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Direct Evidence That Extracellular Giant Hemoglobin is Produced in Chloragogen Tissues in a Beard Worm, *Oligobrachia mashikoi* (Frenulata, Siboglinidae, Annelida)

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In *Oligobrachia mashikoi*, a mouthless and gutless polychaete known as a beard worm, sites of production of extra-cellular giant hemoglobin were examined with whole-mount in-situ hybridization and semi-quantitative RT-PCR. An RNA probe was prepared from mRNA of the A2-globin subunit. Clear signals were obtained from a peritoneal membrane covering the trophosome in the posterior body in all seven individuals examined in this study. In addition, weak signals were observed in the peritoneal membrane covering tissues in the middle part of the body in some individuals. Furthermore, in one individual, signals were obtained in complicated bodies invaginated into the dorsal vessel from a peritoneal membrane that also released signals. The results of RT-PCR regarding the expression levels of four kinds of globin-subunit genes suggest that the main site of hemoglobin production is the peritoneal membrane in the posterior body.

Key words: beard worm, giant hemoglobin, chloragogen tissue, in-situ hybridization, RT-PCR

INTRODUCTION

Frenulata polychaetes (Annelida: Siboglinidae), which lack a mouth and a digestive tract, harbor chemosynthetic bacteria and exist on carbohydrates produced by symbionts (Southward, 1993). This animal group has been found in cold seeps scattered on fiord bottoms and at abyssal depths. Since a small quantity of hydrogen sulfide is always generated in these habitats, the polychaetes have a certain degree of tolerance to this gas (Schulze and Halanych, 2003). They possess a closed blood-vessel system and have a large quantity of extracellular hemoglobin in the blood (Southward, 1993). In *Oligoblachi mashikoi*, the binding affinity of hemoglobin to oxygen has been reported to be extremely high (Nakagawa et al., 2005; Aki et al., 2007). This result seems to reflect the fact that these polychaetes inhabit a weakly reductive environment in which a small quantity of toxic gas is produced (Tervilliger et al., 1987).

Sequence analyses recently revealed that the giant hemoglobin (ca. 400 kDa) of *O. mashikoi* is composed of four kinds of globin subunits (A1, A2, B1, and B2) (Nakagawa et al., 2005). Furthermore, Numoto et al. (2005 a, b) succeeded in analyzing the three-dimensional structure of this hemoglobin by X-ray crystallography, and this analysis suggested the positions of unpaired free cysteine residues to which the sulfur atom in hydrogen sulfide should bind. In addition, we clarified that in *O. mashikoi*, the bacteria oxidize sulfur (Aida et al., 2008). All these data imply that inside the host body, hydrogen sulfide is transported to the chemosynthetic symbiont as a non-toxic molecule bound to free cysteine residues in hemoglobin. Furthermore, we have determined that in *O. mashikoi*, four kinds of globin cDNAs occupy about 24% of a cDNA library synthesized from total RNA from the posterior body, which contains the trophosome (Sasayama et al., 2006, unpublished data). It is thus clear that hemoglobin is physiologically important in *O. mashikoi*.

Similarly, in other siboglinid polychaetes, especially in Vestimentifera (tubeworms), which prefer to inhabit an environment with higher levels of hydrogen sulfide, hemoglobin plays an important role in respiration. Nevertheless, exact and direct information on the location of hemoglobin production sites is lacking for siboglinid polychaetes, except for data obtained in ultrastructural observations. In this study, whole-mount in-situ hybridization with a probe to detect mRNA of the A2-globin subunit was used to identify the production sites of hemoglobin. After the detection of signals, the whole-mounted tissues were embedded in paraffin and sectioned to confirm the exact location of the signals. In addition, the gene expression levels of the four globin subunits were examined by using the reverse-transcription polymerase chain reaction (RT-PCR)

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method in tissues taken from several regions of the body.

MATERIALS AND METHODS

Sampling and fixation

Oligoblachi mashikoi worms were collected from a 25-m depth at the bottom of Tsukumo Bay by using dredge gear as described earlier (Sasayama et al., 2003). Fixation was performed by using the method of Deguchi et al. (2007). The body of the worm was pushed out from its tube with a syringe and fixed in 4% paraformaldehyde/PBS solution at 4°C for 16 hrs. Specimens were rinsed twice with PBT and then dehydrated with 25%, 50%, and 75% ethanol/PBT and 100% ethanol, each step for 5 min on a shaker. These procedures were all conducted at 4°C, and the specimens were then preserved at –20°C. In this study, seven individuals were used.

Synthesis of DIG-labeled RNA probes

A fragment from nucleotide positions 199 to 339 of the A2-globin subunit was amplified by PCR by using a plasmid carrying the A2-globin cDNA (Nakagawa et al., 2005) as a template and a set of primers: an A2-forward primer (5'-AGATAAGCTTGGTAA TGACATCGATTCTTC-3') containing a *Hind* III site (underlined), and an A2-reverse primer (5'-TATAGAGCTCACGAGAGTCATG CTGAGA-3') containing a S*ac* I site (underlined). The nucleotide sequence of the PCR-amplified region codes for the hem pocket, including the proximal and distal histidine residues of the A2-globin, and the sequence similarity to A1-, B1-, and B2-globin cDNA sequences is 52.5%, 51.1%, and 54.6%, respectively. Amplified DNA fragments were cut with *Hind* II and *Sac* I and cloned into pSPT18 (Roche Applied Science, Tokyo) cut with the same restriction enzymes; as a result, the insert comprising nucleotide positions 199 to 339 of the A2-globin cDNA was flanked on either side by the SP6 and T7 promoters in pSPT18. The sequence was confirmed by DNA sequence analysis. A region containing the SP6 promoter and the insert was amplified by PCR using primers 5'-CGACTCTGACGGCAGTTTACGAGA-3' and 5'-ACGAGAGTCATG CTGAGAATTCAAACG-3', and the amplified DNA fragments were used to make a sense DIG-labeled RNA probe. A region containing the T7 promoter and the insert was amplified by PCR using primers 5'-AGTATATACACTCCGCTATCGCTACG-3' and 5'-GGTAATGA CATCGATTCTTCTG-3', and the amplified DNA fragments were used to make an antisense DIG-labeled RNA probe. The DIG-labeled RNA probes were synthesized with a DIG RNA Labeling Kit (SP6/T7) (Roche Applied Science, Tokyo). The sizes of both the sense and antisense probes were confirmed by 1.6% denaturing formaldehyde agarose gel electrophoresis.

Although the antisense DIG-labeled RNA probe might bind to A1-, B1-, and B2-globin mRNAs as well as the A2-globin mRNA in an in-situ hybridization analysis, this would not prevent detection of the sites of synthesis of globin molecules, since the genes for the four subunits must be expressed in the same cells and at the same levels.

Whole-mount in-situ hybridization

Whole-mount in-situ hybridization was conducted according to the method of Deguchi et al. (2007). Preserved specimens were cut into three pieces, each 1 cm long: a forepart including the heart; a middle part anterior to the girdle, not including the trophosome; and a posterior part posterior to the girdle, including the trophosome. Before hybridization, each piece was re-hydrated in a descending ethanol/PBT series and then transferred onto an "in-situ tip" (Aloka, Tokyo). All subsequent procedures were conducted on a shaker with pieces attached to tips. Each piece was treated with a solution of proteinase K (20 μg/mL) in PBT and re-fixed with 4% paraformaldehyde/0.2% glutaraldehyde in PBT. Pre-hybridization and hybridization were then conducted; detailed procedures are described in Deguchi et al. (2007). Each piece was washed and then incubated overnight with anti-digoxygenin-AP antibody. Finally, hybridization signals were developed in an overnight incubation at 4°C, and colorization was stopped with PBT. Each piece was cleared in a glycerol/PBT series. Pieces that showed positive signals were embedded in paraffin and sectioned at a thickness of 8 μm to examine the exact locations of the signals. The sections were counterstained with eosin only.

RT-PCR analysis

To compare the expression levels of four globin-subunit genes in five regions of the body, the body of an adult worm was cut into five pieces: 1) a forepart including the heart and tentacles, 2) an anterior part including a dorsal ciliated portion, 3) a middle part including unpaired papillae, 4) a girdle part, and 5) a posterior part including the trophosome (Fig. 1).

Each part was promptly immersed in ISOGEN solution (Nippon Gene, Toyama, Japan), and total RNA was extracted according to the manufacturer's instructions. To prepare cDNA, total RNA (0.5 μg) was reverse-transcribed in a 20-μl reaction solution containing ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and an oligo-dT(21)-*Hin*dIII primer, 5'-GCGAAGCTTTTTTTTTTTTTTT TTTTTTT-3'. One microliter of the resulting cDNA solution was used in PCR analysis. PCR conditions were 95°C for 2 min, followed by 32 cycles of 95°C for 30 sec, 60°C for 30 sec, and 74°C for 1 min. To detect the cDNA of each globin subunit, the following primers were designed on the basis of globin cDNA sequences (Nakagawa et al., 2005): for the A1-globin cDNA, forward A1 primer 5'-GTAT-GTAACCGACTTGAGCAGATCCTGG-3' and reverse A1-*Kpn*I primer 5'-CTGGTACCTTAGCCGGAAATACCGCTAGCA-3'; for the A-2 globin cDNA, forward A2 primer 5'-GATTGCACTTCCCT-CAATCGCCTGTTTGG-3' and reverse A2-*Kpn*I primer 5'-CTGG-TACCTTAACCAGAAATGCCGCTGACA-3'; for the B1-globin cDNA, forward B1 primer 5'-GAATGCTGCAGTAGAGGTGATGC-CGAGG-3' and reverse B1-*Kpn*I primer 5'-CTGGTACCTTA-CAAGCCTGCACCAATACCA-3'; and for the B2-globin cDNA, forward B2 primer 5'-TCGAGCTGTTGTTCCTCCGAGGCAGG-3' and reverse B2-*Kpn*I primer 5'-CTGGTACCTTACAAGCCTGCT-GAGATGCCG-3'.

Actin gene mRNA was used as an internal reference. To detect the actin cDNA of *O. mashikoi*, primers were designed on the basis of sequence information in an *O. mashikoi* cDNA library (Sasayama et al., 2006, unpublished data): actin forward primer 5'-GCCCCA-GAGGAGCACCCAGT-3' and actin reverse primer 5'-CATCTCCT-GCTCGAAGTCGAG-3'.

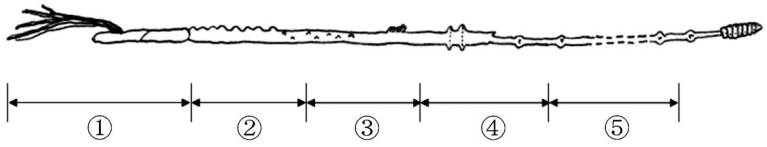

Fig. 1. Diagram showing the regions of *Oligobrachia mashikoi* examined by RT-PCR.

RESULTS

Observations by whole-mount in-situ hybridization

When the sense probe was used, no signals were obtained in any part of the body (Fig. 2A, B). When the anti-sense probe was used, weak signals were detected in one individual, at the root of the tentacles and in subcutaneous regions of the forepart (Fig. 3A). In the middle part, which did not include the trophosome, weak signals were observed in the membranous tissue in some individuals (Fig. 3B). In the posterior part, including the trophosome, intense signals were observed in the membranous tissue in all seven individuals examined (Fig. 3C).

When the whole parts were sectioned, it was determined that signals in the forepart were localized in the peritoneal membrane interspersed with muscle tissue (Fig. 4A). In serial sections, signals were observed in a compact body in the dorsal blood vessel (Fig. 4B, C). We found one portion that suggests that this pocket-like structure originates as the invagination of the peritoneal membrane into the dorsal blood vessel (Fig. 5), and we assumed that this structure was the so-called “heart body” of polychaetes. In the middle part, which did not include the trophosome, paraffin sections confirmed that the weak signals were in the peritoneal membrane (Fig. 6). In the posterior part, including the trophosome, the intense signals were localized in the peritoneal membrane covering the dorsal side of the trophosome (Fig. 7).

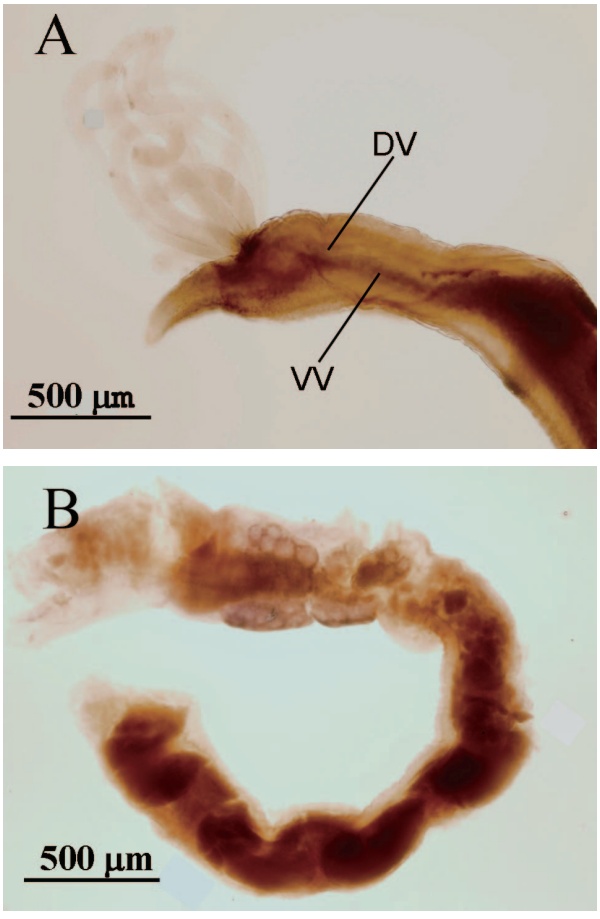

Fig. 2. **(A)** Whole-mount in-situ hybridization of the forepart, using a sense probe. DV, dorsal vessel; VV, ventral vessel. **(B)** Same with a sense probe, but in the middle part, which does not include the trophosome. In both photographs, the dense brown color is blood clotted in the vessels.

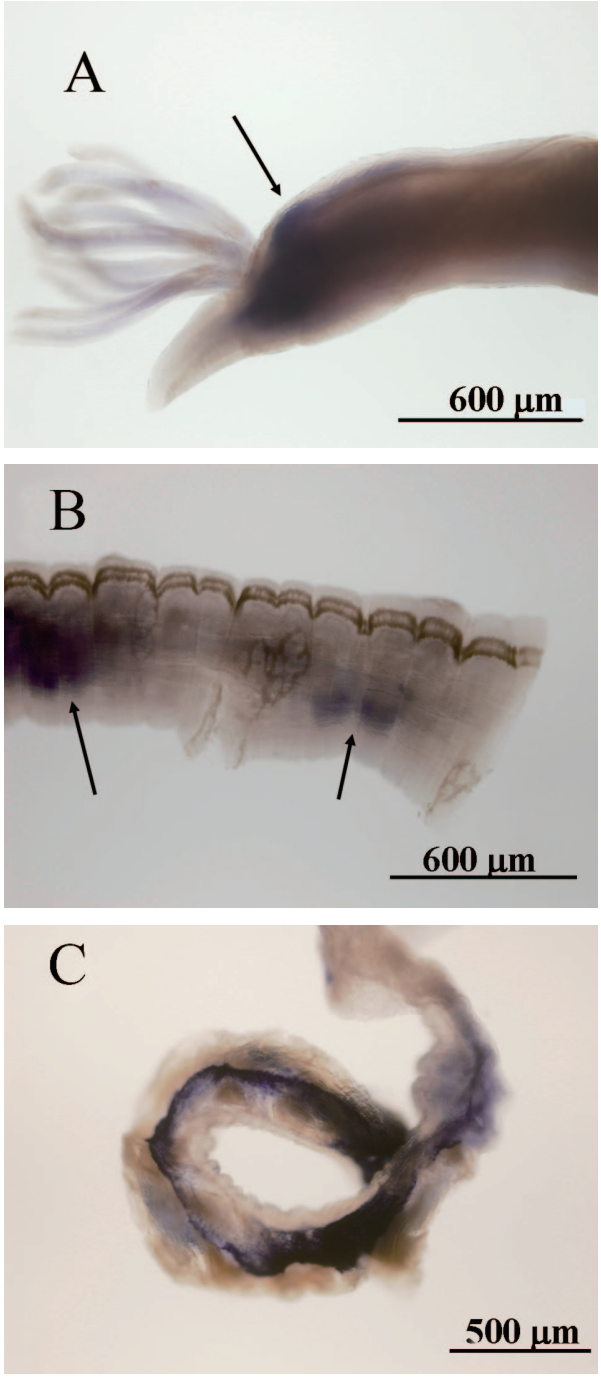

Fig. 3. **(A)** Whole-mount in-situ hybridization of the forepart, using an anti-sense probe. **(B)** Same with an anti-sense probe, but in the middle part. In both photographs, dense purple regions are evident (arrows). **(C)** Same with an anti-sense probe, but in the posterior part that includes the trophosome. Intense signals are evident in the membranous tissue.

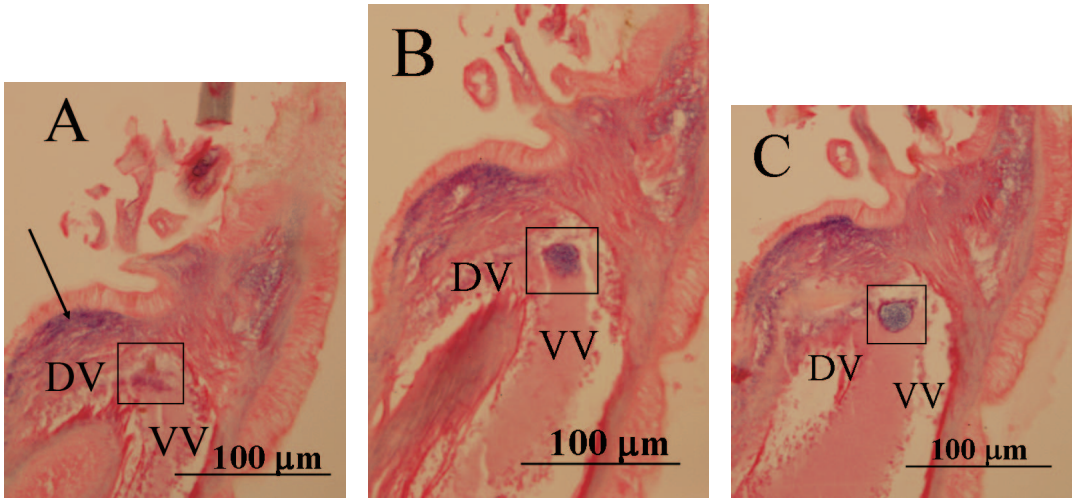

Fig. 4. **(A)** Paraffin section of the forepart of a specimen used for whole-mount in-situ hybridization. The arrow indicates a signal in the peritoneal membrane. In the square, a pocket shows a weak signal that appears to become gradually stronger in subsequent sections **(B, C)**. DV, dorsal vessel; VV, ventral vessel.

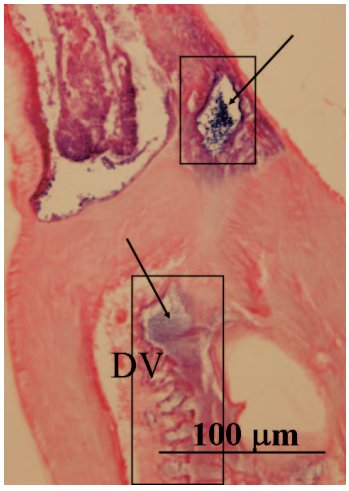

Fig. 5. Section suggesting that the complicated pocket-like regions in the dorsal vessel originate in the peritoneal membrane. The arrows in the squares indicate that the two regions are connected. DV, dorsal vessel.

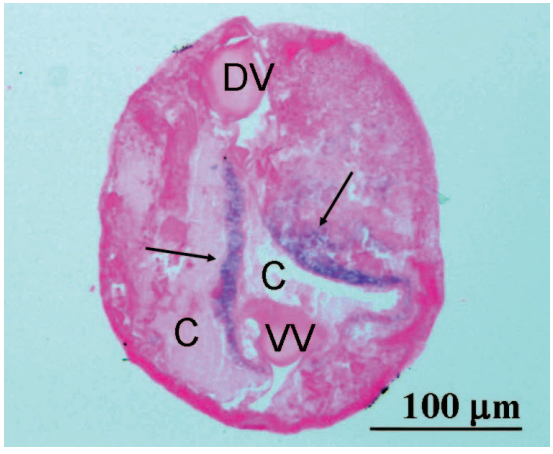

Fig. 6. Section showing signals in the peritoneal membrane (arrows). C, coelom; DV, dorsal vessel; VV, ventral vessel.

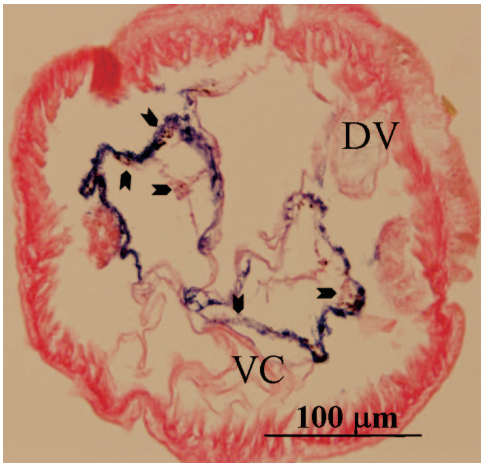

Fig. 7. Section showing intense signals in the peritoneal membrane in the posterior part that includes the trophosome. The arrowheads indicate bacteriocytes. DV, dorsal vessel; VB, ventral vessel.

Comparison of the expression levels of four kinds of globin-subunit genes among five regions of the body

All four globin-subunit genes (A1, A2, B1, and B2) are expressed throughout the body. The expression levels, however, were lower and higher, respectively, in the anterior and posterior parts. Fig. 8 shows the expression levels in each part. The globin-subunit genes did not seem to be expressed in parts 1 or 2; however, after repeatedly conducting RT-PCR, we increased the number of PCR cycles and confirmed that all four genes are expressed in these parts as well. We were unable to distinguish differences in expression levels among the five parts, since amplification of all of the genes attained a plateau. Therefore, we include as Fig. 8 a photograph in which differences are clear. The results of RT-PCR suggest that the heart body and peritonea in the anterior part of *O. mashikoi* play only a minor role

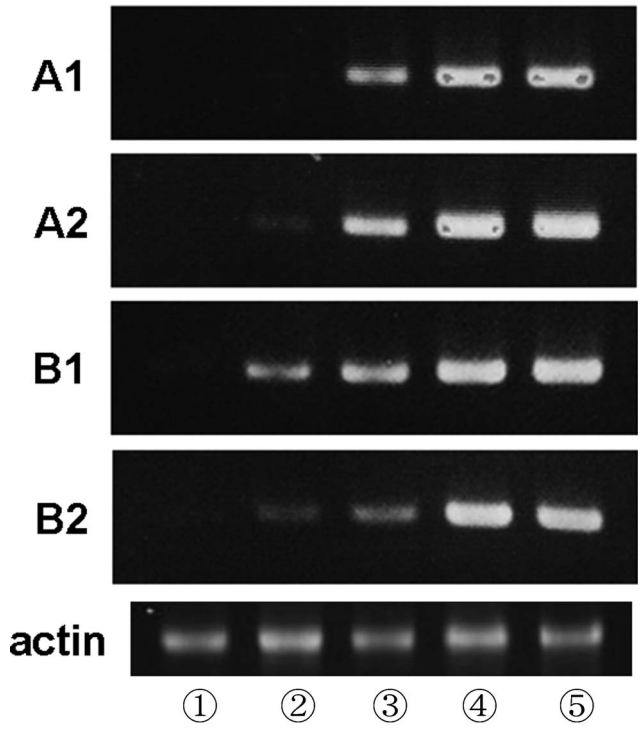

Fig. 8. Electrophoretic pattern showing the expression levels of the four globin-subunit genes (A1, A2, B1, B2) among five regions (1–5) of the body.

in hemoglobin production; instead, the peritoneum covering the trophosome in the posterior part of the body is the main hemoglobin-production site.

DISCUSSION

Hemoglobin-production sites have previously been examined in common polychaetes and earthworms. On the basis of ultrastructural observations, it was surmised that in some species, hemoglobin is made in the chloragogen tissues that cover the digestive tract, also in the heart body, and sometimes in the supraesophageal vessel (Dales and Pell, 1970; Friedman and Weiss, 1980); The heart body originates in the chloragogen tissue (Eisig, 1887), which arises from epithelial cells located on the dorsal side of the visceral peritoneum and covers the digestive tract and dorsal vessel (Barnes, 1974). This tissue plays a role in detoxification and the treatment of waste matter (Roots, 1957). Electron-microscopic observations suggest that, in earthworms, one of the functions of this tissue is to produce hemoglobin (Linthicum et al., 1977). Although these observations are important in identifying the hemoglobin-production sites, there is no direct evidence showing that the chloragogen tissue is an actual production site. After examining ten species of Vestimentifera by using electron microscopy, Schulze (2002) suggested that hemoglobin is synthesized in the intravasal body, which lies along the ventral part of the dorsal vessel and extends from immediately posterior to the heart to the opisthosoma. The intravasal body is regarded as homologous to the heart body (Dall and Pell, 1970). Considering the results of these previous studies, one can conclud that in Frenulata, as in other polychaetes, earthworms, and Vestimentifera, hemoglobin synthesis takes place in tissues originating from chloragogen and/or the heart bodies (Southward et al., 2005).

In *O. mashikoi* (Frenulata), the digestive tract is replaced by the trophosome; therefore, the visceral peritoneum covering the trophosome corresponds to the chloragogen tissue originally covering the digestive tract in polychaetes. In the present study, signals indicating the synthesis of the A2-globin subunit were observed in the heart body and peritoneal membrane.

The results of RT-PCR to examine the expression levels of the four globin-subunit genes also support the observations from in-situ hybridization. All of these globin subunits express weakly in the fore part of the body but strongly in the rear part. Thus, the results of both in-situ hybridization and RT-PCR strongly support the assumptions of former researchers from the viewpoint of molecular biology. Expression of the globin genes in the trophosomal peritoneum suggests that this tissue is important as the production site of hemoglobin, which transports hydrogen sulfide to the symbiotic bacteria and oxygen to the body tissues, respectively. Carney et al. (2007) recently reported that in the hydrothermal vent tubeworm *Ridgeia piscesae*, there are up to 20-fold differences in globin gene expression levels among individuals collected from different sites. In that species, hemoglobin production levels were higher in individuals inhabiting high-temperature environments with high sulfide levels. The habitat of *O. mashikoi* in Tsukumo Bay is limited to a very narrow area (Sasayama et al., 2007). We repeated the RT-PCR experiment using different individuals, but the results were always similar. Therefore, in this species there may be no differences among individuals in globin gene expression levels, since individuals experience uniform environmental conditions.

It is unclear from the present study how hemoglobin flows into the bloodstream. It has been assumed that in some polychaetes, hemoglobin is released directly from the chloragogen tissue into the bloodstream by reverse pinocytosis (Dales and Pell, 1970). The fluid in the coelom of *O. mashikoi* is transparent, in contrast to the blood-red color of the fluid in the dorsal and ventral vessels. This suggests that hemoglobin is not released via the coelomic fluid. Hemoglobin may be secreted from the chloragogen tissue into the capillary blood vessels lining this tissue.

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