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Authors: Ohira, Tsuyoshi, Watanabe, Toshiki, Nagasawa, Hiromichi, and Aida, Katsumi

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Molecular Cloning of a Molt-Inhibiting Hormone cDNA from the Kuruma Prawn *Penaeus japonicus*

Tsuyoshi Ohira[1], Toshiki Watanabe[2]*, Hiromichi Nagasawa[2] and Katsumi Aida[1]

[1]*Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi, Bunkyo, Tokyo 113, Japan*
[2]*Laboratory of Molecular Biology of Marine Organisms, Ocean Research Institute, The University of Tokyo, 1-15-1 Minamidai, Nakano, Tokyo 164, Japan*

ABSTRACT—The crustacean molt-inhibiting hormone (MIH) is released from the X-organ sinus gland complex and suppresses ecdysteroid synthesis by the Y-organ. In the present study, we have isolated a cDNA which encodes a MIH (Pej-SGP-IV) of the kuruma prawn *Penaeus japonicus* in order to study its expression and characterize the structure of its precursor. A cDNA fragment was isolated using RT-PCR with two degenerate oligonucleotide primers that were designed based on the peptide sequence of Pej-SGP-IV, and this fragment was used as a probe to screen an eyestalk cDNA library. In a positive cDNA clone (814 base pairs (bp)), an open reading frame of 315 bp was found; the conceptually translated protein consists of a putative signal peptide (28 residues) and Pej-SGP-IV (77 residues). In Northern blot analysis using a cDNA probe, specific hybridization to a transcript of 0.95 kb was seen in RNA extracted from the eyestalk but not from hepatopancreas, abdominal muscle, brain, thoracic ganglia or abdominal ganglia. The level of the *Pej-SGP-IV* mRNA in the eyestalk did not change significantly during the molt cycle.

INTRODUCTION

In crustaceans, various neuropeptide hormones are released from the X-organ sinus gland complex which is located in the medulla terminalis of the eyestalk (reviewed by Keller, 1992). Several of these hormones, crustacean hyperglycemic hormone (CHH), gonad (vitellogenesis)-inhibiting hormone (GIH or VIH), molt-inhibiting hormone (MIH) and mandibular organ-inhibiting hormone (MOIH) are structurally related and thereby form a peptide family referred to as the CHH family (reviewed by Keller, 1992). The CHHs have been shown to regulate carbohydrate metabolism (Kegel *et al*., 1989; Yang *et al*., 1995, 1997). GIH inhibits the onset of vitellogenesis (Soyez *et al*., 1991) and MOIH inhibits methyl farnesoate (MF) synthesis by the mandibular organ (Wainwright *et al*., 1996).

MIHs have been isolated from the lobster *Homarus americanus* (Chang *et al*., 1990), the shore crab *Carcinus maenas* (Webster, 1991), the kuruma prawn *P. japonicus* (Yang *et al*., 1996) and the crayfish *Procambarus clarkii* (Nagasawa *et al*., 1996). MIHs are presumed to inhibit molting by suppressing secretion of the molting hormone ecdysteroids from the Y-organ except at the premolt stages when ecdysteroids are released to trigger molting (reviewed by Lachaise *et al*., 1993). Along these lines, the MIHs in *C. maenas*, *P. japonicus* and *P. clarkii* have been shown to suppress ecdysteroid secretion from the Y-organ *in vitro* (Webster and Keller, 1986; Terauchi *et al*., 1996; Yang *et al*., 1996). These observations have led to the postulate that the titer of the MIH in hemolymph decreases at the premolt stage. As an approach to address this postulate, we have isolated a cDNA encoding a precursor of the MIH (Pej-SGP-IV) from *P. japonicus* (Yang *et al*., 1996), and used this cDNA to study changes in levels of the *Pej-SGP-IV* mRNA in the eyestalk during the molt cycle. Levels of mRNA did not decrease significantly at the premolt stages, suggesting that the synthesis, modification and/or secretion of Pej-SGP-IV are regulated post-transcriptionally.

MATERIALS AND METHODS

Animals and molting stages

Live kuruma prawns *P. japonicus* caught in Hamanako Lake (Shizuoka, Japan) were purchased from a commercial source, and kept in 500 l tanks at the Fisheries Research Laboratory, Faculty of Agriculture, The University of Tokyo (Maisaka, Shizuoka, Japan). Adult prawns of both sexes were employed in the present study. Prawns were grouped the following five molting stages according to Drach and Tchernigovtzeff (1967) as modified by Hong (1977): early postmolt (stage A), late postmolt (B), intermolt (C), early premolt (D_{0-2}) and late premolt (D_3).

* Corresponding author: Tel. +81-3-5351-6534;
FAX. +81-3-5351-6488.

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Isolation of RNA from the eyestalk, hepatopancreas, abdominal muscle, brain, thoracic ganglia, and abdominal ganglia

Total RNA was isolated from the eyestalk as described by Ohira *et al.* (1997). Preparation of total RNA from the hepatopancreas, abdominal muscle, brain, thoracic ganglia, and abdominal ganglia was performed as described for hepatopancreas total RNA according to Ohira *et al.* (1997). Poly(A)$^+$ RNA was prepared from total RNA using Oligotex™-dT30 super (Roche Japan).

RT (reverse transcription)-PCR

Two degenerate oligonucleotide primers were designed based on the amino acid sequence of Pej-SGP-IV: primer 1 (the sense strand; 5'-GAYATHTAYAARAARGTNGT-3') and primer 2 (the anti-sense strand; 5'-RAACCAYTCRTTRTARAARC-3'). These were derived from the sequences of amino acid positions 15-21 and 44-50, respectively (Fig. 1; Yang *et al.*, 1996, 1997). cDNA was synthesized by annealing 1 pmol of primer 2 to 1 μg of the total RNA from the eyestalk and carrying out a RT reaction using SuperScript™ reverse transcriptase (GIBCO BRL). The RT reaction product was mixed with 100 pmol of each of the two primers, and the following program was used for PCR amplification: 1 min (3 min only for the first cycle) at 94°C, 1 min at 50°C, 2 min at 65°C, for 40 cycles.

Screening of an eyestalk cDNA library

The product of the RT-PCR reaction was subcloned into the pCR™II plasmid using TA Cloning® Kit (Invitrogen). The *Eco* RI fragment (124 bp) of this plasmid was self-ligated using T4 DNA ligase to generate longer fragments, and labeled with [α-^{32}P]dATP by using Multiprime™ DNA labeling systems (Amersham). Approximately 2.1 $\times 10^5$ clones of an eyestalk cDNA library (Ohira *et al.*, 1997) were screened with the labeled DNA. Hybridization was performed at 42°C in 50% formamide, 6 × SSPE (1.08 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, pH 7.4), 1% SDS, 10 μg/ml of denatured salmon sperm DNA. The radio-labeled cDNA probe was added to 5×10^5 cpm/ml. Following hybridization, filters were washed in 2 × SSPE and 0.1% SDS (2 × 15 min at room temperature), 1 × SSPE and 0.1% SDS (2 × 15 min at room temperature), 1 × SSPE and 0.1% SDS (15 min at 42°C), and finally 0.5 × SSPE and 0.1% SDS (15 min at 42°C). Positive plaques were isolated in another round of screening, and the recombinant pBluescript SK- phagemids were rescued from the bacteriophage clones according to the instructions of the manufacturer (Stratagene). Dideoxy sequencing reactions were carried out using a Thermo Sequenase Cycle Sequencing Kit (Amersham), and electrophoresis and signal detection were done on an automatic sequencer SQ-3000/32 (HITACHI).

Northern blot analysis

RNA samples were quantitated by taking the OD_{260} of RNA solutions. Poly(A)$^+$ RNAs (1 μg/lane) from the eyestalk (intermolt stage), hepatopancreas, abdominal muscle, brain, thoracic ganglia, and abdominal ganglia (indiscriminate molting stages) were run on 1.5% formaldehyde-agarose gels, transferred to a NYTRAN membrane (Schleicher & Schuell), and probed with a *Pej-SGP-IV* cDNA fragment (corresponding to nucleotides 1-643 in Fig. 1) which was radiolabeled with [α-^{32}P]dATP. The activity of the probe in the hybridization buffer was 5×10^6 cpm/ml. The final wash of the membrane was done in 0.1 × SSPE and 0.1% SDS at 65°C. RNA ladders (0.16-1.77 and 0.24-9.4 kb purchased from GIBCO BRL) were used as molecular weight markers.

A Northern blot of poly(A)$^+$ RNA of the eyestalk at the early postmolt (stage A), late postmolt (B), intermolt (C), early premolt (D_{0-2}) and late premolt (D_3) stages was probed, as described above, with the *Pej-SGP-IV* cDNA (corresponding to nucleotides 1-643 in Fig. 1), and subsequently with an RT-PCR product M108 (see below). Levels of hybridized transcripts were determined by using a Fujix BAS 1000 computerized densitometer scanner (Fuji Film).

M108 was generated by subjecting total eyestalk RNA to RT-PCR using two degenerate oligonucleotide primers which were derived from two conserved regions of the vertebrate and invertebrate actins (data not shown). An amplified sequence (396 bp) was conceptually translated into 132 amino acids, which exhibited 80-90% identity to actins in other arthropods (unpublished observation). The *Eco* RI restriction sequence (GAATTC) was included in the two primers, and the *Eco* RI fragment of a subcloned RT-PCR product was used as the probe for Northern hybridization.

RESULTS AND DISCUSSION

Total RNA isolated from the eyestalk was subjected to RT-PCR to obtain a cDNA fragment corresponding to amino acid position 15-50 of Pej-SGP-IV (Yang *et al.*, 1996, 1997). The reaction products were subcloned and analyzed for nucleotide sequence. In one of the subclones, the conceptually translated sequence of the insert was identical to that of amino acid in positions 15-50 of Pej-SGP-IV (data not shown). This RT-PCR product that was considered likely to be part of a

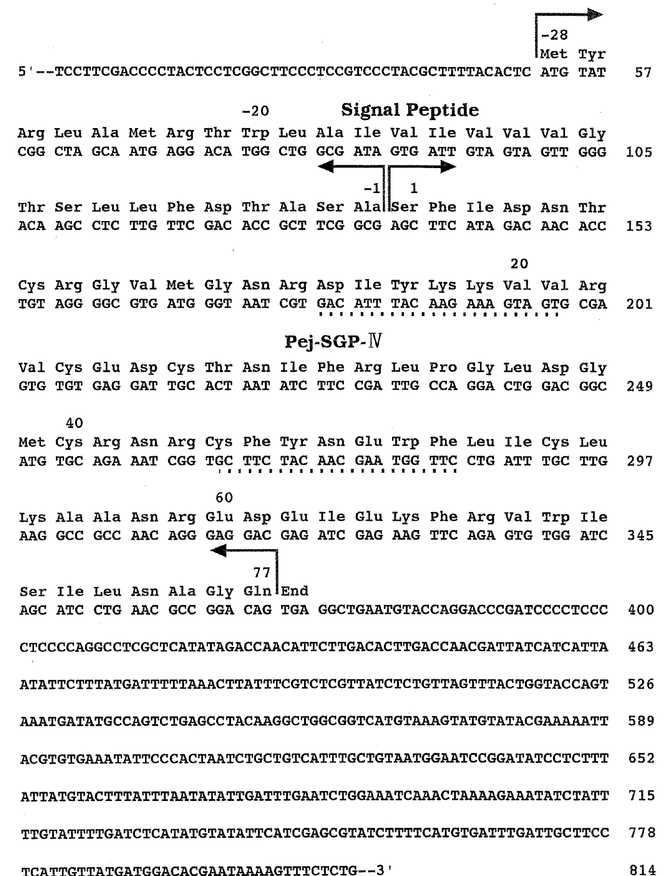

Fig. 1. Nucleotide sequence of a cDNA clone and the deduced amino acid sequence of a Pej-SGP-IV precursor in the kuruma prawn *Penaeus japonicus*. Amino acid numbering starts at the N-terminal residue of the Pej-SGP-IV with the presumptive signal peptide indicated by negative numbers. The deduced amino acid sequence of the Pej-SGP-IV precursor is shown above the nucleotide sequence. The consensus polyadenylation signal is underlined. The dotted lines indicate positions of the primers used for RT-PCR (see text). The cDNA sequence has been deposited in the DDBJ/EMBL/GenBank databases (accession no. AB004652).

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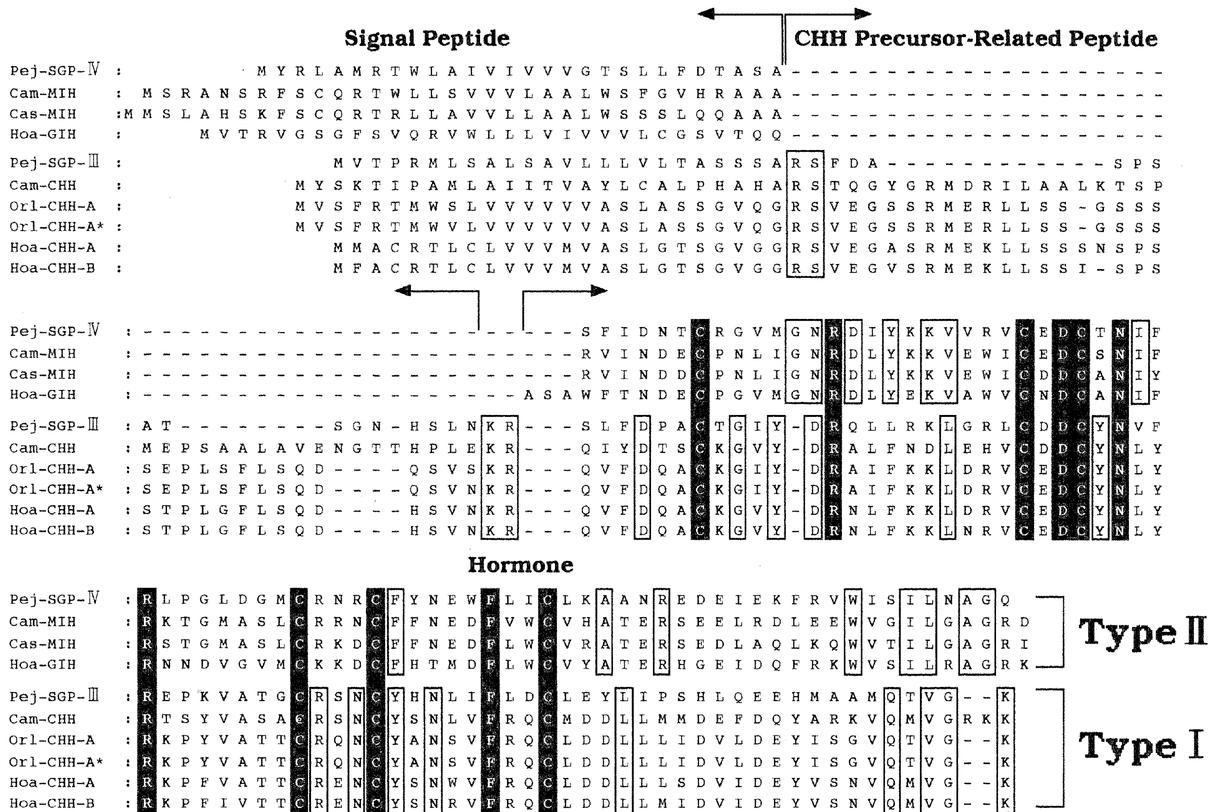
cDNA encoding Pej-SGP-IV, was therefore used as a probe to screen an eyestalk cDNA library. About 20 positive clones were identified, from which ten were randomly selected for further analysis.

Sequence analyses demonstrated that two of these clones encoded a Pej-SGP-IV precursor. We have also sequenced five of the remaining eight clones; these clones did not contain coding sequences for CHH family peptides (unpublished observation). The nucleotide sequence of one of the two which encoded a Pej-SGP-IV precursor is shown in Fig. 1. An open reading frame (ORF) of 315 bp was found, and this ORF was conceptually translated into a putative protein of 105 amino acid residues. The sequence of the 77 residues on the C-terminus side (Ser^1-Gln^{77}) was identical to that of Pej-SGP-IV.



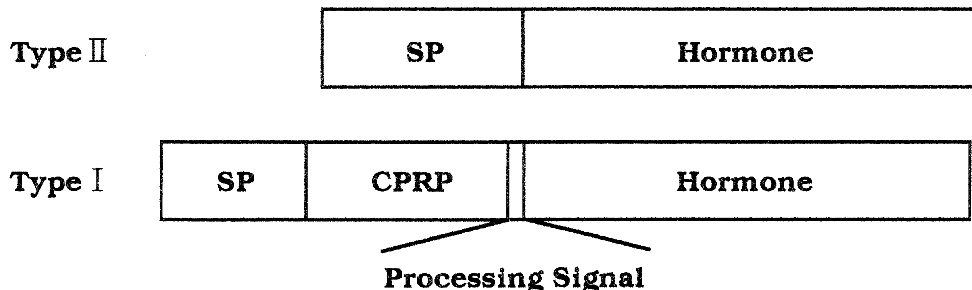


Fig. 2. Alignment of the CHH-family peptide precursors and schematic representation of their organization. (**A**) The amino acid sequences of the precursors of the following CHH family peptides were aligned using the Clustul V program (Higgins *et al.*, 1992): *Penaeus japonicus* MIH (Pej-SGP-IV), *Carcinus maenas* MIH (Cam-MIH), *Callinectes sapidus* MIH (Cas-MIH), *Homarus americanus* GIH (Hoa-GIH), *P. japonicus* CHH (Pej-SGP-III), *C. maenas* CHH (Cam-CHH), *Orconectes limosus* CHHs (Orl-CHH-A and -A*), and *H. americanus* CHHs (Hoa-CHH-A and -B). The filled boxes indicate the amino acids conserved among all precursors. The open boxes indicate the amino acids conserved within either type I or II. (**B**) Schematic representation of the organizations of type I and II CHH-family peptides. SP, signal peptide; CPRP, CHH precursor-related peptide.

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This segment was flanked N-terminally by Met^{-28}-Ala^{-1} and followed by a stop codon. Segment Met^{-28}-Ala^{-1} has a highly hydrophobic core (Trp^{-20}-Val^{-12}) and is therefore likely to constitute a signal peptide (Von Heijne, 1986). This cDNA also includes 5' and 3' untranslated regions (51 and 437 bp, respectively). The 3' region contains a consensus polyadenylation signal (AATAAA) 10 bp upstream from the poly(A) tail (Fig. 1). The nucleotide sequence of the other positive clone was identical to the one shown in Fig. 1, except that it lacked the first 10 bp of the 5' untranslated region.

In crustacean to date, cDNAs encoding the precursors of nine CHH family peptides have been cloned (Weidemann *et al.*, 1989; Klein *et al.*, 1993; De Kleijin *et al.*, 1994a, b, 1995; Lee *et al.*, 1995; Ohira *et al.*, 1997): we aligned the amino acid sequence of the Pej-SGP-IV precursor against those of the nine precursors (Fig. 2A). In the Pej-SGP-IV precursor, no region was found which exhibited sequence similarity to CHH precursor-related peptides (CPRPs), which have been found in the precursors of type I CHH family peptides (see below). Thus, the result of the sequence analysis suggests that the Pej-SGP-IV precursor consists of only the signal peptide and mature hormone.

The CHH family peptides are grouped into two types (type I and II) based on two considerations: the extent of sequence similarity of the mature hormone and the presence/absence of a Gly residue at position 12 of the mature hormone (Yang *et al.*, 1996). Results of the sequence analyses of the nine precursors have shown that the precursors of the two types have distinct organizations: the type I precursors consist of a signal sequence, CPRP and mature hormone, whereas the type II precursors lack a CPRP (Fig. 2B; De Kleijn and Van Herp, 1995). The result of the present study is consistent with the previous results, since CPRP was not found in the precursor of Pej-SGP-IV, a type II peptide (Fig. 2A).

In Northern blot analysis, a cDNA probe hybridized to a band in poly(A)$^+$ RNA from the eyestalk, but not from hepatopancreas, abdominal muscle, brain, thoracic ganglia or abdominal ganglia (Fig. 3). The autoradiogram was over-exposed to show that the *Pej-SGP-IV* mRNA was not detected in the latter five tissues. In a shorter exposure, hybridization was observed as a seemingly single band of approximately 0.95 kb.

We also investigated changes in levels of *Pej-SGP-IV* transcripts in the eyestalk during the molt cycle. The *Pej-SGP-IV* mRNA was detected on a Northern blot of poly(A)$^+$ RNA from eyestalks at five different molting stages (Fig. 4A). The level of hybridization did not change significantly during the molt cycle, as the minimal level observed at the late postmolt stage was about half of the maximal level at the early postmolt and late premolt stages (Table 1). To ensure that the amounts of the loaded RNA samples were comparable, the filter was rehybridized with probe M108 which contained a partial coding

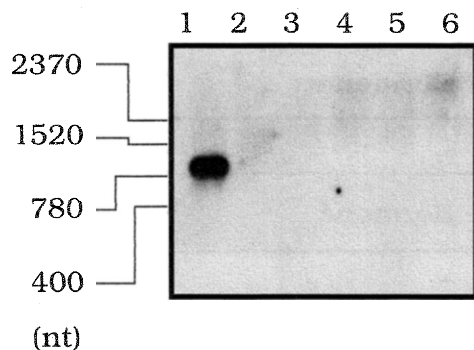

Fig. 3. Tissue specific expression of the *Pej-SGP-IV* mRNA. A Northern blot of poly(A)$^+$ RNA from the eyestalk (lane 1), hepatopancreas (lane 2), abdominal muscle (lane 3), brain (lane 4), thoracic ganglia (lane 5) and abdominal ganglia (lane 6) was hybridized with a radiolabeled *Pej-SGP-IV* cDNA probe. Position of four molecular weight markers (2370, 1520, 780 and 400 bp) are indicated.

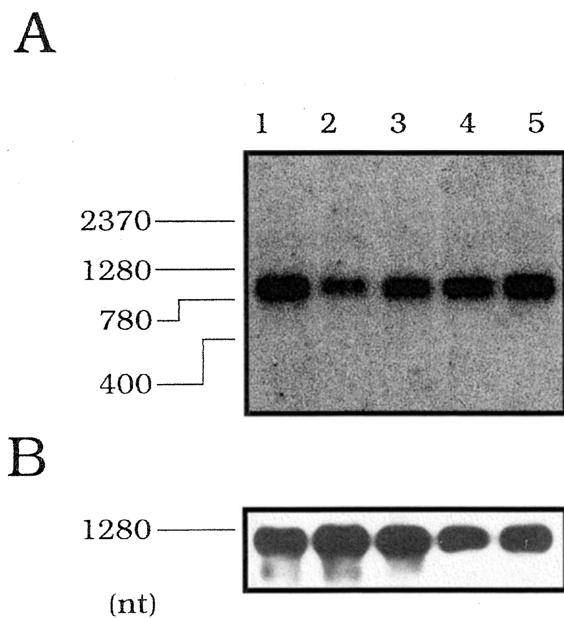

Fig. 4. Changes in levels of *Pej-SGP-IV* mRNA during the molt cycle. A Northern blot of poly(A)$^+$ RNA from the eyestalk during the early postmolt (lane 1), late postmolt (lane 2), intermolt (lane 3), early premolt (lane 4) and late premolt (lane 5) periods was hybridized with a *Pej-SGP-IV* cDNA probe (**A**). The same filter was rehybridized with probe M108 which contained a partial coding sequence of an actin (or actin homologue) of *P. japonicus* (**B**). Positions of four molecular weight markers (2370, 1280, 780 and 400 bp) are indicated.

Table 1. Quantification of relative levels of the *Pej-SGP-IV* mRNA

Molting stage	Relative amount of Pej-SGP-IV mRNA
Early postmolt	100.0
Late postmolt	49.9
Intermolt	65.5
Early premolt	80.3
Late premolt	97.3

Levels of the *Pej-SGP-IV* mRNA in the Northern blot shown in Fig. 4 were quantified (Materials and Methods). The level of *Pej-SGP-IV* mRNA at the early postmolt stage was arbitrarily set at 100.

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sequence of an actin (or an actin homologue) in *P. japonicus* (see Materials and Methods). As shown in Fig. 4B, almost equal levels of hybridization was seen in all five samples.

Pej-SGP-IV has been shown to suppress secretion of ecdysteroids from the Y-organ *in vitro* (Terauchi *et al.*, 1996). Pej-SGP-IV, therefore, is presumed to inhibit molting by suppressing secretion of ecdysteroids except for a premolt stage when ecdysteroids are released to trigger molting. In the present study, we did not observe any decrease in the level of *Pej-SGP-IV* transcripts at the early and late premolt stages. This observation suggests that the synthesis, modification and/ or secretion of Pej-SGP-IV are regulated post-transcriptionally. However, the results of the present study do not exclude the possibility that transcription of the *Pej-SGP-IV* mRNA is repressed for a short duration prior to molting. The length of the duration of the premolt ecdysteroid surge has not been determined in *P. japonicus*. If the surge is brief, then levels of *Pej-SGP-IV* transcripts may decline for a short period, and individuals representing such a stage may not have been represented in the RNA samples (each of the five RNA samples are derived from 5-10 individuals).

It remains to be solved how synthesis, modification and/ or secretion of Pej-SGP-IV are regulated, and how such regulation is related to secretion of ecdysteroids by the Y-organ in crustaceans.

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