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Molecular Cloning of Silkworm Paralytic Peptide and Its Developmental Regulation

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The silkworm paralytic peptide (PP) is a member of the ENF peptide family that exerts multiple biological activities involved in defense reaction and growth regulation. We isolated its cDNA and examined mRNA expression profiles. cDNA encoded 131 amino acids from which the 23-residue PP sequence was found at the C-terminal portion. Immunoblot analysis and paralytic activity assay indicated that inactive proprotein in larval hemolymph was processed into active peptide immediately after bleeding. In the last larval instar, 0.6-kb PP mRNA was expressed in various tissues, of which the fat body was predominant. Its expression in the fat body decreased during the feeding period and then increased during metamorphic process. Juvenile hormone and 20-hydroxyecdysone upregulated its expression. At the embryonic stage, 1.5-kb mRNA, in addition to 0.6-kb mRNA, was expressed from 1 day after oviposition to hatching. PP was thus expressed stage-specifically under hormonal control.

Key Words: **paralytic peptide; ENF peptide family; silkworm; *Bombyx mori;* juvenile hormone; ecdysteroid; embryogenesis; metamorphosis.**

Insect hemolymph contains a variety of biologically active peptides. The paralytic peptide (PP) identified from the larval hemolymph of the silkworm, *Bombyx mori*, is a 23-residue peptide that causes rapid, rigid paralysis when injected into the larval hemocoel (1). PP presumably exists in hemolymph as a biologically inactive precursor, and once bleeding occurs, PP is activated to immobilize larva and prevent hemolymph loss (1, 2).

B. mori PP has counterpart molecules in other lepidopteran insects. They were originally identified based on distinct functions, but were very similar in primary sequence (>70%) and now are referred to as the ENF peptide family based on the consensus sequences of their amino-termini (Glu-Asn-Phe-) (3). They include growth-blocking peptide (GBP) of *Pseudaletia separata* that inhibits larval growth and delays the larval-pupal transformation (4), plasmatocyte-spreading peptide (PSP) of *Pseudoplusia includens* that stimulates hemocytes (5), cardioactive peptide of *Spodoptera eridania* that increases frequency of the heart beat (6), and a number of paralytic peptides isolated from several moth species (2, 7, 8). In addition to these activities, *Pseudaletia* GBP stimulates proliferation of both mammalian and insect cells (9) and *Antheraea yamamai* PP induces egg diapause in *B. mori* (10). Recent work has shown that many ENF peptides have multiple overlapping functions (3, 6, 10, 11). They are thus assumed to play multiple important roles in defense reactions, growth regulation, and homeostasis of lepidopteran insects and are regarded as an insect cytokine.

We found that *B. mori* PP also has multiple biological activities, including mitogenic, hemocyte-spreading, and growth-blocking activities, in addition to paralytic activity (unpublished results). To assess the physiological functions of PP in more detail, we cloned its cDNA and examined mRNA expression profiles. The cDNA sequence indicated that PP was synthesized as the carboxyl-terminal portion of a larger precursor protein as are other ENF peptide (9, 11–13). PP mRNA was expressed during embryonic and postembryonic stages under the control of JH and ecdysteroids.

MATERIALS AND METHODS

Insects, plasma sampling, and hormonal treatments. Silkworm larvae of an F1 hybrid between C145 and N140 and a nondiapausing strain pnd-w1 were reared on an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) under a 12 h light:12 h dark photoperiod at 25 ± 1°C. pnd-w1 was used only for mRNA expression analysis during the embryonic development. In the larval stage, only females were used except for testis collection.

Hemolymph from newly molted 5th instar larvae was collected into an ice-cold 1.5 ml plastic tube and hemocytes were removed by centrifugation at 500*g* for 1 min. Obtained plasma was incubated at

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25°C up to 60 min and then heated at 95°C for 5 min. After centrifugation at 10,000*g*, supernatant was subjected to paralytic activity assay and Western blot analysis.

On day 0 of the last larval instar, 100 ng of a juvenile hormone analog, fenoxycarb (Sankyo Co., Tokyo, Japan), dissolved in 5 μl acetone was applied topically to silkworms with a micropipet along the dorsal midline. This dose is sufficient to induce nonspinning permanent larvae (14). The same volume of acetone was applied as a control. On day 5 of the last larval instar, 50 μg of 20-hydroxyecdysone (Sigma, St. Louis, MO) dissolved in 20 μl distilled water was injected into the silkworm through the first abdominal leg with a microsyringe. The same volume of distilled water was injected as a control.

Bioassay for paralytic activity. Plasma samples were serially diluted in 20 μl of 20 mM phosphate buffered saline (0.15 M NaCl, pH 7.0) and injected into newly molted 5th instar larvae through the last abdominal leg. Paralytic activity was expressed as a reciprocal of the minimal plasma concentration necessary to cause local contraction within 1 min of injection.

PP cDNA cloning. The cDNA fragment coding for *B. mori* PP was first amplified by reverse transcription (RT)-PCR using degenerate primers. Total RNA was extracted by acid guanidinium-phenol-chloroform method (15) using TRIzol (Gibco BRL, Rockville, MD) and reverse-transcribed with an oligo (dT) primer using Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Forward primer F1 5′-GRNYTNTTYGGNRANATHCA-3′ was designed based on the conserved region among several ENF peptide precursors (9, 11–13) (Fig. 1B). Reverse primers R1 5′-CCRTCNGCNGTNCKYTTRAA-3′ and R2 5′-CANCCNCCNACRAARTTYTC-3′ were designed based on the amino acid sequence of PP (1) (Fig. 1B). The first PCR was conducted with F1 and R1 primers and then second nested PCR with F1 and R2 primers. Annealing was at 50°C in 35 cycles. PCR products were cloned into pGEM-T vector (Promega, Madison, WI) and were then sequenced.

To obtain full-length cDNA, rapid amplification of cDNA ends (RACE) was applied. Poly (A)-rich RNA was extracted from the fat body of newly molted last instar larvae using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). 5′RACE and 3′RACE were conducted using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Reverse primer PP-31 (351–376) was used for 3′RACE and forward primer PP-51 (154–179) for 3′RACE. PCR products were cloned into pGEM-T vector.

A series of the resulting clones were sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) by an automatic DNA sequencer (model 310, Applied Biosystems). Sequence data were edited and analyzed by Genetyx-Mac software (Software Development Co., Tokyo, Japan).

Production of anti-PP antibody and Western blot analysis. *B. mori* PP was synthesized by solid-phase Fmock method (1). Rabbit anti-PP antibody was raised against synthetic PP conjugated with keyhole lympet hemocyanin (Nihon Biotest Co., Tokyo, Japan). For Western blot analysis, protein samples were separated by tricine-SDS-PAGE on 16.5% gel and transferred to PVDF membrane (Bio-Rad, Hercules, CA). The membrane was incubated with anti-PP antiserum in 1:6000 dilution after blocking with 5% skim milk, and then with goat anti-rabbit IgG conjugated with horse radish peroxidase (Zymed, San Francisco, CA). Signals were detected by chemiluminescence using ECL-Plus chemiluminescence detection (Amersham Pharmacia Biotech) and a LAS-1000 luminoimage analyzer (Fujifilm Co., Tokyo, Japan).

In vitro transcription/translation and expression of recombinant protein of PP precursor. The full length of PP cDNA was amplified by PCR and cloned into pGEM-T vector (Promega). Capped RNA was synthesized with Ribomax systems (Promega), and translation was carried with Biotin *in vitro* translation Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

B. mori cell line NISES-BoMo-DZ (16), a kind gift from Dr. Shigeo Imanishi, was cultured at 25°C in IPL-41 medium (Gibco BRL) with 10% (v/v) fetal bovine serum (Gibco BRL). The full coding region of PP cDNA (56–451) was amplified by PCR and inserted into *Kpn*I and *Sac*II sites of the expression vector pIZT/V5-his (Invitrogen, Groningen, Netherlands) that uses the baculovirus IE2 promoter for foreign gene expression. Cells were seeded at 1–5 $\times$ 10^5/well in 24-well plates and transfected with 1 μg of the plasmid/well mediated by a cationic ion lipid, Tfx-10 (Promega) (17). The empty vector pIZT/V5-his was transfected as a control. One week later, the medium was collected and subjected to Western blot analysis.

Northern blot hybridization. Total RNA was extracted using TRIzol (Gibco BRL), 20 μg of each RNA was separated on a guanidine thiocyanate 1% agarose gel (18) and transferred to a Hybond NX nylon membrane (Amersham Pharmacia Biotech). Membranes were hybridized with DNA probes randomly labeled with alpha [α-^{32}P]dATP by a Strip-EZ DNA kit (Ambion, Austin, TX). The RT-PCR clone (134–399) was used as a probe for PP. Hybridization, washing, and stripping of blots were based on the manufacturer's instructions. Signals were detected by a Molecular Imager imaging analyzer (GS-250, Bio-Rad).

RESULTS

Cloning of PP cDNA and sequence comparison. A 266-bp PP cDNA fragment was amplified from the fat body cDNA pool of the newly molted last instar larvae by RT-PCR using degenerate primers. As the deduced amino acid sequence of this PCR product showed predicted similarities to corresponding regions of precursors of GBP/PSP/PP, 5′RACE and 3′RACE were then conducted and the entire cDNA sequence was determined to be 583-bp long (Fig. 1A). Two putative polyadenylation signals (ATTAAA) were located upstream of the poly (A) tail.

The longest open reading frame preceded by in-frame terminator codons encoded 131 amino acids with a calculated molecular mass of 14.3 kDa. The 23 amino acid sequence of active PP was found at its carboxyl-terminus. Active PP is expected to be cleaved off from the precursor protein after the arginine residue as are other ENF peptide (9, 11–13) (Fig. 1B) and many insect neuroendocrine peptides (19). The amino-terminus of the deduced PP precursor protein contained a number of hydrophobic residues, characteristic of a secretion signal, and the most likely cleavage site predicted by the SignalP program (20) (http://www.cbs.dtu.dk/services/SignalP-2.0/) was between residues Ala_{23} and Gly_{24}. The secreted proPP is expected to have a length of 108 amino acids with a calculated molecular mass of 11.9 kDa.

Comparison of putative prepro-protein sequences of PP and other ENF peptides showed that only the C-terminal active peptides and their flanking regions were highly conserved (Fig. 1B). Overall sequence identities between prepro-proteins of PP and other ENF peptides are 30–38%, whereas identities among the active peptides are 74–91%.

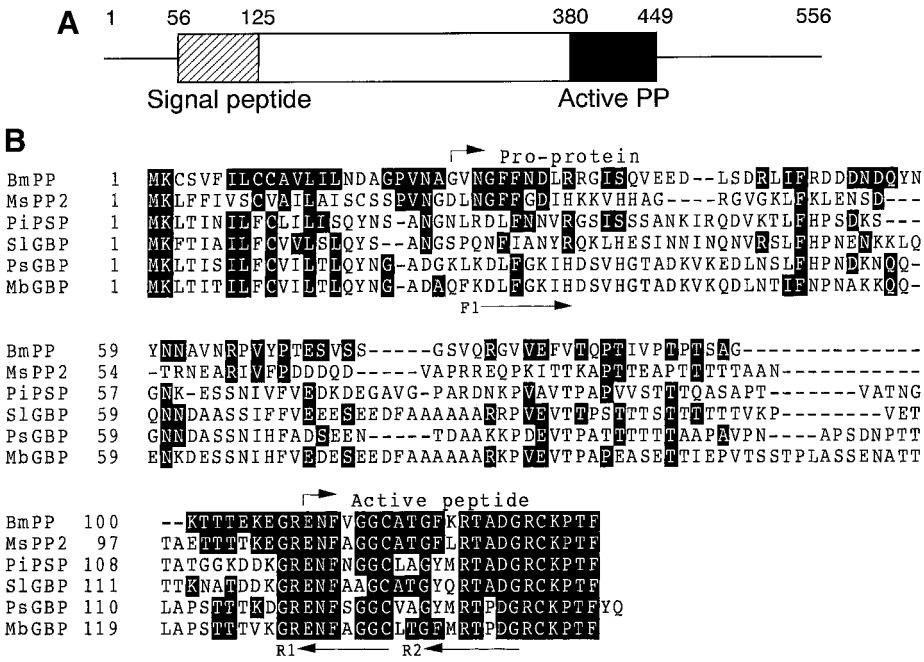

FIG. 1. *B. mori* PP structure. (A) Schematic representation of cDNA and predicted protein sequences of PP. The nucleotide sequence was determined by combining 3 different cDNAs, 5′RACE product (1–350), RT-PCR product (154–379), and 3′RACE product (180–583). Numbers above the figure show positions in cDNA. The putative signal peptide and active 23-residue PP sequence are boxed. Thin lines at both ends are untranslated regions. The sequence data have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence database under the Accession No. AB064522. (B) Sequence comparisons among precursors of *Bombyx* PP (BmPP) and members of the ENF peptide family. Amino acids shared with BmPP are highlighted. Dashes represent gaps introduced to align sequences. Cleavage sites for secretion signals predicted by the SignalP program (20) are conserved among all sequences and depicted as starting positions of pro-proteins. Cleavage sites for active peptides are also well conserved. Arrows indicate positions of degenerate primers used in RT-PCR. MsPP, *Manduca sexta* PP2 (Accession No. AF122899); PiPSP, *Pseudoplusia includens* PSP 1 (Accession No. AF062489); SlGBP, *Spodoptera litura* GBP (Accession No. AB015466); PsGBP, *Pseudaletia separata* GBP (Accession No. S80564); MbGBP, *Mamestia brassicae* GBP (Accession No. AB015465).

Immunoblot analysis and paralytic activity assay of PP molecules. Anti-PP rabbit antiserum reacted strongly with a protein band that migrated at Mr = 15 kDa in the plasma sample prepared immediately after bleeding in Western blot analysis (Fig. 2A). When plasma samples were left at room temperature, this protein decreased rapidly and a smaller immunoreactive protein with an estimated molecular mass of 4 kDa appeared. The amount of 4-kDa protein reached the maximum 10 min after bleeding.

In parallel, temporal change in the paralytic activity in collected plasma was assayed. Freshly collected plasma caused no paralysis in injected larva. Paralytic activity appeared several minutes later and peak activity was observed 10–20 min after bleeding. Thus, generation of paralytic activity coincided well with the amount of 4-kDa immunoreactive protein. These results strongly suggest that this protein is active 23-residue PP, although its estimated molecular mass is larger than the calculated value (2.5 kDa). One hour later, paralytic activity decreased markedly, whereas considerable amount of 4-kDa protein was detected. PP may have been inactivated in plasma by modification of amino acid residues or binding with other proteins.

The full coding region of PP cDNA was expressed in a *B. mori* cell line under the control of baculovirus IE2 promoter. One week after transfection, a broad protein band immunoreactive with anti-PP antibody was detected in the medium by Western blot analysis (Fig. 2B). No immunoreactive protein was detected in cell lysate, indicating that most parts of the synthesized protein were secreted into the medium via its own secretion signal. This secreted recombinant protein should have lost the signal peptide during secretion and therefore correspond to the pro form of PP. Its electrophoretic mobility in SDS–PAGE was very close to that of the 15-kDa immunoreactive protein in fresh plasma, indicating that the plasma 15-kDa protein was proPP, although its estimated molecular mass was also larger than the calculated one (11.9 kDa). The estimated molecular mass (15 kDa) of preproPP synthesized by *in vitro* transcription/translation was well consistent with the calculated size (14.3 kDa) (Fig. 2B).

Expression profiles of PP mRNA. At the beginning of the last (5th) larval instar, the PP probe strongly hybridized with a 0.6-kb message, which coincided well with the PP cDNA (583 bp) in size (Fig. 3). A minor 1.5-kb transcript was also detected in some tissues. PP

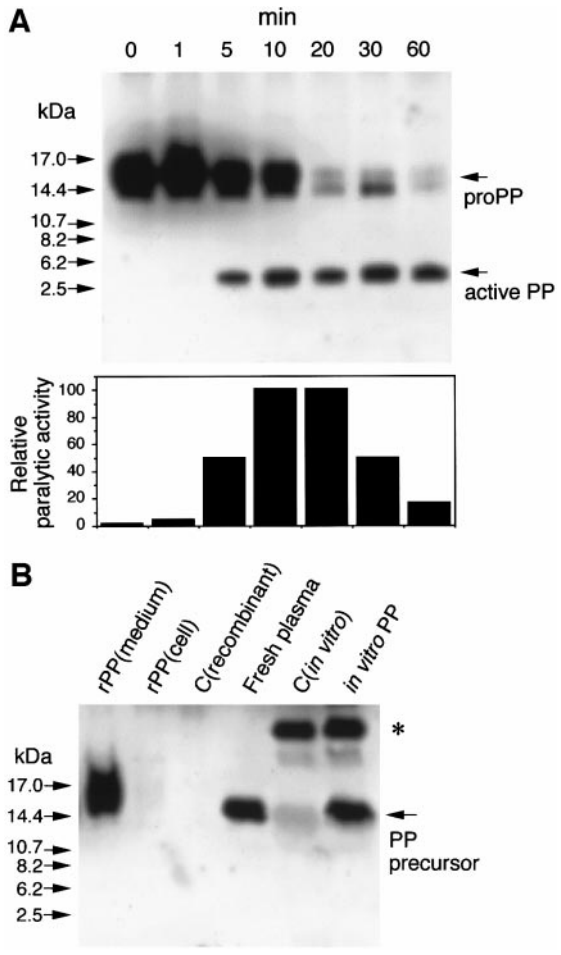

FIG. 2. Immunoblot analysis for PP molecules. (A) Temporal changes in PP processing and paralytic activity in plasma after bleeding. Collected plasma was left at room temperature for the indicated time. Relative paralytic activity is referenced to the value at 10 min (n = 2). (B) Western blot analysis of recombinant PP precursor expressed in a *Bombyx* cell line and *in vitro* translated PP precursor. Empty vectors were used as controls in each experiment. Asterisk indicates nonspecific bands. rPP (medium), recombinant PP precursor secreted in the medium; rPP (cell), recombinant PP precursor in the cell lysate; C (recombinant), medium after empty vector was transfected; C (*in vitro*), control of *in vitro* translation; *in vitro* PP, *in vitro* translated PP precursor.

mRNA was most abundantly expressed in the fat body among the 14 tissues investigated. High expression was also observed in the gonads (ovary and testis), wing disc, central nervous system, muscle, and epidermis. In contrast, little expression was detected in the 3 parts of the silk gland, midgut, and Malpighian tubules. These expression profiles are similar to those of ENF peptides in other moth species (11–13).

Temporal expression profiles of PP mRNA were studied in the fat body, wing disc, and midgut during the last larval instar (Fig. 4). In the midgut, little mRNA was observed throughout the last larval instar. In the wing disc, PP mRNA was expressed most strongly in newly molted larvae and decreased afterward. In the fat body, the highest PP expression was also detected at the beginning of the instar. PP mRNA then decreased until day 7 and increased after the wandering stage.

As juvenile hormone (JH) and molting hormone, 20-hydroxyecdysone (20E), regulate expression timing of many genes (21), we assessed their effect on PP expression in the fat body. When a JH analogue, fenoxycarb, was administered to the last instar larvae on day 0, high PP expression was maintained until day 6 (Fig. 5A). In controls, PP expression continuously decreased as in untreated larvae (Fig. 4). When 20E was injected into day 5 last instar larvae, PP mRNA increased in the fat body 1 day later (Fig. 5B). In contrast, PP expression continuously decreased in water-injected larvae. Thus, both JH and 20E enhanced PP mRNA expression.

PP mRNA expression in embryo was examined in the non-diapausing strain, pnd-w1 (Fig. 6). PP expression was not detected immediately after oviposition, indicating an absence of maternal PP mRNA. One day after oviposition, 0.6- and 1.5-kb mRNAs appeared and were expressed continuously until hatching. Contrary to the larval stage, the signal of 1.5-kb mRNA was stronger than that of 0.6 kb. The expression of 1.5-kb mRNA peaked from 1 to 3 days after oviposition, whereas 0.6-kb mRNA was expressed most abundantly just before hatching.

DISCUSSION

The primary cDNA structure indicated that *Bombyx* PP is synthesized as the carboxyl-terminal portion of a larger precursor protein and secreted into hemolymph as are other ENF peptides (9, 11–13). Indeed, a large

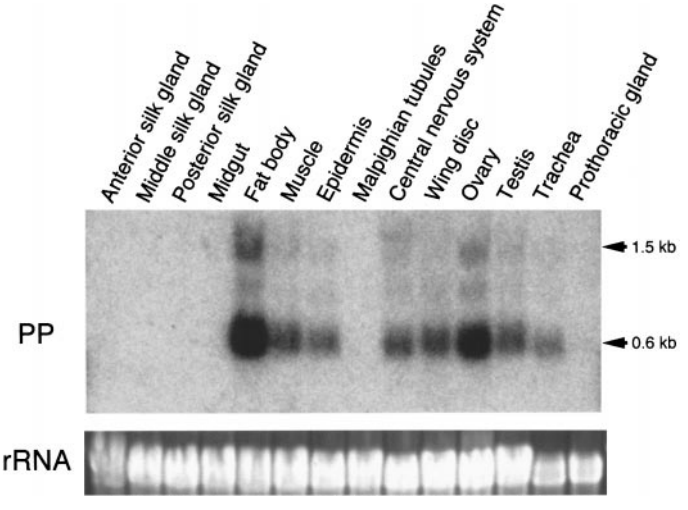

FIG. 3. Tissue-specific expression of PP mRNA at the beginning of the last larval instar. Ethidium bromide staining of rRNA is shown as a control for equal loading.

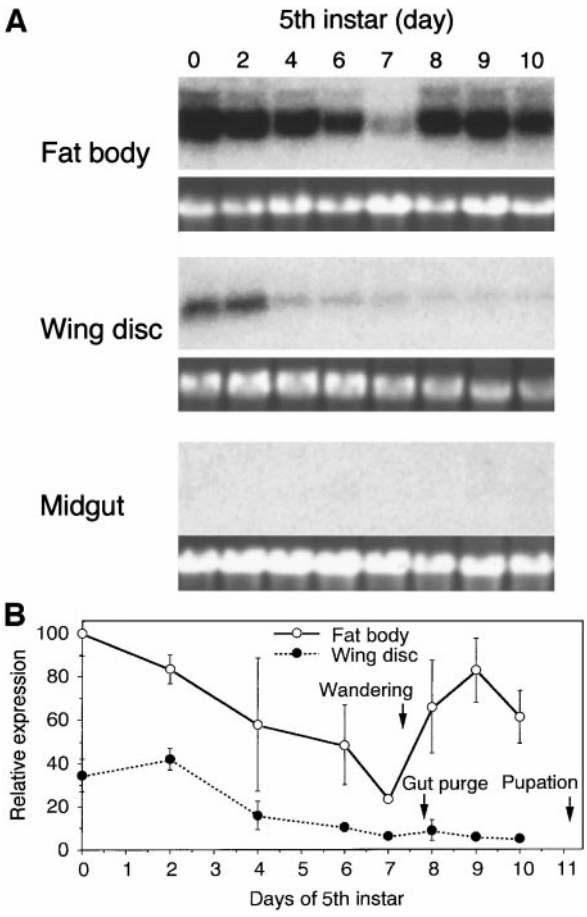

FIG. 4. Temporal changes in PP mRNA in 3 tissues during last larval instar. (A) Representatives of Northern blot hybridization. Ethidium bromide staining of rRNA is shown as a control for equal loading. (B) Expression profiles of 0.6-kb PP mRNA in the fat body and wing disc. Relative expression is referenced to the signal strength in the fat body on day 0 ($n = 2$). Bars represent SD.

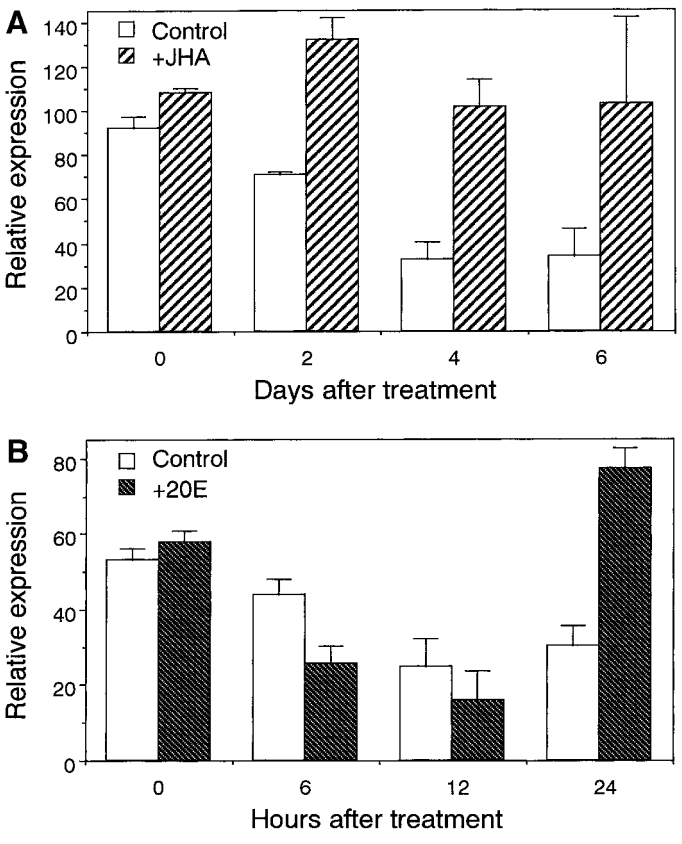

FIG. 5. Effect of JH and 20E on PP expression. (A) Last instar larvae of day 0 were applied topically with 100 ng of fenoxycarb (+JHA) or acetone (control). (B) Last instar larvae of day 5 were injected with 50 μg of 20-hydroxyecdysone (+20E) or distilled water (control). In each experiment, relative expression is referenced to the signal strength in the fat body on day 0 ($n = 2$). Bars represent SD.

amount of inactive proPP was present in plasma and processed rapidly into active PP after bleeding, indicated by Western blot analysis and paralytic activity assay. Sequence comparisons among known ENF peptide precursors showed that all active peptides are cleaved off after either Lys-x-Gly-Arg or x-Lys-Gly-Arg residues of precursors. These conserved sequences likely serve as recognition sites for processing endoproteinases. The lack of similarity in the first two-thirds of pro-proteins suggests that they do not encode biologically active peptides other than ENF peptides.

We assume that the same processing of PP as observed in collected plasma occurs in the hemocoel of injured larvae. When the silkworm is injured, inactive proPP is processed into active 23-residue PP near the wound by specific endoproteinases in hemolymph activated by bleeding. PP then causes local contraction of muscles to prevent hemolymph loss. As PP stimulates spreading of plasmatocyte *in vitro* and cell proliferation of several *Bombyx* cell lines (unpublished results), adhesion and spreading of plasmatocyte over the injured body wall and proliferation of epidermal cells may also be induced to promote wound healing.

PP mRNA was expressed most abundantly in the fat body in the last larval instar. Because the fat body occupies a large part of larval tissues, the primary source of proPP in hemolymph should be this tissue. In

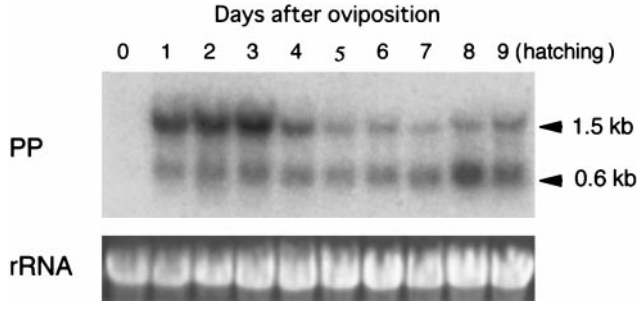

FIG. 6. Temporal changes in PP mRNA in 3 tissues during embryonic development. Ethidium bromide staining of rRNA is shown as a control for equal loading.

all larval tissues where PP expression was detected, 0.6 kb mRNA was the predominant transcript. In contrast, mRNA size for *Pseudaletia* GBP and *Pseudoplusia* PSP differed among tissues (12, 13). These results suggest that tissue-specific regulatory mechanisms underlying ENF peptide transcription are different among moth species. During early embryonic development, 1.5 kb of mRNA was expressed more abundantly than 0.6 kb PP mRNA. This longer mRNA is likely another transcript for PP with additional non-coding regions as is the nerve-specific mRNA for *Pseudaletia* GBP (22). Its molecular characterization should provide information on transcriptional and translational control of the PP gene.

JH and 20E enhanced expression of PP mRNA during the larval stage. These hormones are well known to control proliferation and differentiation of cells during molting and metamorphosis (21). As *B. mori* PP also has mitogenic activity in several silkworm cell lines (unpublished results), we assume that PP acts as a growth factor functioning downstream of the JH and 20E signaling pathways. In the wing discs, a high level of DNA synthesis was observed on day 0–1 of the last larval instar (23) when PP was expressed strongly. PP expressed in the wing disc may regulate cell proliferation in an autocrine manner. Experiments to test this hypothesis are now underway.

During embryonic development, both the 0.6- and 1.5-kb mRNA appeared 1 day after oviposition, when the germ anlage was completely enveloped by the serosa and the yolk cells began to be formed (24), and were expressed until hatching. As titers of several ecdysteroids increase during the early embryonic stage (25), they may regulate PP mRNA expression. This embryonic PP should not be involved in the known biological functions such as local contraction and hemocyte-stimulation. Because the *B. mori* embryo is protected by the hard chorion, such defense reactions do not appear to be biologically significant against injury. Otherwise, we assume that PP plays some role in embryogenesis. Many vertebrate growth factors, such as members of EGF and FGF families, have been shown to play essential roles in embryonic development (26, 27). The tertiary structures of the ENF peptides, *P. includens PSP*, *P. separata* GBP and *Manduca sexta* PP, were recently found to adopt a very similar structure to the C-terminal subdomain of mammalian EGF (28–30). Further, realizing that PP has growth factor activity, it is reasonable to assume that *Bombyx* PP also is involved in regulating cell proliferation and differentiation during embryogenesis. Especially, a product of 1.5-kb mRNA may play specific roles in early embryonic development.

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