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Isolation of Small Prothoracicotropic Hormone—Like Gene in *Drosophila melanogaster*

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The prothoracicotropic hormone (PTTH) produced by the neurosecretory cells in insects is involved in molting and metamorphosis by activating the prothoracic (ring) glands to secrete ecdysone (or related ecdysteroids). In the present study, the small PTTH-like gene was isolated by screening of cDNA library using the bombyxin (corresponding to small PTTH in *Bombyx mori*) gene probe in *Drosophila melanogaster*. It showed 50-60% sequence homology to bombyxin gene. The expression patterns of this gene showed developmental stage- and tissue-dependent manners. The mRNA was detected only in the late third instar larval—prepupa which is stages showing the highest hormonal activity to secrete ecdysteroids, and detected in the brain part of the late third instar larvae.

KEY WORDS: *Drosophila melanogaster*, prothoracicotropic hormone (PTTH).

The prothoracicotropic hormone (PTTH) is the brain peptide which stimulates the prothoracic glands to secrete ecdysone (or related ecdysteroids). It was discovered by Kopec in 1922 by ligation and transplantation experiments which demonstrated the endocrine role of the brain in pupation of the gypsy moth *Lymantria dispar*. Virtually most of work of PTTH has been conducted using the *Bombyx mori* and *Manduca sexta*. The PTTH exists in multiple molecular forms which, based upon difference in molecular weights, fall into two major groups: big PTTHs (20-29 kDa) and small PTTHs (4-7 kDa). There are also molecular variations within each size class. For example, the big and small PTTH activities in *Manduca* pupal brains are each composed of at least two acidic peptides (Bollenbacher *et al.*, 1984).

Substantial progress has been made in characterization of bombyxin, corresponding to small PTTH in *Bombyx mori* (Ishizaki *et al.*, 1983, 1987; Nagasawa *et al.*, 1984, 1986; Jhoti *et al.*, 1987; Mizoguchi *et al.*, 1987; Iwami *et al.*, 1989). Bombyxin molecules are heterodimers consisting of A- and B-chains whose amino acid sequences show considerable homology to vertebrate insulin-family peptides (Nagasawa *et al.*, 1984, 1986; Jhoti *et al.*, 1987; Maruyama *et al.*, 1988; Kataoka *et al.*, 1991). Recently, six genes which encode different bombyxin molecules have been characterized (Iwami *et al.*, 1989, 1990; Adachi *et al.*, 1989; Kawakami *et al.*, 1989). These genes are classified into three (A-, B- and C-) family. The copy number of bombyxin family A- and B-genes was estimated by genomic Southern hybridization to be 12 and 10, respectively (Kawakami *et al.*, 1989).

Little is known about the molecular identity of

PTTH in *Drosophila* either structurally or functionally. This is largely due to their small size and the complexity of their endocrine glands. The recent experiment, shown the ability to stimulate ecdysteroids synthesis in the larval ring glands with the presence of PTTH, provides a basis for the molecular characterization of Drosophila PTTH (Henrich *et al.*, 1987; Pak *et al.*, 1992).

In the present study, a small PTTH-like gene was isolated from a *Drosophila* cDNA library using bombyxin gene as a probe. For the cloned gene, DNA sequence and expression patterns were investigated.

Materials and Methods

Preparation of bombyxin gene probe

The plasmid p4KGEM20, containing bombyxin cDNA (gifted from Dr. M. Iwami at Nagoya University: Iwami *et al.*, 1989), was used as probe for screening of *Drosophila* cDNA library. The A-chain region of bombyxin gene (0.15 kb length) was isolated by digestion of p4KGEM20 with restriction enzymes *Pst I* and *Hha I*. The probes were labeled with α-^{32}P-dCTP (spec. act. 3000 Ci/mmol, Amersham) using random priming method (Boehringer Mannheim).

Screening of λgt11 *Drosophila* cDNA library

The λgt11 Drosophila (Canton-S wild type) cDNA library (mRNA source: whole bodies of wandering third instar larvae: Clontech) was plated about 8,000 pfu/150 mm plate with *E. coli* Y1090r- as a host cell. The nitrocellulose filters (BA85 type: Schleicher & Schuell) were used to obtaining replicas, and then prehybridized in prehybridization solution (6X SSC, 1X Denhardt's reagent, 0.1% SDS and 10 μg/ml salmon sperm DNA) at 45°C for 8 hrs and hybridized in hybridization solution (6X SSC, 1X Denhardt's reagent and 1 μg/ml salmon sperm DNA) containing α-^{32}P-dCTP labeled bombyxin gene as a probe at 42°C for 12-24 hrs. Bacteriophage DNA was isolated using LambdaSorb Phage Adsorbent (Promega) containing of a conjugate of fixed *Staphylococcus aureus* cells and rabbit polyclonal antibodies directed against lambda phage particles. *Drosophila* cDNA was eluted from phage DNA and then inserted into pUC19 vector.

DNA sequencing

DNA sequencing was performed by the chain termination method (Sanger *et al.*, 1977) using Sequenase (Version 2.0, USB Biochemical). Double-stranded plasmids (about 4 μg/reaction) were denatured in solution of 0.2 N NaOH and 0.2 mM EDTA. Then they were labeled with α-^{35}S-dATP (spec. act. 3,000 Ci/mmol, Amersham) and extended by addition of each dideoxynucleotide. The reacted DNAs were electrophoresed on buffer-gradient (top buffer, 0.5X TBE buffer; bottom buffer, 2.5X TBE buffer) 6% polyacrylamide gel containing 8 M urea. The samples were run at constant power of 50 W for 4-12 hrs. The gels were placed onto Whatman 3MM paper, dried and autoradiographed for 2 days.

The nested sets of deletions in the target DNA were generated by 20 second interval-exonuclease III digestion using the Erase-a-Base system (Promega). At this condition, the plasmids of approximately 200 bp-length intervals were produced.

Northern blot analysis

Total RNA was isolated from whole bodies of particular staged 20-50 animals as described by Chomczynski and Sacchi (1987). The RNAs were separated on 1% formaldehyde agarose gel, and then transferred onto nitrocellulose filters. The filters were prehybridized with prehybridization solution (5X SSC, 50% formamide, 5X Denhardt's solution, 0.1% SDS and 100 μg/ml salmon sperm DNA) at 42°C for 4 hrs, and then hybridized with hybridization solution (5X SSC, 50% formamide, 1X Denhardt's solution, 0.1% SDS and 100 μg/ml salmon sperm DNA) containing α-^{32}P-labeled gene probe at 42°C for 24 hrs.

For slot blot hybridization, RNA samples were solubilized with the buffer (7.4% formaldehyde, 6X SSC) and denatured by heating at 60°C for 15 min and then rapidly cooled in ice. The samples were applied onto NC filter. Wells were rinsed

twice with 15X SSC, then the filters were hybridized with ^{32}P-labeled DNA probes by the same method of the Northern blot analysis.

Results and Discussion

Screening of cDNA library

Approximately 4 × 10^5 plaques of *Drosophila* λgt11 cDNA library were screened using bombyxin gene as a probe to isolate a small PTTH gene from *D. melanogaster*. From three times of screenings, six plaques were obtained as positives (λsP10, λsP13, λsP20, λsP31, λsP67 and λsP69). The positive plaques were amplified by the method of plate lysate, and then phage DNAs were isolated using a conjugate of *Staphylococcus aureus* cells and rabbit polyclonal antibodies directed against bacteriophage particles.

The phage DNAs were digested with *EcoR* I restriction enzyme to elute *Drosophila* cDNA. The sizes of the cDNA were ranged from 0.5 to 1.3 Kb. The isolated *Drosophila* cDNA fragments were subcloned into *EcoR* I site of pUC19 plasmid vector, which were named psPTTH10, psPTTH13, psPTTH20, psPTTH31, psPTTH67 and psPTTH69.

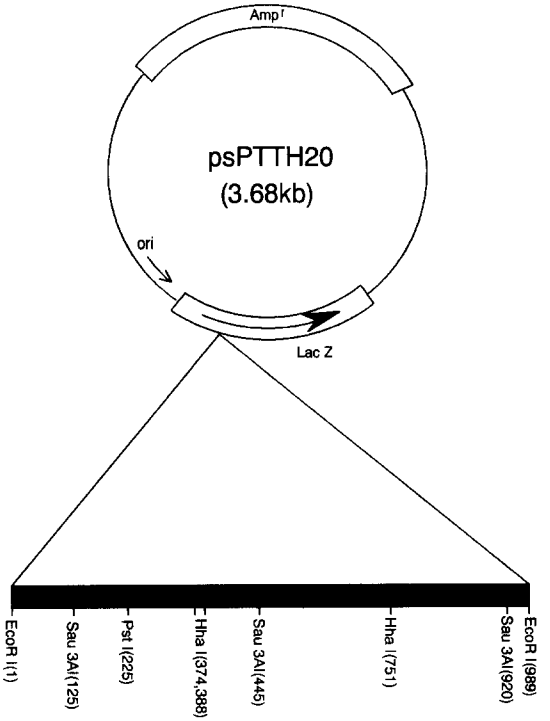

Fig. 1. Restriction map of plasmid psPTTH20. *Drosophila* cDNA fragments (0.98 kb) were isolated from bacteriophage λsP20, and then inserted into *EcoR* I site of pUC19 vector.

Sequence analysis

When the sequencings were carried out for the six recombinant plasmids, cDNA from plasmid psPTTH20 (originated from bacteriophage λsp20) was the most homologous with the bombyxin gene. Therefore, further study was performed only to the plasmid psPTTH20. The *Drosophila* cDNA was named to 'small PTTH-like gene' (Fig. 1).

The length between estimated start codon (ATG, designated to 1) and stop codon (TGA) was 246 base pairs. When amino acid sequence was deduced from the cDNA sequence, a polypeptide consisted of 81 amino acids was produced, which is slightly shorter than bombyxin having 91 amino acids (Fig. 2). It showed about 50-55% DNA and about 30% amino acid sequences homology to bombyxin gene family, respectively (Fig. 3). It was slightly more homologous to C family of bombyxin gene than any other two families. The C family is the most distant from other two families. The sequence homology shown between bombyxin and small PTTH-like gene is low. When three families of bombyxin were compared, C-family showed amino acid sequence homology of less than 50% to other families (Iwami *et al.*, 1990).

The *in situ* hybridization of the small PTTH-like gene with giant chromosomes showed two hybridized bands at positions 1A and 100D (data not shown). It implicates that *Drosophila* genome carries this gene more than two copies. The existence of several isoforms was previously suggested on the study of *Drosophila* PTTH activity (Pak *et al.*, 1992).

Expression of small PTTH-like gene

The mRNA of small PTTH-like gene was very weakly detected for the first time in the late third instar larva, as shown in Fig. 4A. The transcripts were sharply decreased as ongoing the

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      +1
5' - ATG CGG CAT AAC TAC ATT CGC CTG CGT GGC CGT ACC TGG TTT TTA ACC TTA    51
     Met Arg His Asn Tyr Ile Arg Leu Arg Gly Arg Thr Trp Phe Leu Thr Leu    17

     TAC GGG ATT TTA AGT ACG CTA TTT GTT GGA TTT TTC GTC ATT TAT TGC ACT   102
     Tyr Gly Ile Leu Ser Thr Leu Phe Val Gly Phe Phe Val Ile Tyr Cys Thr    34

     ATT CTA TTT GAC TTT TTA ATT CGA GTG CTT GGT GGT TAT TTG ACG AGT TTT   153
     Ile Leu Phe Asp Phe Leu Ile Arg Val Leu Gly Gly Tyr Leu Thr Ser Phe    51

     GGC AGT TAT CTT CAA ACG AAT GTT TGG CAG TCA TCC AAC CTT GAT GAT GTG   204
     Gly Ser Tyr Leu Gln Thr Asn Val Trp Gln Ser Ser Asn Leu Asp Asp Val    68

     CTA AAC ATG CCA AAA CAG TGT TTA TGC CAT CAC AGA TGT TGA GTATTTTTGGG   257
     Leu Asn Met Pro Lys Gln Cys Leu Cys His His Arg Cys ***                81

     CGCTTATCTCTTGTCACCGGCATCTTTATGTTAATCTTATTTCTTTTTCTGGAAAAAAATTGGTCAT   324
     TGGGTAAAGTGTATGATTGGTATCAAACATTTCGTGACTTAACCCGAAACACCAACCGTTTTGCCAT   391
     GGTAAGAGAAGTAGATGCCACTTACAAA                                          419
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Fig. 2. DNA sequence of small PTTH-like gene and deduced amino acid sequence. DNA sequencing was carried out by chain termination method. The amino acid sequence was deduced from the DNA sequence.

development. After the early pupal stage, the expression of the small PTTH-like gene was not observed.

To examine whether this gene is tissue-specifically expressed, the late third instar larvae which showed the highest amount of the mRNA were sectioned into two parts: anterior and posterior. RNA was isolated from each part, and slot blot analysis was carried out. As shown in Fig. 4B, the transcripts were detected only in the anterior part containing brain.

If the small PTTH-like gene is the same as *Drosophila* small PTTH gene, it must be expressed most highly in the late third instar larval—prepupal stage, and expressed only in the brain. Also, the degree of expression should be very low, because *in situ* hybridization of bombyxin mRNA at the *Bombyx* brain showed only four pairs of the neurosecretory cells hybridized (Iwami, 1990). When enormous RNA (150 μg/lane) was loaded, the transcripts were weakly detected only in the late third instar larva—prepupa. Also, the gene expression was shown only at the anterior part containing brain from the late third instar larvae. The expression patterns are well agreed with the previously known characteristics of PTTH.

Speculation on the Evolution of PTTH genes

Recently, big PTTH cDNA (Kawakami *et al.*, 1990), bombyxin cDNA and genomic DNA (Iwami *et al.*, 1989, 1990; Adachi *et al.*, 1989; Kawakami *et al.*, 1989) were cloned in *Bombyx mori*. However, no PTTH genes were cloned from any other insects except for *Bombyx*. Therefore, it is limited to compare *Drosophila* small PTTH-like gene with PTTH genes from other insects. However, it appears that the small PTTH genes in insects are less conserved genes. For the reasons: (1) the homology between bombyxin and small PTTH-like gene is low (approximately 30% amino acid sequence homology), (2) immunohistochemistry and immunoblot analysis for *Drosophila* third instar larval brain using bombyxin monoclonal antibody did not detect *Drosophila* PTTH (data not shown), which indicates non-immunoreactivity between bombyxin and small PTTH in *Drosophila* (for these experiments, the bombyxin monoclonal antibody, anti-bombyxin (1-10) and *Bombyx* big PTTH monoclonal antibody, anti-PTTH (1-15) were gifted from Dr. H. Ishizaki at Nagoya University: Mizoguchi *et al.*, 1987, 1990) and (3) intra-species amino acid sequence homology between bombyxin A- and B-families showed high

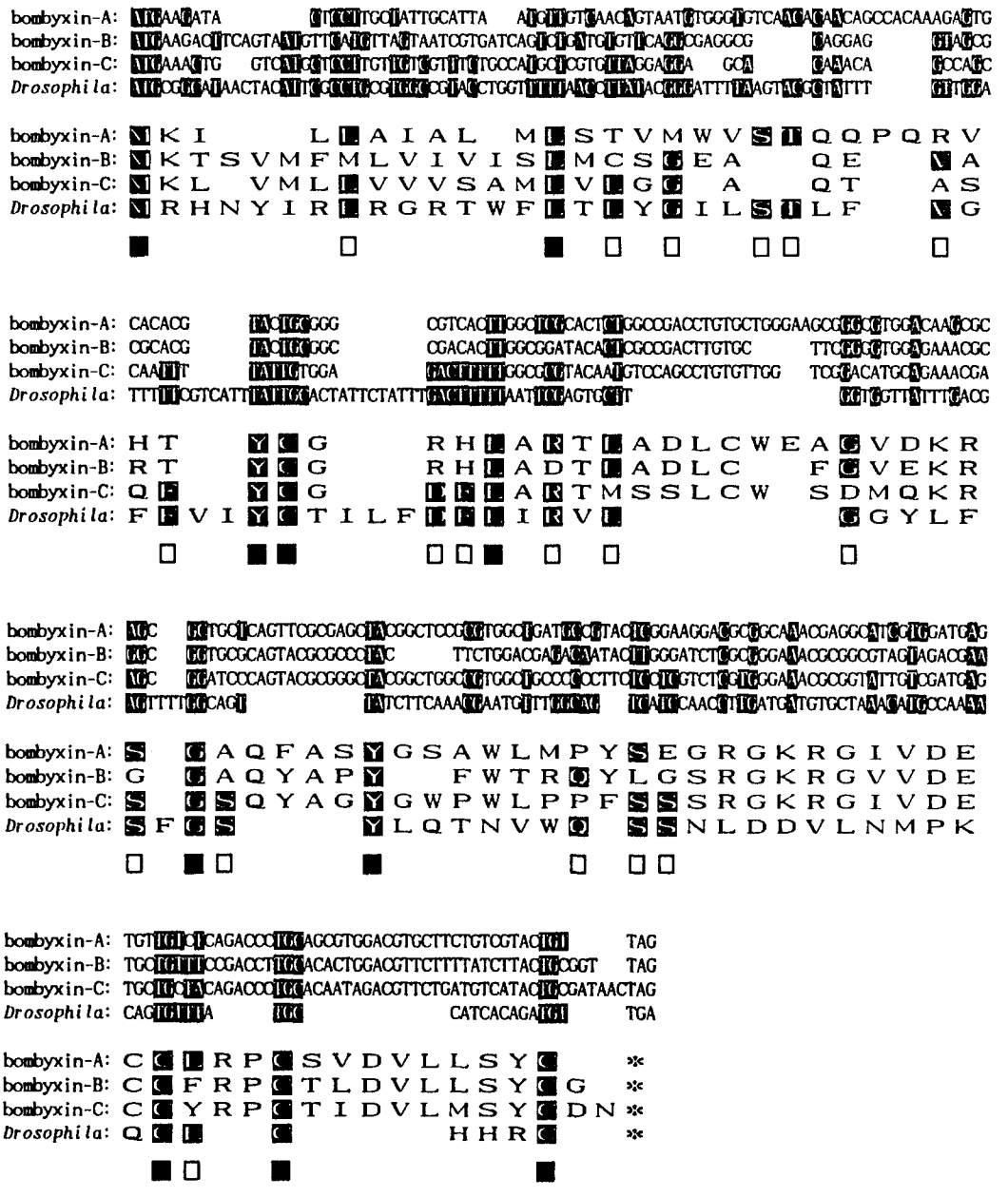

Fig. 3. Comparison of DNA and amino acid sequences between bombyxin genes and *Drosophila* small PTTH-like gene. Gaps are introduced for maximum alignment. The amino acid residues which are completely conversed throughout the four peptides shown and those conserved between small PTTH-like gene and one or two of bombyxin gene families are indicated by the solid and open squares, respectively. Homology data concerning to bombyxin gene families are obtained from Iwami *et al.* (1990).

homology (about 60%), but homology of C-family with A- and B-families is significantly low (44% and 36%, respectively) in *Bombyx mori* (Iwami *et al.*, 1990).

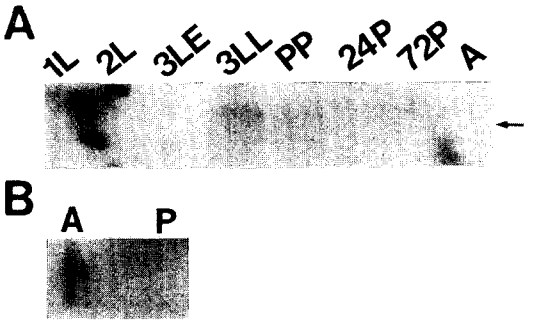

Fig. 4. Expression of small PTTH-like gene. (A) Developmental expression of the gene. All lanes were loaded with 150 μg of total RNA on 1% formaldehyde agarose gel, then electrophoresed. 1L, first instar larvae; 2L, second instar larvae; 3LE, early third instar larvae; 3LL, late third instar larvae; PP, prepupae; 24P and 72P, 24 hr- and 72 hr-pupae; A: adults. (B) Tissue-specific expression fo the gene. The late third instar larvae were sectioned two parts, anterial and posterial parts. Total RNAs were isolated from each part, then slot-blotted (100 μg/slot). A, anterial part containing brain; P, posterial part containing tail.

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초파리에서 전흉선자극호르몬 유사 유전자의 재조합

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전흉선자극호르몬(PTTH)은 곤충의 신경분비세포에서 만들어지는데, 전흉선을 자극하여 변태호르몬(ecdysone 또는 여타 ecdysteroids)의 분비를 촉진시킴으로서 변태를 유도한다. 본 연구에서는, 노랑초파리의 cDNA library로 부터 bombyxin(누에의 small PTTH) gene probe를 사용하여 small PTTH 유사 유전자를 분리하였다. 이 유전자는 bobyxin 유전자와 50-60%의 염기서열 유사성을 보였으며, 발생단계 및 조직 특이적인 발현양상을 보였다. 즉, 변태호르몬의 분비가 가장 왕성한 시기인 후기 3령기 유충—변데기 시기에서만 그 mRNA를 발견할 수 있었으며 후기 3령기 유충에서도 머리부위에서만 발현되었다.