolism exist in zebrafish. Together with previous report (Xu *et al.*, 2000, Dickmeis *et al.*, 2001), we suggest that a creatine synthesis and transport system as in mammal may similarly exist in fish. Since zebrafish is easy to maintain, manipulate and observe in the lab, it may provide a model system for human disease study (Dodd *et al.*, 2000, Dooley and Zon, 2000). Further functional analysis of these molecules in zebrafish should be useful for understanding the mechanism of human creatine deficiency syndromes.

Experimental Procedures

Cloning, sequence alignments and phylogenetic analyses

The RT-PCR reactions were carried out on RNA extracted from 24 hpf zebrafish embryos according to manufacturer's instructions ˇPromegaˇ. The primers were as follows:

agat 5'-TACGGCGGTCAGACATTC-3' and
5'-GGTAATCCTGGTCGTAGAGC-3';

gamt 5'-CGTTCACGCACTCCGCATTTG–3' and
5'-AGGGTTGGGGCGACCTCTTCC-3';

ct1 5'-AACTCCACATTCGGCAACCT-3' and
5'-GAGCCACGGGCATCATAGA-3'.

The PCR product was 355 bp corresponding to position 379-733 of the cDNA (AAH56747) for *agat*; 417 bp and located in the region corresponding to 5-421 of the cDNA (NM_205741) for *gamt*; 625 bp corresponding to position 625-1249 of the cDNA (XM_690840) for *ct1*. PCR products were subcloned into pGEM-T Easy vectors (Promega, Madison, WI) and sequenced. Alignments of sequences and phylogenetic analyses were performed with ClustalX 1.81 and Treepuzzle 5.0 software.

Probes synthesis and whole mount in situ *hybridization*

Sense and anti-sense RNA probes were synthesized using the digoxigenin-UTP (DIG) *in vitro* transcription kit with SP6 and T7 polymerases (Roche Applied Science, Indianapolis, IN) on the above-mentioned cDNA clones. *In situ* hybridizations were performed as described (see http://zfin.org/zf_info/zfbook/chapt9/9.82.html), followed by sectioning.

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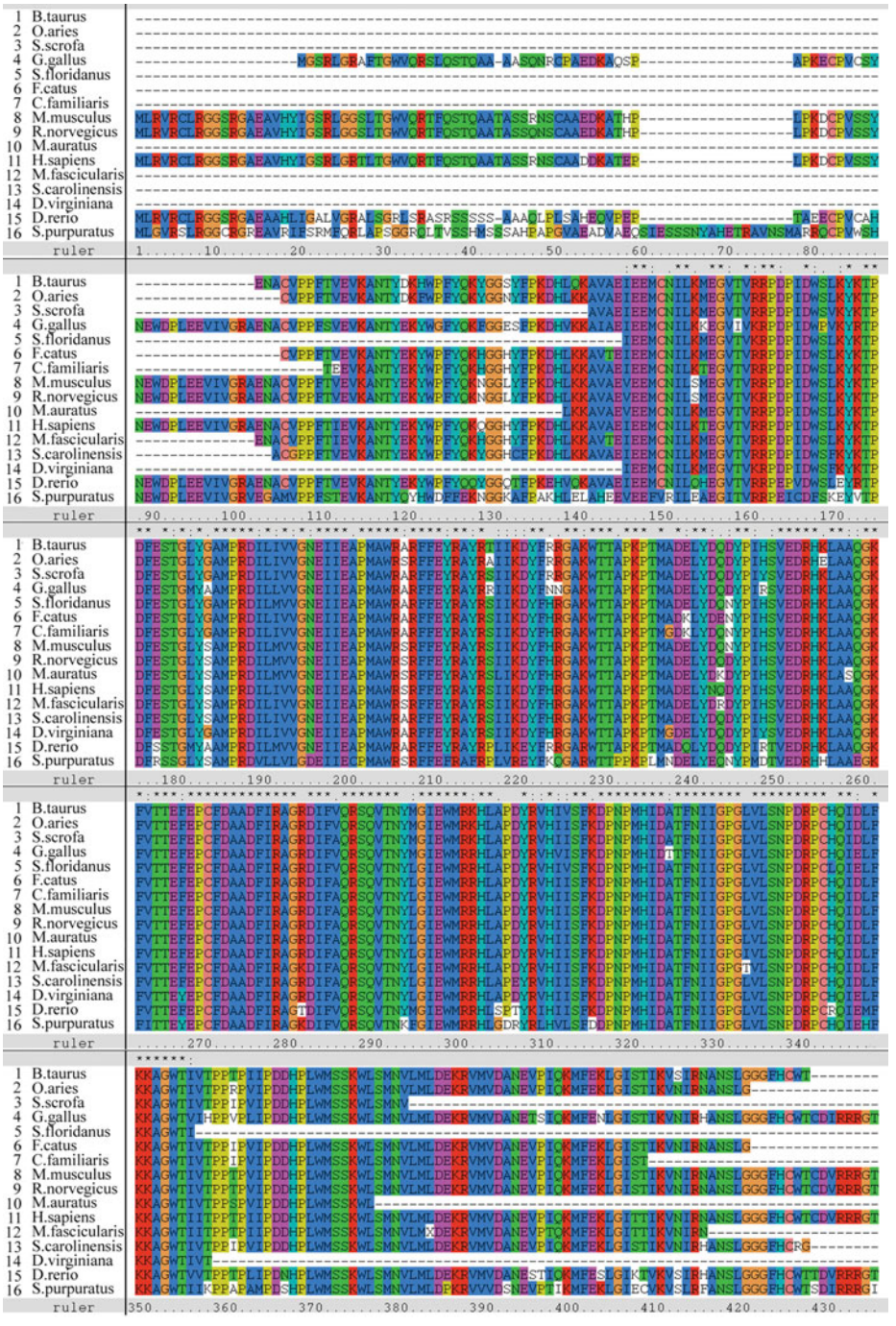
APPENDIX I

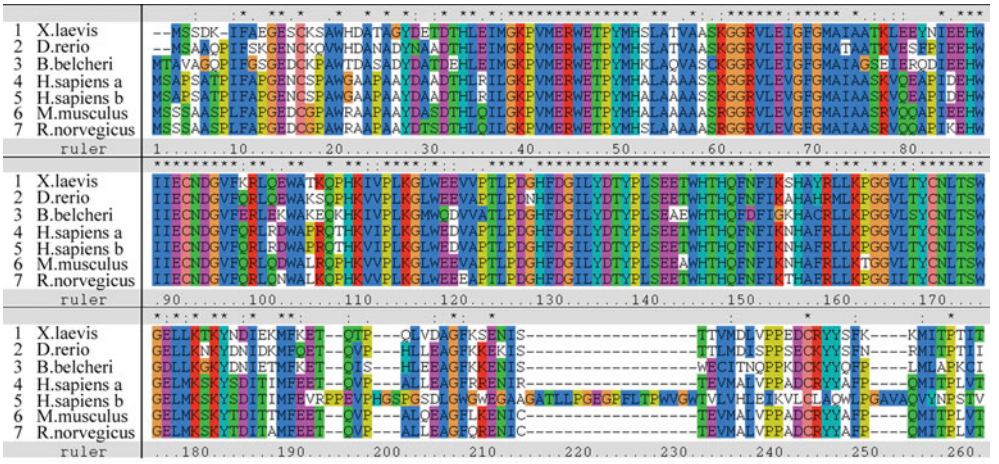
Amino acid sequence alignment of AGAT (A), GAMT (B) and CT1 (C) in zebrafish and other species using ClustalX software

A

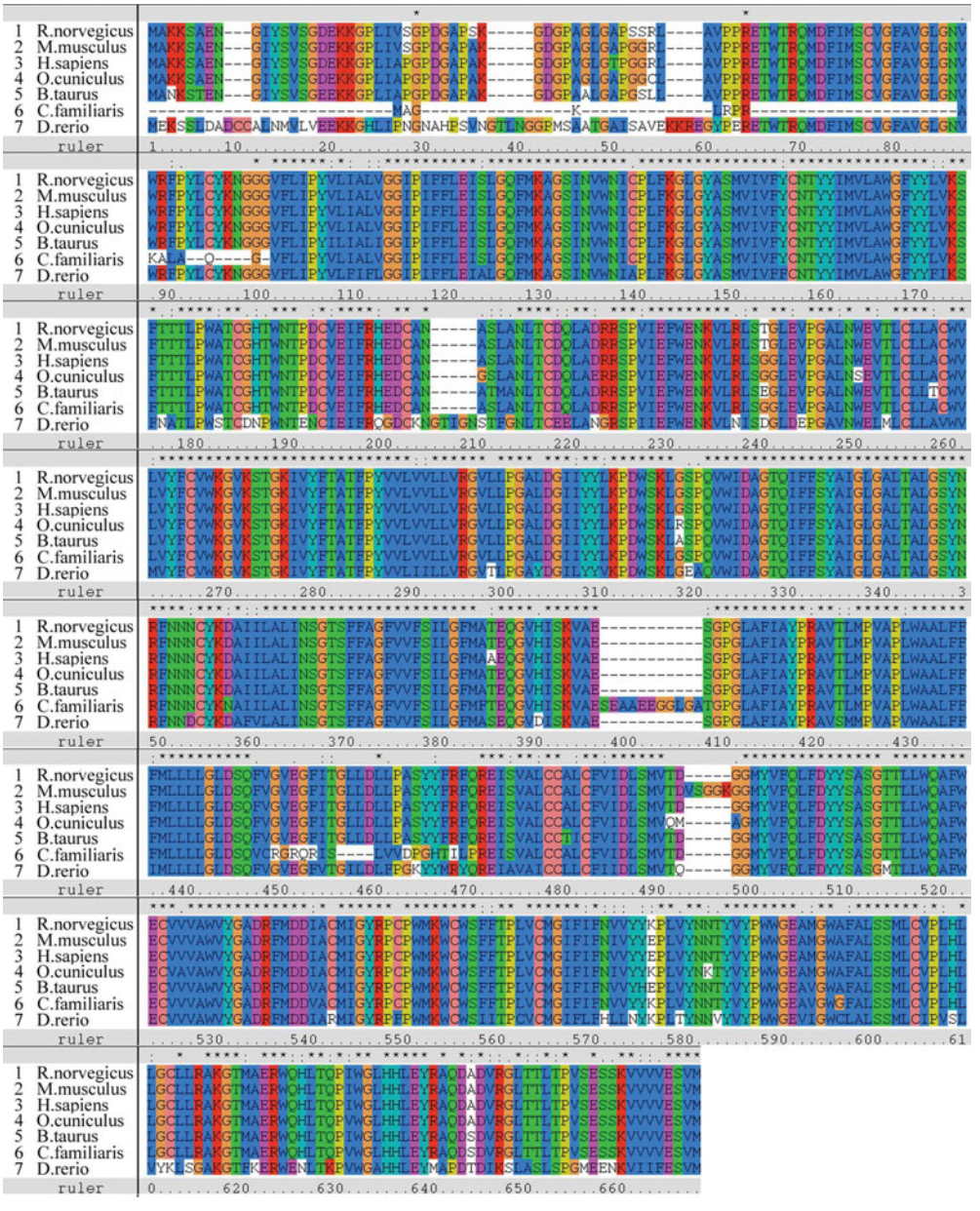


Color background indicates the amino acids that match the consensus. The asterisks indicate identical amino acids among all species. The following are the accession numbers of amino acid sequences. For AGAT: AAT39889 (Bos Taurus), *AAT39898* (Ovis aries), *AAT39894* (Sus scrofa), *NP_990076* (Gallus gallus), *AAT39896* (Sylvilagus floridanus), *AAT39888* (Felis catus), *AAT39890* (Canis familiaris), *AAH03879* (Mus musculus), *NP_112293* (Rattus norvegicus), *AAT39891* (Mesocricetus auratus), *AAB29892* (Homo sapiens), *AAT39892* (Macaca fascicularis), *AAT39899* (Sciurus carolinensis), *AAT39895* (Didelphis virginiana), *AAH56747* (Danio rerio), *XP_786560* (Strongylocentrotus purpuratus). *For GAMT: AAH45001* (Xenopus laevis), *NP-991304* (Danio rerio), *ABA00513* (Branchiostoma belcheri), *NP-0000147* (Homo sapiens isoform a), *NP-620279* (Homo sapiens isoform b), *NP-034385* (Mus musculus), *NP_036925* (Rattus norvegicus). *For CT1: P28570* (Rattus norvegicus), *Q8VBW1*(Mus musculus), *P48029* (Homo sapiens), *P31661*(Oryctolagus cuniculus), *O18875* (Bos taurus), *XP_549362* (Canis familiaris), *XP_695932* (Danio rerio).

B


1 X.laevis
2 D.rerio
3 B.belcheri
4 H.sapiens a
5 H.sapiens b
6 M.musculus
7 R.norvegicus
ruler

C


1 R.norvegicus
2 M.musculus
3 H.sapiens
4 O.cuniculus
5 B.taurus
6 C.familiaris
7 D.rerio
ruler