

# Glutamine synthetase gene expression during the regeneration of the annelid *Enchytraeus japonensis*

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**Abstract** *Enchytraeus japonensis* is a highly regenerative oligochaete annelid that can regenerate a complete individual from a small body fragment in 4–5 days. In our previous study, we performed complementary deoxyribonucleic acid subtraction cloning to isolate genes that are upregulated during *E. japonensis* regeneration and identified glutamine synthetase (*gs*) as one of the most abundantly expressed genes during this process. In the present study, we show that the full-length sequence of *E. japonensis* glutamine synthetase (EjGS), which is the first reported annelid glutamine synthetase, is highly similar to other known class II glutamine synthetases. EjGS shows a 61–71% overall amino acid sequence identity with its counterparts in various other animal species, including *Drosophila* and mouse. We performed detailed expression analysis by *in situ* hybridization and reveal that strong *gs* expression occurs in the blastemal regions of regenerating *E. japonensis* soon after amputation. *gs* expression was detectable at the cell layer covering the wound and was found to persist in the epidermal cells during the formation and elongation of the blastema. Furthermore, in the elongated blastema, *gs* expression was detectable also in the presumptive regions of the brain, ventral nerve cord, and stomodeum. In the fully formed intact head, *gs* expression was also evident in the prostomium, brain, the anterior end of the ventral nerve cord, the epithelium of

buccal and pharyngeal cavities, the pharyngeal pad, and in the esophageal appendages. In intact *E. japonensis* tails, *gs* expression was found in the growth zone in actively growing worms but not in full-grown individuals. In the nonblastemal regions of regenerating fragments and in intact worms, *gs* expression was also detected in the nephridia, chloragocytes, gut epithelium, epidermis, spermatids, and oocytes. These results suggest that EjGS may play roles in regeneration, nerve function, cell proliferation, nitrogenous waste excretion, macromolecule synthesis, and gametogenesis.

**Keywords** Glutamine synthetase · Gene expression · Regeneration · Oligochaete annelid · *Enchytraeus japonensis*

## Introduction

Some types of annelids exhibit remarkable regenerative abilities that are comparable to planarians. However, the regeneration mechanisms are thought to be quite different between planarians and annelids. Whereas planarians regenerate via totipotent stem cells (neoblasts) that are widely distributed throughout their bodies (Reddien and Alvarado 2004), annelid regeneration is thought to occur mainly through the dedifferentiation and subsequent redifferentiation of cells without any contribution from totipotent stem cells (Thouveny and Tassava 1998). In this respect, annelids regenerate in a manner similar to that of amphibians (Carlson 1998) but on a much larger scale. The elucidation of the annelid regeneration mechanisms is thus expected to provide valuable information that may allow us in the future to explore strategies to enhance the regenerative capabilities in vertebrates.

In our recent study, we have proposed the oligochaete annelid *Enchytraeus japonensis* as a new model system for

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regeneration studies (Myohara et al. 1999). *E. japonensis* has striking features that are highly advantageous in this regard: (1) It reproduces asexually by dividing its body into several fragments, which then regenerate a complete individual within 4–5 days, (2) artificially amputated fragments can also regenerate new individuals in the same manner, and (3) both asexual and sexual reproduction can be artificially induced in the laboratory (Myohara et al. 1999).

As an initial strategy toward the elucidation of the molecular mechanisms underlying annelid regeneration, we previously performed complementary deoxyribonucleic acid (cDNA) subtraction cloning using regenerating fragments of *E. japonensis* and intact animals (Myohara et al. 2006). Filter array screening subsequently revealed that about 38% of the forward-subtracted cDNA clones we isolated contained genes that were upregulated during regeneration. These clones hybridized with probes derived from regenerating fragments but not with probes from intact worms. Of these clones, 279 were then sequenced and found to contain 165 different sequences (79 known and 86 unknown). Among these 279 regeneration-upregulated clones, as many as 13 (4.7%) were found to encode glutamine synthetase, a ubiquitous and key enzyme in nitrogen metabolism (Kumada et al. 1993).

Because of its abundance in our subtracted cDNA library, we cloned the full-length sequence of *E. japonensis* glutamine synthetase (EjGS). By reverse transcriptase–polymerase chain reaction (RT-PCR), we subsequently revealed that the expression levels of the glutamine synthetase gene (*gs*) were consistently high during the regenerative process, with a gradual decline seen only in the very late stages, and our preliminary analysis by whole mount in situ hybridization further revealed strong *gs* expression in the blastemal tissues of the regenerating fragments (Myohara et al. 2006). As these previous results suggested an important role for *gs* in annelid regeneration, in the present study, we have examined the detailed expression pattern of *gs* in regenerating fragments and intact worms and considered the possible roles of this enzyme in the biology of *E. japonensis*. This is the first detailed report of the sequence and expression of *gs* in annelids.

## Materials and methods

### Animals

*E. japonensis* worms were reared at 24°C in 1.1% (w/v) plain agar medium in disposable Petri dishes and were fed with rolled oats as previously described (Myohara 2004). Under these conditions, the worms grow continuously to about 10 mm long, consisting of 50–60 segments, and reproduces asexually by fragmentation every 2 weeks. To induce sexual reproduction, starved worms were cultured in

wet leaf mold and refed with rolled oats as previously described (Myohara 2004).

### Molecular analysis

The full-length cDNA of EjGS (accession number AB109095) has been isolated in our previous study (Myohara et al. 2006). Multiple alignments of the entire amino acid sequences of the glutamine synthetase proteins from representative species, which were obtained through GenBank/EMBL/DDBJ Data Bank and chosen to cover a wide range of phylogenetic groups, were performed using ClustalW software (Thompson et al. 1994). To avoid errors resulting from mixing of possible pseudogenes, only data originating from full-length messenger ribonucleic acid (mRNA) sequences were used.

### In situ hybridization

Both sense and antisense digoxigenin (DIG)-labeled RNA probes were synthesized using the DIG RNA labeling kit (SP6/T7, Roche) as previously described (Myohara et al. 2006). Whole-mount in situ hybridizations were also performed as previously described (Myohara et al. 2006) with some modifications to enhance the signals. Briefly, hybridization was carried out overnight with 1 µg/ml of *gs* DIG-labeled RNA probe at 55°C. An anti-DIG alkaline phosphatase (AP) conjugate antibody (Roche) was preabsorbed by incubation for 2.5 h with fixed and rehydrated worms and used at a dilution of 1:2,000. In situ hybridization of paraffin sections (6 µm) was carried out according to Hoshino et al. (1999) and Abe et al. (2003) with a few modifications. Briefly, sections were hybridized with DIG-labeled probes at a concentration of 100 ng/ml in probe diluent (Genostaff, Tokyo, Japan) at 60°C for 16 h. Washes were then carried out using 5× Hybri-wash (Genostaff), and an anti-DIG AP conjugate antibody (Roche Applied Science, Tokyo, Japan) was then applied at a 1:1,000 dilution in Tris-buffered saline with 0.1% Tween 20 for 2 h. After an overnight coloring reaction with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Roche), the sections were counterstained with a Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan), dehydrated, and mounted in Malinol (Muto Pure Chemicals).

## Results and discussion

Comparisons between *E. japonensis* glutamine synthetase and other known glutamine synthetases

A full-length 2,717-bp cDNA encoding *gs* was previously isolated from regenerating *E. japonensis* in our laboratory (Myohara et al. 2006). The sequence of this gene was found

to contain a putative open reading frame of 360 amino acids with a calculated molecular mass of 40,509 Da. Because this was the first report in annelids of a full-length sequence for *gs*, we performed a database search in the present study to determine whether similarities existed with the *gs* genes from other organisms. BLASTP analysis revealed that the deduced glutamine synthetase protein in *E. japonensis* (EjGS) was highly similar to known class II glutamine synthetases along its entire amino acid sequence (Fig. 1), which is well conserved across species (Kumada et al. 1993). Within the EjGS sequence, we identified a beta-grasp (catalytic) and a noncatalytic domain, five conserved regions that are common to both prokaryotes and eukaryotes, ligands for Mn<sup>2+</sup> ions, and an adenosine triphosphate-binding site (Pesole et al. 1991). An amino acid alignment between EjGS and the *gs* proteins from five invertebrates, sea anemone (Cnidaria), *C. elegans* (Nematoda), Pacific oyster (Mollusca), fruit fly (Arthropoda), sea urchin (Echinodermata), and four vertebrates (zebrafish, clawed frog, chicken, and mouse) showed that the overall amino acid identities between EjGS and its counterparts in various other animal species were within a range of 61–71% (Fig. 1).

#### Expression of the *gs* gene in the blastema of regenerating *E. japonensis*

By whole-mount in situ hybridization analysis, we detected strong *gs* expression in the blastemal region in regenerating fragments of *E. japonensis* (Fig. 2a). This expression was detectable from the early stages of the regenerative process in the cell layer covering the wound (Fig. 2b,k) and was found to persist in the epidermal cells during the formation and elongation of the blastema (Fig. 2c,d). There was also a notable concentration of this transcript in the region corresponding to the prostomium anlagen in the anterior blastema (Fig. 2e) and in the growth zone in the posterior blastema (Fig. 2l). In situ hybridization analyses with paraffin-embedded sections additionally revealed that the *gs* expression pattern was mainly restricted to the epidermal cells in the early blastema (Fig. 2h) but did occur also in the inside tissues of the elongated blastema, where it was detected in the presumptive regions of the brain, ventral nerve cord, and buccal cavity (stomodeum), in addition to the prostomium and epidermal cells (Fig. 2i).

In the later stages of *E. japonensis* regeneration, by which time segmentation and organogenesis have almost completed, we observed that the *gs* expression levels in the blastemal regions had become reduced (Fig. 2f,m). In the fully formed intact head, however, the expression of this gene was detectable in the prostomium, brain, anterior end of the ventral nerve cord, epithelial cells lining the buccal and pharyngeal cavities, pharyngeal pad, and esophageal appendages (Fig. 2g,j; for *E. japonensis* morphology, see

Schmelz et al. 2000). In intact tails, *gs* expression was also evident in the growth zone in actively growing worms (Fig. 2n) but was absent in full-grown individuals in which the growth zone had become indistinguishable (Fig. 2o). These observations are in agreement with the RT-PCR data included in our previous report, which showed that the expression levels of *gs* were maintained at high levels during the early stages of regeneration, decreased temporarily in the later stages of this process, and were upregulated again in the fully formed intact worms (Myohara et al. 2006).

#### Expression of the *gs* gene in nonblastemal regions

In accordance with the abovementioned RT-PCR results, we found by in situ hybridization analysis in the present study that *gs* expression was not restricted to the blastemal regions (Figs. 2a and 3a) but that less intense but clear expression could be observed also in the nephridia (Fig. 3a,b), chloragocytes (Figs. 2a, 3a–c), gut epithelium (Fig. 3c), epidermis of the nonblastemal regions of regenerating fragments (Fig. 3a), and also in intact worms (Fig. 3d,e). Nephridia are the excretory organs of oligochaete annelids and the chloragocytes (chloragogen cells) are specialized peritoneal cells of the intestine that participate in the metabolism, storage, and excretion of glycogen and lipids, thus resembling the vertebrate liver in terms of function (Jamieson 1981; Edwards and Bohlen 1996). The *gs* expression levels in these tissues were observed to be variable among the specimens examined and also among regions within the same specimen (Figs. 2a and 3c). We were unable to find any correlation between this pattern and a specific gut region or specific condition of our specimens, although an uneven distribution of glutamine synthetase has been reported previously in the mammalian and fish digestive tracts (James et al. 1998; Mommsen et al. 2003). Intense *gs* expression was also found in the spermatids in the seminal vesicles and in oocytes in the ovisacs in sexually mature worms (Fig. 3e).

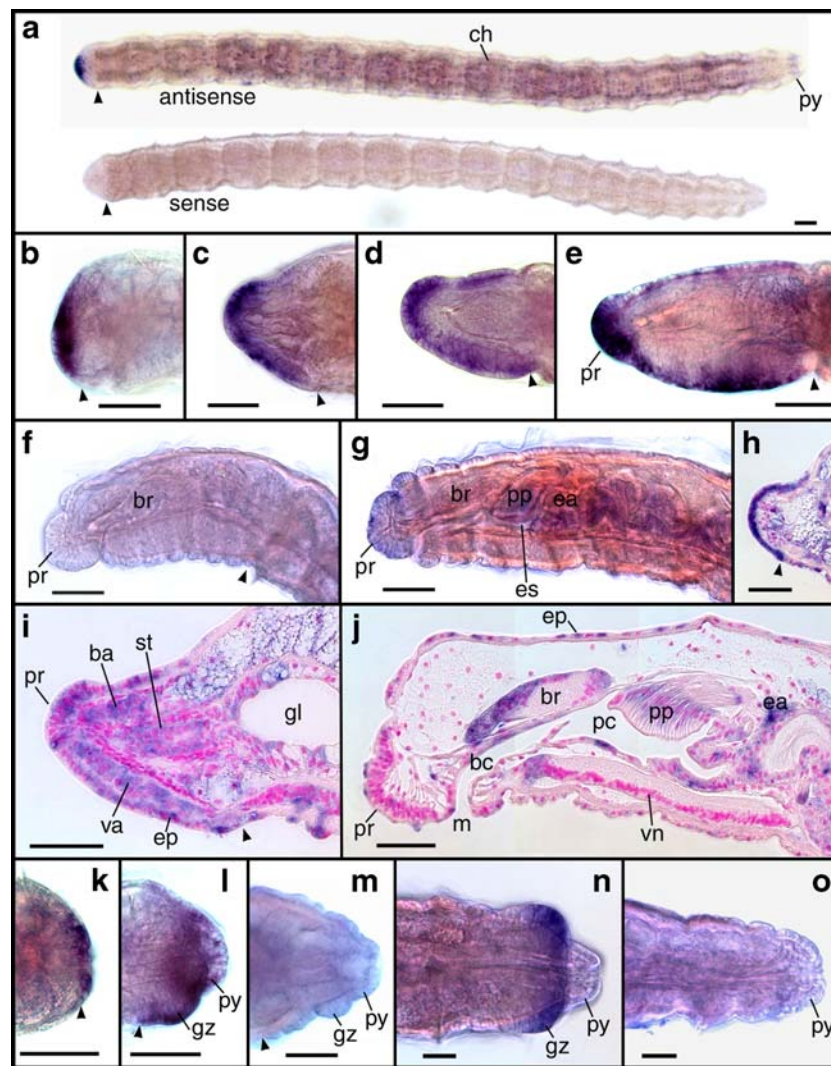
#### General comparisons between the distribution of glutamine synthetase in different organisms

Glutamine synthetase is found in a wide range of organisms, including microorganisms, plants, and animals and is known to be expressed in a tissue-specific and developmentally controlled manner. In murine organs, glutamine synthetase activity is highest in the liver, followed in descending order by the epididymis, testes, uterine tube, stomach, kidney, brain, and adipose tissues (van Straaten et al. 2006). High glutamine synthetase expression in the brain, kidney, and liver has also been reported in fish (Murray et al. 2003). In *Xenopus*, *gs* transcripts have been detected in the ovary, kidney, eye, muscle, and embryo but

<i>E. japonensis</i>	-----MAEVLDKAITRHYLSLPQDPGKVVQVYVWIDGTGENLRCKTRTVDFIPKKAEELEPVWNFDGSSTGQAEGNSDVIYL
sea anemone	---MSLHDKRVTSDKTCVLERYSKLDQGT--SVQALYIWIWIDGTGEGIRCKTKTLEKRPDSIEDLPIWNFDGSSTAQAEGHNSDVIYL
nematode	---MAQSLRSQFATDKQVLQKFLNLDQKG--MYQALYIWIWIDGSGENIRAKTRTFDFEPTDPEKLPFWNFDSSTGQAEGADSDVIYL
Pacific oyster	-----MTD-----KSGKTVLQRYMRIPODDRVMCYELWVDGSGEGIRSKCRTLDPEPKPKCEPVWNYDGSSTYQAEGNSDMYL
fruit fly	MHSAILEDSPNARINKTILDRYLSLPLQENIVQATYVWIDGTEDLRCKDRFLDFIPQSPKELPVWNYDGSSTYQAEGNSDVIYL
sea urchin	-----MGEY--SAATDKTVSDRYLTLDTDG--HCQVMYCWVDGSGENLRACKTKTMDVPEKASDCPVWNFDSSTDQSEGSNSDMFL
zebrafish	-----MATSASSKLSKAMKQYMDLPQGE--KVQVMIYIWIWIDGTGEGLRCKTRTLDSEPKSVEELPEWNFDSSTYQAEGNSDMYL
clawed frog	-----MSVSHSRLNKGVREQYMKLPQGE--KVQVTVVWIDGTGEGVRCRTRTLDQEPKTIDELPEWNFDSSTHQAEGNSDMYL
chicken	-----MATSASSHLSKAIKHYMKLPQGE--KVQAMYIWIWIDGTGELRCKTRTLDHEPKSLEELPEWNFDSSTFQAEGNSDMYL
mouse	-----MATSASSHLNKGIKQMYMSLPQGE--KVQAMYIWIWIDGTGELRCKTRTLDCEPKVEELPEWNFDSSTFQSEGSNSDMYL
<hr/>	
<i>E. japonensis</i>	HPVALFNDPFRFRGNKIALCETYKYNNKPTETNHRKSCKEVME--RAQAEHPWFGIEQEYTLTDVDDKHPYGWPKLGCPPGQPPYY
sea anemone	HPVAIFKDPFRGGENILVLCETYTHDHPKTHNNRKTCMNIMNDPEVKSSHPWFGIEQEYTLTDIDGHPLGWPKGGFPQPQPPYY
nematode	KPVAIYDPFRQGNKLMVCETYDNKKKPTATNYRQRCKEVME--KAADQHPWFGMEQEYTLTDIDGHFPWPKNGFPQPQPPYY
Pacific oyster	HPVALYSDPFRMGKNKLMCEVFKYNGKPAESNNRKSCEVCE--KAAEEIPWFGIEQEYTLTDVDDKHPWPKNGFPQPQPPYY
fruit fly	YPVAIYKDPFRFRGNILVMCDTYKFDGTPDTNKRKTCLEVAN--KCAEEEPWFGIEQEYTLDFDGHPLGWPKNGFPQPQPPYY
sea urchin	KPCALFRDPFRFRGNKLMCEVYKYDWRPAETNYRKTCEIMT--RAADTEPWFGEQEYTLTGSDKWPFGWPKGGFPQPQPPYY
zebrafish	FPQAMFRDPFRKDPNKLVLCDVLYKNHKEPAETNLRQSCCKIMD--MVQNQHPWFGMEQEYTLTGTDGHPFGWPSNGFPQPQPPYY
clawed frog	IPVQMFDPFCCLDPNKLVMCEVLKYNRKSATNLRHTCKKIME--MVNDHRPWFGEQEYTLTGINGHPYGPWENGFPQPQPPYY
chicken	RPAAMFRDPFRKDPNKLVLCEVFKYNRQSADTNLRHTCRIMD--MVSNOHPWFGMEQEYTLTGTDGHPFGWPSNCFQPQPQPPYY
mouse	HPVAMFRDPFRKDPNKLVLCEVFKYNRKAETNLRHICKRIMD--MVSNOHPWFGMEQEYTLTGTDGHPFGWPSNGFPQPQPPYY
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<i>E. japonensis</i>	CGVGADKVIYGRDVEAHYRACLAYAGVKICGTNAEVMPAQWVQVGPCEGIEIGDHLWMAFLLHRVAEDFGVVVTLDPKPMSS--NW
sea anemone	CGVGTNKVFGREVVEAHYRACLAYAGVKIAGTNAEVMPAQWVQVGPCEGIEIGDHLWISRYILLHRVAEDFHVVSFDPKPMPGDW
nematode	CGVGANKVYGRDIVEAHYRACLAYAGIKISGTNAEVMPQWVQVGPCEGIEIGDQLWVARYLLQVAAEFVGVASFDCKPIKGDW
Pacific oyster	CGVGANKVYGRDIVEAHYRACLAYAGKVIAGCNAEVMPAQWVQVGPCEGIEISVGDLLWVARYILLHRVAEDFGVIVTFDPKMPGDW
fruit fly	CGVGANKVYARDIVDAHYRACLAYAGIKVSGTNAEVMPAQWVQVGPCEGIEISGDDLLWMAFLLHRVIEEFVGVIVSTLDPKMPGDW
sea urchin	CGVGANKVYGRDIVESHYRACLAYAGVKIAGSNAEVMPAQWVQVGPCLGIEIGDHLWMSRFLHRVCEDFGVIAITLDPKMEGDW
zebrafish	CGVGADKAYGRDVEAHYRACLAYAGVKICGTNAEVMPAQWVQVGPCEGIEIGDHLWVAFLLHRVCEDFGVVASFDPKPIPGNW
clawed frog	CGVGADKVIYGRDVEESHYKACLAYAGIKICGTNAEVMPAQWVQVGPCEGIEIGDHLWVAFLLHRVCEDFGVVATLDPKMPGTGNW
chicken	CGVGADKAYGRDIVEAHYRACLAYAGVKIAGTNAEVMPAQWVQVGPCEGIEIGDHLWVAFLLHRVCEDFGVIVSFDPKPIPGNW
mouse	CGVGADKAYGRDIVEAHYRACLAYAGVKITGTNAEVMPAQWVQVGPCEGIEIGDHLWVAFLLHRVCEDFGVIAITLDPKPIPGNW
<hr/>	
<i>E. japonensis</i>	NGAGAHTNYSTESMRQDGGIKAEIAEIKLSLEHDRHIRMYDPRGGDNRRLVGSFETSSISEFSAGVANRGASIRIPRQVETD
sea anemone	NGAGAHCNYSVTGMRENGMRLIYEAIDKLEKNHDYHIKKYDPKQGDNARRLTGRHETSSIIYQFSGVANRGASVRIPRQCAED
nematode	NGAGCHTNFSTDKMRNPGGIDEIMSAINKLSLVHPQHAIAYDPHGGKDNERRLTGLHETASIDKFSYGVASRASSIRIPRSTDDD
Pacific oyster	NGAGAHTNYSTVMKREDGGIKHIEDAIEIKLSKCHKIKAYDPKGEEDNKRRLTGLHETSSINDFSAGVANRGASIRIPRQVQND
fruit fly	NGAGAHTNYSTKAMREDGGIRDIEKAVAKLSKCHERHIRAYDPKQGDNARRLTGRHETSSINDFSAGVANRGASIRIPRQVND
sea urchin	NGAGAHCNFSTLAMREDGGIDVMEAIKKLGRKHEHIRAYDPKNGEDNARRLTGRHETSSIDDSSGVANRGASIRIPRQCGID
zebrafish	NGAGCHTNFSTKEMREEGGLKHEIEESIEIKLGRHQYHIRAYDPKGGLDNARRLTGHETSININEFSAGVANRGASIRIPRQVQGE
clawed frog	NGAGCHTNFSTESMRVEGGLKHEIEAIEIKLGRHDIYHICVYDPRGGKDNERRLTGQHETSSIIHFSAGVANRGASIRIPRQVQGE
chicken	NGAGCHTNFSTKMRREDGGLKHEIEAIEIKLSKRHQYHIRAYDPKGGLDNARRLTGFHETSSIIHFSAGVANRGASIRIPRNVGHE
mouse	NGAGCHTNFSTKAMREENGLKCEIEAIDKLSKRHQYHIRAYDPKGGLDNARRLTGFHETSININDFSAGVANRGASIRIPRQVQGE
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<i>E. japonensis</i>	KGKYLEDRRPSNCDPYSVTEAIVRTTLLKWD----- 360 aa 100
sea anemone	KGKYLEDRRPSNCDPYSVTEAIVRTTIKNTYC----- 369 aa 67
nematode	GYGYFEDRRPSNCDPYTVTGTALVRTVCLGAERKLSMMYVPSQAPNITKH 385 aa 61
Pacific oyster	KGKYLEDRRPSNCDPYSVVEVLIRTTCLDEC----- 361 aa 71
fruit fly	KGKYLEDRRPSNCDPYSVVEALIRTTCLDE----- 369 aa 70
sea urchin	KGKYMEDRRPSNCDPYRVTEALVRSVLDWET----- 365 aa 68
zebrafish	KKGYFEDRRPSANCDPYAVTEALIRTTCLLNEEGEVPEKYM----- 372 aa 71
clawed frog	GYGYFEDRRPAANCDPYAVTEALVRTTILNETGSETKDYKN----- 373 aa 70
chicken	KKGYFEDRGPANCDPYAVTEALVRTTCLLNETGDEPFYKYN----- 373 aa 69
mouse	KKGYFEDRRPSANCDPYAVTEAIVRTTCLLNETGDEPFQYKN----- 373 aa 69

**Fig. 1** Amino acid alignment of *E. japonensis* glutamine synthetase (EjGS) with representative GSs from various other species. The species and protein ID of the GSs listed here are as follows: *Enchytraeus japonensis* (BAE93509.1), sea anemone *Aiptasia pallida* (AAR36878.1), nematode *Caenorhabditis elegans* (NP\_001041010.1), Pacific oyster *Crassostrea gigas* (CAD90162.1), fruit fly *Drosophila melanogaster* (AAZ41780.1), sea urchin *Paracentrotus lividus* (AAC41562.1), zebrafish *Danio rerio* (AAH66735.1), clawed frog *Xenopus laevis* (AAH46681.1), chicken *Gallus gallus* (AAA48783.1), and mouse *Mus*

*musculus* (AAH15086.1). Amino acid residues are indicated in blue when greater than or equal to 70% of these residues are identical in the ten representative species. The beta grasp domain, catalytic domain, and the regions that are conserved in prokaryotes and eukaryotes are underlined by broken single lines, broken double lines, and thick single lines, respectively. Ligands for  $Mn^{2+}$  ions are indicated by arrowheads. The ATP-binding sites are boxed. The protein length and overall amino acid identities with EjGS are indicated at the end of each sequence

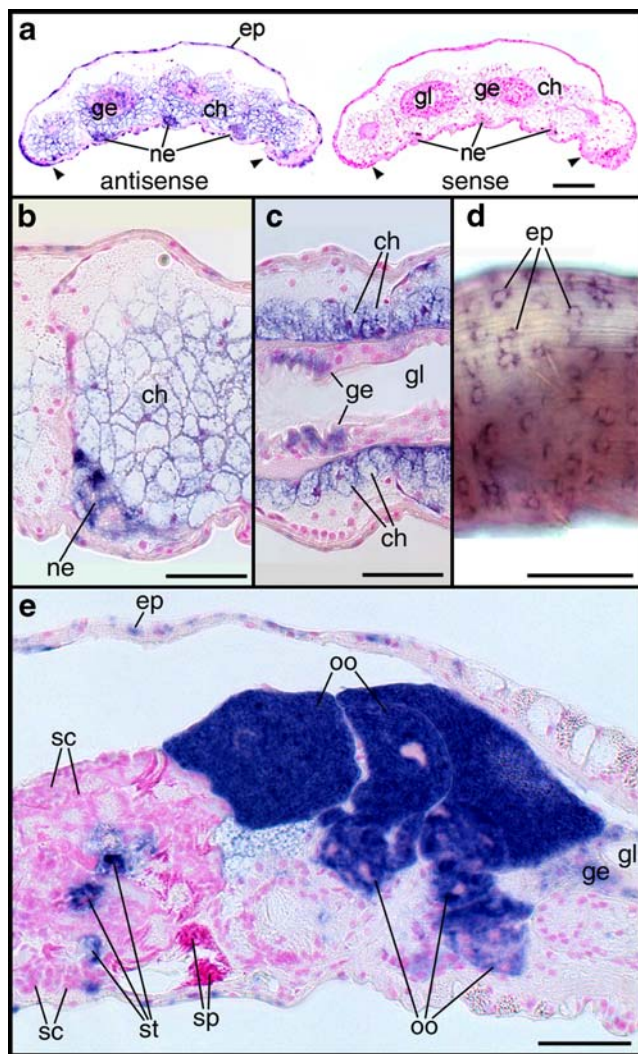


**Fig. 2** Glutamine synthetase mRNA expression during regeneration in *E. japonensis*. Transcripts were detected using whole-mount in situ hybridization (a–g and k–o) or in situ hybridization analysis of paraffin-embedded sections (h–j) using an antisense *gs* riboprobe, with the exception of the lower specimen shown in a. The anterior is to the left, and the ventral side is down in each image. Arrowheads indicate the amputated sites. a Whole-body images of regenerating fragments at about 1 day after amputation with the anterior blastema and an intact tail. The lower specimen was hybridized with a sense probe. b–f Lateral views of anterior blastemas at about 1 (b), 1.5 (c), 2 (d), 2.5 (e), and 4 days (f) after amputation. g Lateral view of an intact

head. h–i Sagittal sections of anterior blastemas at about 1.5 (h) and 2 days (i) after amputation. j Sagittal section of an intact head. k–m Lateral views of posterior blastemas at about 0.5 (k), 1 (l), and 1.5 days (m) after amputation. n–o Ventral views of intact tails in an actively growing (n) and a full-grown individual (o). ba Brain anlage, br brain, bc buccal cavity, ch chloragocyte, ea esophageal appendage, ep epidermal cell, es esophagus, gl gut lumen, gz growth zone, m mouth, pc pharyngeal cavity, pp pharyngeal pad, pr prostomium, py pygidium, st stomodeum, va ventral nerve cord anlage, vn ventral nerve cord. Scale bars, 50  $\mu$ m

not in the brain, heart, liver, stomach, or testis (Hatada et al. 1995). In the case of invertebrates, little information is currently available regarding the expression profile of this enzyme and is restricted to arthropods; *gs* expression has been reported in mosquito midgut epithelium, ovary, and fat body (Smartt et al. 2001) and in crustacean neural tissues (Linser et al. 1997; Allodi et al. 2006; Sullivan et al. 2007). Our present study, therefore, provides novel information regarding the expression of this enzyme in nonarthropod invertebrates, and our results are in general agreement with

those of previous reports; that is, in full-grown *E. japonensis*, *gs* is expressed in (1) chloragogen tissues, which correspond to the vertebrate liver in terms of functionality (Jamieson 1981), (2) nephridia, which are the annelid counterparts of the kidney and uterine tube in vertebrates (Jamieson 1981), (3) the brain and ventral nerve cord, (4) the prostomium where large numbers of sensory cells including photoreceptor cells are present (Jamieson 1981; Edwards and Bohlen 1996), (5) the epithelium of the digestive tract, and (6) spermatids and oocytes.



**Fig. 3** Glutamine synthetase mRNA expression in nonblastemal tissues. Transcripts were detected by in situ hybridization on paraffin-embedded sections (**a–c** and **e**) or by whole-mount in situ hybridization (**d**) using an antisense *gs* riboprobe, except for the *right-hand* specimen in **a**. The anterior is to the *left*, and the ventral side is *down* in each image. **a** Sagittal sections of regenerating fragments at about 0.5 days after amputation. The *right-hand* specimen was hybridized with a sense probe. *Arrowheads* indicate the amputated sites. **b–c** Sagittal sections of intact worms. **d** Whole-mount in situ hybridization analysis of an intact worm showing *gs* expression in the epidermal cells. **e** Sagittal section of a sexually mature worm. *ch* Chloragocyte, *ep* epidermal cell, *ge* gut epithelium, *gl* gut lumen, *ne* nephridium, *oo* oocyte, *sc* spermatocyte, *st* spermatid, *sp* sperm. *Scale bars*, 50  $\mu$ m

#### Possible functions of glutamine synthetase in *E. japonensis*

**Regeneration** The possible involvement of glutamine synthetase in the regeneration of damaged tissue has been reported in only a few cases. These include the observed increase in *gs* expression after peripheral motor nerve injury in mouse (Toki et al. 1998) and also in *Xenopus* tail regeneration in which *gs* expression was found to be upregulated and could be detected in the terminal vesicle of

the regenerating spiral cord (Tazaki et al. 2005). Our present results, together with our previously reported findings, strongly suggest the involvement of glutamine synthetase also in annelid regeneration. In the previous study, we have shown that glutamine synthetase is one of the most abundantly expressed upregulated genes during regeneration in *E. japonensis* (Myohara et al. 2006), and in the present study, our detailed expression analysis by in situ hybridization has revealed strong *gs* expression in the blastemal regions of regenerating *E. japonensis*. It has been reported previously in *Xenopus* that embryos abundantly express glutamine synthetase in the developing nervous system during the gastrula and neurula stages (Hatada et al. 1995). In accordance with this finding, we observed in the present analyses that *gs* is expressed in the presumptive regions of the brain and ventral nerve cord anlagen in the blastema during *E. japonensis* regeneration. This further suggests the involvement of glutamine synthetase in annelid neural regeneration.

**Nerve function** Glutamine synthetase has been shown to play an important role in glutamate transmitter degradation in the nervous system (Derouiche and Frotscher 1991) and is used as a specific glial cell marker in the neural retina (Vardimon et al. 1993). In intact *E. japonensis*, the expression of this enzyme was evident in the brain and ventral nerve cord, suggesting that it has a nervous system function. Its expression in the prostomium may also be attributable to the distribution of numerous sensory cells, probably including photoreceptor cells, in this region (Jamieson 1981; Edwards and Bohlen 1996).

**Cell proliferation** Glutamine synthetase is the only enzyme that can synthesize glutamine, which provides nitrogen for the synthesis of amino acids, purines, pyrimidines, and other compounds required in many critical cellular events including proliferation (Meister 1980). In the rat intestinal epithelium, high concentrations of glutamine synthetase protein and mRNA have been found in the highly proliferative crypt region (Roig et al. 1995). Hence, the high *gs* expression levels that we observed in blastemal cells and in the growth zone in actively growing worms could be explained, at least in part, by a high demand for glutamine during the intense cell proliferation that would be ongoing in these areas.

**Detoxification** Glutamine synthetase is also important for the detoxification of excess glutamate and ammonia by converting them to glutamine, which is nontoxic and can be stored in tissues or circulate throughout the body without causing any harm (Meister 1980). A comparison between the cellular concentrations of glutamine synthetase in various murine organs in a previous report has also revealed

that the highest concentrations of this enzyme positively correlate with the cells that have detoxifying functions (van Straaten et al. 2006). The *gs* expression that we observed in the nephridia and chloragocytes in *E. japonensis* herein could therefore be ascribed to the involvement of these tissues in excretion of nitrogenous waste in oligochaete annelids (Jamieson 1981).

**Macromolecule synthesis** Glutamine is known to be required for the synthesis of certain macromolecules that are present in mucus, such as the hexosamines that lubricate and protect the gut mucus (Weiss et al. 1999). The mucus secreted by epidermal cells covering the earthworm body is also essential for locomotion, gas exchange, and protection in these animals (Jamieson 1981), and hexosamines have been found to be a component of their cuticles (Muir and Lee 1970). We speculate therefore that the *gs* transcripts in the *E. japonensis* epidermal cells have a role in mucus and/or cuticle synthesis in intact and regenerating individuals. In addition, the *gs* expression found in epithelial cells of the buccal and pharyngeal cavities may be attributed to cuticle synthesis in these tissues (Jamieson 1981).

**Gametogenesis** Glutamine synthetase expression in the ovary has been reported in *Xenopus* (Hatada et al. 1995) and in the yellow fever mosquito (Smartt et al. 2001). In oligochaete annelids, the testes and ovaries are small organs located in the ventral part of the genital segments (Edwards and Bohlen 1996). The spermatogonia that are formed in the annelid testes pass into the seminal vesicles, which contain male cells at all stages of development. Oogonia in annelids are formed in the ovaries and divide to form oocytes, which are shed from the ovaries into the ovisacs. In *E. japonensis*, *gs* transcripts were detectable in spermatids in the seminal vesicles and in oocytes in the ovisacs but not in the testes or ovaries, suggesting that glutamine synthetase may have a role in the later stage of gametogenesis.

In summary, in the present study, we have characterized an annelid *gs* gene and shown that it is expressed in a tissue-specific manner in regenerating and intact *E. japonensis*. To further elucidate the actual functions of this gene, additional studies including RNAi gene knockdown experiments will be needed in the future. Ongoing investigations in our laboratory of other regeneration-associated upregulated genes, several of which are novel (Myohara et al. 2006), should also shed important new light on the network of biological pathways involved in the regenerative processes in *E. japonensis*.

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