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Review

The brain secretory peptides that control moulting and metamorphosis of the silkmoth, *Bombyx mori*

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ABSTRACT Progress made toward the elucidation of molecular features of the prothoracicotropic hormone (PTTH) of the silkmoth *Bombyx mori* is reviewed. PTTH stimulates the prothoracic glands to synthesize and release ecdysone, and is therefore a key hormone for the regulation of insect moulting and metamorphosis. *Bombyx* PTTH is a 30 kDa homodimeric glycoprotein, whose carbohydrate moiety is not essential for the biological function. The *Bombyx* genome contains a single copy of the PTTH gene. PTTH is produced by four dorsolateral neurosecretory cells of brain. Another *Bombyx* brain peptide exerting prothoracicotropic activity to a heterologous moth *Samia cynthia ricini* but no activity to *Bombyx* has been identified and termed bombyxin. Bombyxin is a 5 kDa heterodimeric peptide that shows a high similarity to insulin in the amino acid sequence. The bombyxin gene structure also shows a high similarity with the insulin gene structure. The *Bombyx* genome contains more than 30 copies of the bombyxin gene. Bombyxin is synthesized by eight dorsomedial neurosecretory cells of brain.

KEY WORDS: *Bombyx mori, insect metamorphosis, neuropeptide, prothoracicotropic hormone, bombyxin*

Introduction

In 1922, Kopec found that a humoral factor from the insect brain was responsible for the induction of larva-to-pupa moulting, by neck ligation and brain transplantation experiments using the gypsy moth *Lymantria dispar*. This was the first demonstration of the presence of the hormone in invertebrates, and he named this hormone the brain hormone. Kopec's conclusion had long been ignored, however, until 1940 when Wigglesworth confirmed, by using the blood-sucking bug *Rhodnius prolixus*, that the brain hormone originated from the neurosecretory cells of the brain. Before that time nobody believed or even imagined that nerve cells might produce a hormone molecule, and indeed Wigglesworth's contribution was the first, not only in insects but also throughout the animal kingdom, to define neurosecretion, a phenomenon of endocrine function played by a special type of large neurons, neurosecretory cells. In 1947, Williams proved that the brain hormone turns on the prothoracic glands which secrete a hormone directly responsible for stimulating the development of peripheral tissues, now known as ecdysone. As numerous neurohormones were later discovered from the insect brain, the term brain hormone has come to be replaced by the term prothoracicotropic hormone (PTTH), which is now generally being used. Although the biological background of PTTH was established early in the history of endocrinology and knowledge about PTTH contributed much to the formation of important concepts in endocrinology, progress in purification and structure elucidation of PTTH was very slow, as was the case for invertebrate neuropeptides in general, compared to that of vertebrate neurohormones.

One of the authors of this article, H. Ishizaki, began purification of PTTH from the silkmoth *Bombyx mori* in 1960. After a 10-year struggle by this biologist alone, Prof. (now Prof. Emeritus) Saburo Tamura of the University of Tokyo, a biochemist famous in the organic chemistry of naturally occurring biologically active substances, offered to cooperate with him in purifying PTTH. A. Suzuki, the other author of this paper, soon succeeded to Tamura, and tight cooperative working relations between the Nagoya biology group and the Tokyo chemistry group persisted over 20 years. The present article summarizes the progress made by this joint research, which has explored the outlines of the molecular features of *Bombyx* PTTH. Similar reviews have appeared previously (Ishizaki, 1986; Ishizaki and Suzuki, 1988, 1992).

We first mention the quite unanticipated discovery of a novel PTTH-like peptide, bombyxin, that stemmed from the choice of assay animal for PTTH. At the onset of the PTTH purification study, *Bombyx mori* was chosen as the source of PTTH. *Bombyx* is

Abbreviations used in this paper: PTTH, prothoracicotropic hormone; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SBRP, *Samia* bombyxin-related peptide.

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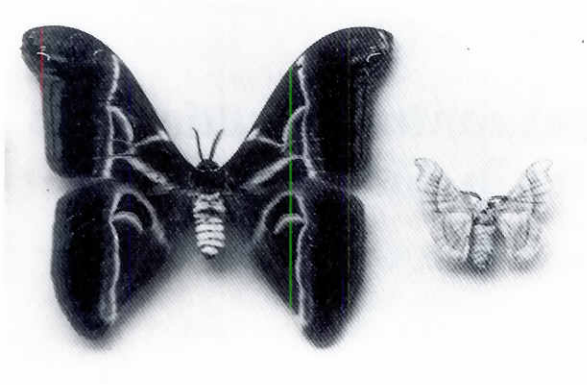

Fig. 1. Adults of *Samia cynthia ricini* (left) and *Bombyx mori* (right).

commercially available and is the only insect that can be easily supplied in huge numbers. In fact, we have used more than $2x10^7$ *Bombyx* heads up to now. As a bioassay animal for PTTH purification, on the other hand, the animal adopted was the saturniid moth *Samia cynthia ricini* (Fig. 1), with which the biological background of PTTH function had long been studied in H.I.'s laboratory. When *Samia* pupae are surgically deprived of their brains shortly after pupation, they remain as pupae for a long period of time without undergoing adult development (Ishizaki and Ichikawa, 1967). Transplantation of live *Bombyx* brains and injection of *Bombyx* brain extracts into *Samia* debrained pupae both caused the resumption of adult development, in exactly the same way as *Bombyx* brainless pupae responded to them. From this fact we concluded that *Bombyx* PTTH activated the *Samia* prothoracic glands in a species non-specific manner. Simply because of various technical advantages in using *Samia* pupae compared with *Bombyx* pupae, *Samia* brainless pupae were chosen as an assay animal for the purification of PTTH extracted from *Bombyx*.

After a long tedious process of purifying *Bombyx* PTTH by using this assay system, we finally obtained it in a pure form to permit sequence analysis (Suzuki *et al.*, 1982). Then we injected the purified PTTH into *Bombyx* brainless pupae to reconfirm its activity on *Bombyx*. To our surprise, the pure PTTH was totally inactive on *Bombyx* pupae. Careful reexamination of the purification steps, by which each fractionated sample was assayed with both *Bombyx* and *Samia* brainless pupae, readily revealed that the crude extracts from *Bombyx* brains contained two distinct molecules, a 5-kDa peptide which was active when tested with *Samia* but inactive on *Bombyx*, and a 30-kDa peptide exhibiting the activity on *Bombyx* but not on *Samia* (Ishizaki *et al.*, 1983a). The crude extracts contained these two peptides, and this fact had led to the erroneous assumption that a single *Bombyx* PTTH molecule was active in both species. The molecule we had pursued up to that time by the *Samia* assay was obviously the 5-kDa peptide, while the true PTTH of *Bombyx*, which should activate its own prothoracic glands, was the 30-kDa peptide that had been discarded during the 5-kDa peptide purification. Then tedious work started again to purify the 30-kDa true *Bombyx* PTTH. In earlier publications we referred to the 5-kDa peptide as S-PTTH (S represents the initial of *Samia*) or 4K-PTTH (according to an apparent molecular mass estimated by Sephadex gel filtration), but it has been renamed as bombyxin (Mizoguchi *et al.*, 1987) which is in current use. The 30-kDa peptide was similarly once called B-PTTH (the initial of *Bombyx*), but it is now called simply *Bombyx* PTTH.

The historical background stated above resulted in a far more advanced progress of the bombyxin study than the PTTH study. Although the function of bombyxin for *Bombyx* has not yet been fully defined, bombyxin is undoubtedly a physiologically important peptide for *Bombyx*. The following descriptions are therefore made first for bombyxin, and then for PTTH.

Primary structure of bombyxin

A purification scheme consisting of 15 successive procedures for bombyxin from *Bombyx* heads has been established (Suzuki *et al.*, 1982; Nagasawa *et al.*, 1984a). Various difficulties in purification that we met and had to overcome have been documented in a review article (Ishizaki and Suzuki, 1984). Bombyxin comprises many molecular forms which could be satisfactorily resolved only by high performance liquid chromatography (HPLC). Bombyxin-I, one of the heterogeneous molecular forms of bombyxin, was obtained as a single peak on an HPLC at the terminal step of purification, with a recovery of 8% and a purification fold of $2x10^6$ (Suzuki *et al.*, 1982; Nagasawa *et al.*, 1984a). Fifty μg of bombyxin-I was obtained from 650,000 *Bombyx* heads and 0.1 ng of this pure material was able to evoke adult development when injected into a *Samia* brainless pupa ($3x10^{-11}$ M in hemolymph). When incubated *in vitro* with a prothoracic gland taken from freshly ecdysed *Samia* pupa, bombyxin-I enhanced ecdysone release at a concentration of $1x10^{-11}$ M. So far, other molecular forms named bombyxin-II, -III, -IV and -V have been purified to homogeneity (Nagasawa *et al.*, 1984b, 1986, 1988; Jhoti *et al.*, 1987; Maruyama *et al.*, 1988).

When the N-terminal 19 amino acid residues of bombyxin-I, -II, and III were determined, a surprising, unexpected fact was revealed: the sequences of bombyxins showed significant similarity with the N-terminal portion of the A-chain of insulin (Nagasawa *et al.*, 1984b. At that time bombyxin was still called 4K-PTTH). When the complete sequence of bombyxin-II was determined (Nagasawa *et al.*, 1986), the similarity to insulin family peptides became even clearer. Bombyxin is a heterodimer consisting of two chains which we named the A- and B-chains, and these chains are ~50% and ~30% similar to the A- and B-chains of human insulin, respectively (Fig. 2). Bombyxin resembles relaxin in having a pyroglutamic acid residue at the B-chain N-terminus. So far, bombyxin-IV has also been sequenced fully (Maruyama *et al.*, 1988; Nagasawa *et al.*, 1988) and bombyxin-I, -III and -V have been partially sequenced (Nagasawa *et al.*, 1984b; Jhoti *et al.*, 1987). Two inter- and one intra-chain disulfide bonds are formed at the same positions as in insulin (Maruyama *et al.*, 1988). Bombyxin-II and -IV have been chemically synthesized and the synthetic bombyxins showed the same biological activity as natural bombyxins (Nagasawa *et al.*, 1988; Maruyama *et al.*, 1990). Molecular modeling for the three-dimensional structure of bombyxins has further shown that bombyxins resemble insulin in adopting the core structure similar to that of insulin (Jhoti *et al.*, 1987). The presence of insulin-like molecules in insects had frequently been suggested by indirect evidence (reviewed by Kramer, 1985), but our finding of the amino acid sequence similarity between bombyxins and insulin was the first to demonstrate unequivocally the presence of insulin-related peptides in insects.

The cephalic neuroendocrine system of insects consisting of the brain, corpora cardiaca, and corpora allata has often been argued for its analogy to the hypothalamo-hypophysial system of vertebrates, from the anatomical and physiological view points (e.g.

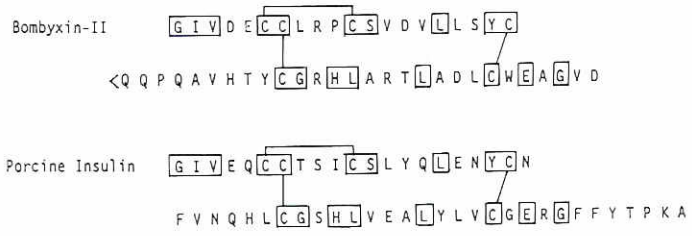

Fig. 2. Amino acid sequences of bombyxin-II and porcine insulin. *The homologous residues are boxed. Cys residues forming disulfide bonds are connected by lines. <Q denotes pyroglutamate.*

Scharrer and Scharrer, 1944). It had therefore been suspected that insect brain secretory peptides might be evolutionarily related to vertebrate hypothalamic neurohormones. The fact was not so, however: the *Bombyx* brain peptide bombyxin has now been shown to share a common ancestral molecule with the vertebrate pancreatic peptide, insulin. This finding was thus surprising, but at the same time was not surprising, in view of the existing knowledge that the peptides originally found in the central nervous system have often been found in the gastro-entero-pancreatic endocrine system, and *vice versa* (Barrington, 1982; Costa and Furness, 1982; Fujita *et al.*, 1983; Endo, Y., *et al.*, 1990).

Structure of cDNAs and genes coding for bombyxin

Using the synthetic oligonucleotide probes designed on the basis of the known amino acid sequences of bombyxins, two cDNAs and a gene of *Bombyx* coding for bombyxins have been isolated (Adachi *et al.*, 1989; Iwami *et al.*, 1989). The results showed that the bombyxin cDNAs and gene encoded a precursor protein for bombyxin consisting of the signal peptide, B-chain, C-peptide (connecting peptide, comparable to that of proinsulin), and A-chain (Fig. 3). The C-peptide is flanked by the dibasic residues, Lys-Arg, suggesting that the C-peptide is excised proteolytically after disulfide bond formation, to generate mature bombyxin. These structural features of preprobombyxin are precisely the same as those of preproinsulin (Steiner *et al.*, 1985). Thus, the notion that bombyxin and insulin share a common ancestral molecule has further been substantiated on the gene level.

By using the isolated bombyxin gene as probe, four additional bombyxin genes have been cloned (Kawakami *et al.*, 1989). Subsequently, by probing with various bombyxin genes, as many as 29 bombyxin gene copies have been cloned and characterized (Iwami, 1990; Iwami *et al.*, 1990; Kondo *et al.*, in preparation). These bombyxin gene copies have been classified into A, B, C, and D families according to the degree of the amino acid sequence similarity. They are localized in the *Bombyx* genome forming clusters with a characteristic arrangement where two genes belonging to different families are apposed with opposite transcriptional directions. The largest gene cluster so far characterized extends over a 50-kilo base-pair genomic DNA segment that contains 21 bombyxin gene copies (Kondo *et al.*, in preparation).

All of the genes encoding preprobombyxin lack introns, in contrast with the vertebrate insulin genes, which have one or two introns (Steiner *et al.*, 1985). It has been proposed that bombyxin genes are the functional processed genes that have been generated by the reverse transcription of processed mRNAs and subsequent insertion into the genome (Iwami *et al.*, 1989, 1990; Iwami, 1990). This fact, together with the presence of multiple gene copies in the *Bombyx* genome, which contrasts with the presence of a single or two copies of vertebrate insulin genes in the genome (Steiner *et al.*, 1985), suggests that different mechanisms underlie the evolution of insulin-family peptide genes in vertebrates and invertebrates.

By using the bombyxin gene isolated from *Bombyx*, the genes of *Samia* coding for precursor proteins for bombyxin-like peptides designated as *Samia* bombyxin-related peptides (SBRPs) have been cloned (Kimura-Kawakami *et al.*, 1992). Six SBRP gene copies have been characterized, and their structure and the arrangement in the *Samia* genome have been shown to resemble those of the bombyxin genes of *Bombyx*. Southern analysis showed that the *Samia* genome contained more SBRP gene copies. Two SBRPs, SBRP-A1 and -B1, that were chemically synthesized based on the amino acid sequences deduced from the corresponding genes exhibited the prothoracicotropic activity when tested with brain-removed *Samia* pupae (Nagata *et al.*, in preparation).

Along with our finding of bombyxin, reports on insulin-related peptides occurring in invertebrates appeared in succession. Thus, the sponge *Geodia cydonium* (Robitzki *et al.*, 1989), the mollusc *Lymnaea stagnalis* (Smit *et al.*, 1988; Geraerts *et al.*, 1991), and the locust *Locusta migratoria* (Lagueux *et al.*, 1990) have been shown to possess the genes or gene transcripts coding for insulin-related peptides. The structures of these genes are much more divergent than the insulin genes of vertebrates, confirming further that an insulin protogene evolved in invertebrates under mechanisms considerably different from those which have operated in vertebrates.

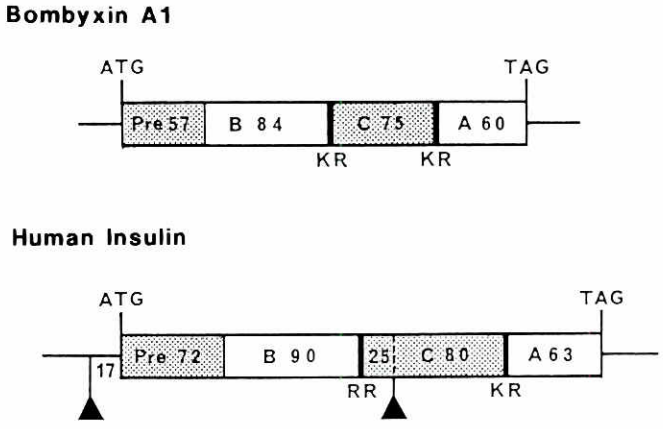

Fig. 3. Schematic representation of the genes encoding prepropeptides for bombyxin A1 and human insulin. *Open boxes represent the domains that contribute to the formation of mature peptides. Hatched boxes designate the domains coding for the peptide regions that are excised after translation. Pre, signal peptide; B, B-chain; C, C-peptide; A, A-chain. Numerals indicate the nucleotide numbers. Bold boundaries with KR or RR represent the posttranslational proteolytic cleavage sites. Triangles indicate introns.*

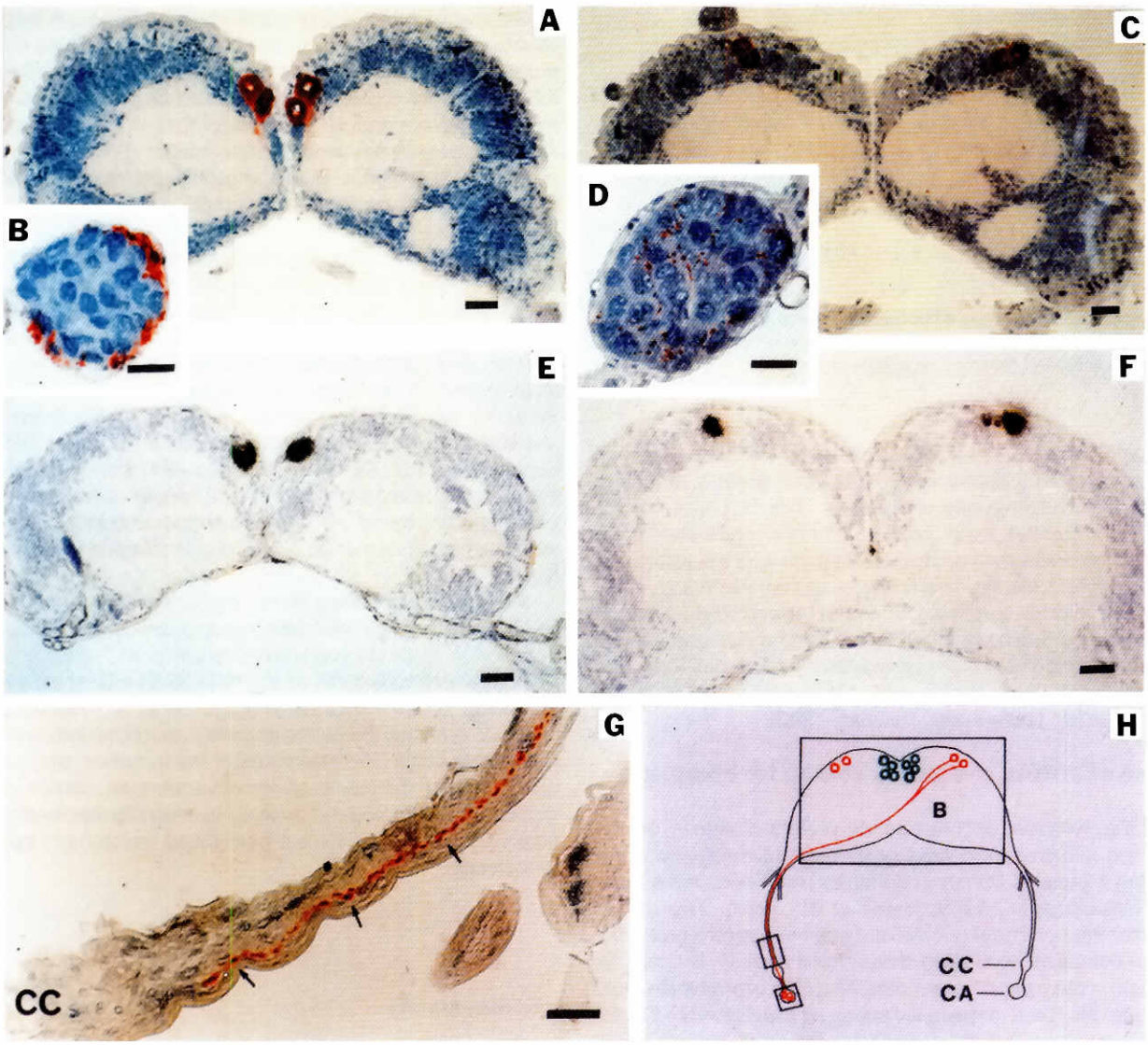

Fig. 4. Immunohistochemistry for bombyxin and PTTH, and *in situ* hybridization for their mRNA in the cephalic endocrine tissues of *Bombyx* fifth-instar larvae. (A) *Brain immunostained with the bombyxin antibody. Four dorsomedial neurosecretory cells are seen here, but serial sections demonstrate eight (four in each brain hemisphere) immunoreactive cells.* **(B)** *Corpus allatum with bombyxin-immunoreactive nerves in the peripheral region.* **(C)** *Brain immunostained with the PTTH antibody. Two pairs of dorsolateral neurosecretory cells are positive.* **(D)** *Corpus allatum immunostained with the PTTH antibody. The positive axons are distributed throughout the allatum.* **(E)** *In situ hybridization of brain for bombyxin mRNA, as probed with a bombyxin gene. The same cells as the immunoreactive cells are positive.* **(F)** *In situ hybridization of brain for PTTH mRNA, as probed with PTTH cDNA.* **(G)** *Retrocerebral nerves leading to the corpus cardiacum (CC), to show the pathway of PTTH from the brain perikaryon to the corpus allatum via corpus cardiacum. Arrows point to the bead-like immunoreactive granules.* **(H)** *Schematic drawing of a Bombyx larval brain (B), corpora cardiaca (CC), and corpora allata (CA). Green circles, bombyxin cells. Red circles, PTTH cells. The axon pathway down to the corpus allatum is shown only for the two PTTH cells in the right brain-hemisphere. Boxes show trimmings of the photographs for brain, retrocerebral nerve leading to CC, and CA. Bars, 50 μm.*

Bombyxin-producing cells

We raised a monoclonal antibody against a synthetic peptide corresponding to the N-terminal 10-amino acid sequence of the bombyxin-I A-chain [the bombyxin-I-(1-10) antibody] (Mizoguchi *et al.*, 1987). This antibody was proved to recognize bombyxin by competitive enzyme-linked immunobinding assay and immunoblotting, only after denaturation of bombyxin by heating or dithiothreitol treatment. Immunoblotting of brain extracts on native polyacrylamide gel electrophoresis (PAGE) resolved eight immunoreactive bands, which appeared as a single band on SDS-PAGE, indicating that this antibody recognized many molecular forms of bombyxin (Mizoguchi *et al.*, 1990). Immunohistochemically, four pairs of large dorsomedial neurosecretory cells of the *Bombyx* brain have been shown to contain bombyxin (Fig. 4A) (Mizoguchi *et al.*, 1987, 1990). The immunoreactive material could be traced down to the corpora allata, through the axons that emerge from the neurosecretory cells and cross at the central line of brain so that the

axons reach the contralateral corpus allatum, suggesting that bombyxin is released into hemolymph from the corpora allata. The axon endings were localized at the periphery of the allata (Fig. 4B).

Developmental change in the bombyxin content in *Bombyx* brain was examined by immunoblotting, immunohistochemistry, and bioassay for bombyxin activity with *Samia* brainless pupae (Mizoguchi *et al.*, 1990). The bombyxin level in brain in the fourth larval instar was high in the first half, while low in the latter half. It became high again at the time of ecdysis to the fifth (final) instar, but gradually decreased afterward until larvae began wandering. After wandering the bombyxin content increased gradually until pupation when a level as high as that in the newly ecdysed fifth-instar larvae was regained.

An intriguing fact was disclosed by immunohistochemistry: there was a differential immunoreactivity of two two-cell groups among the four bombyxin-producing neurosecretory cells within a brain hemisphere. During the late fifth-instar and prepupal periods, it was often observed that two cells were heavily immunostained whereas the other two cells stained only weakly. The possibility has been discussed that the production and/or release of bombyxin is oscillatory in nature and the two two-cell groups have different phases of oscillation (Mizoguchi *et al.*, 1990). Pulsatile secretion of peptide hormones is a rather general phenomenon (Rasmussen *et al.*, 1989; Smith *et al.*, 1989), and the cell group-dependent difference in bombyxin accumulation might represent a cytological manifestation of such a pulsatile secretion.

The bombyxin-I(1-10) antibody was thus useful for immunoblot and immunohistochemical studies, but was not applicable to radioimmunoassay or affinity purification of bombyxin, because the antibody was incapable of recognizing undenatured, bioactive bombyxin. After efforts to prepare an antibody recognizing native bombyxin, we obtained such a monoclonal antibody using as an antigen natural bombyxin-II that had been purified to homogeneity (Mizoguchi, 1990; Saegusa *et al.*, 1992). This antibody (bombyxin-II antibody) has been proven to bind native bombyxin in extracts of *Bombyx* brains, but about one fourth of the total bombyxin activity in the extract remained unbound (Saegusa *et al.*, 1992). This is not unexpected because bombyxin comprises many heterogeneous molecular forms. By using this antibody, a radioimmunoassay for bombyxin has been developed, and the developmental fluctuation of the immunoreactive material in the *Bombyx* hemolymph was examined from the newly ecdysed first-instar larva to newly ecdysed adult (Saegusa *et al.*, 1992). Noticeably, very high immunoreactivity titres were detected during the early and middle periods of pupa-adult development, and the titres in the female hemolymph were twice as high as the male titres (Fig. 5). Thus, it seems likely that bombyxin regulates a sex-associated process(es) in adult development.

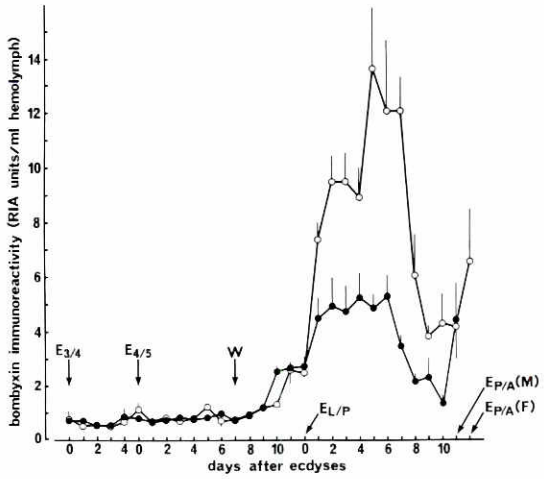

Fig. 5. Developmental change in the titre of the bombyxin immunoreactivity in *Bombyx* hemolymph. *Abscissa, days from third- to fourth-instar larval ecdysis ($E_{3/4}$), fourth- to fifth-instar larval ecdysis ($E_{4/5}$), and pupal ecdysis ($E_{L/P}$). W, initiation of wandering. $E_{P/A}$ (M) and $E_{P/A}$ (F) represent adult ecdyses of males and females, respectively. Open circles, female titres; closed circles, male titres.*

Primary structure of *Bombyx* PTTH

When it became clear that bombyxin was not the true PTTH of *Bombyx* but that the true PTTH is a 30-kDa peptide as stated in the Introduction, work started again on purification of the 30-kDa PTTH. Kataoka *et al.* (1987, 1991) established a scheme for PTTH purification consisting of 16 procedures which included five different HPLC procedures as the terminal steps. The increase in the specific activity was as much as 5×10^6 fold, and only 5.4 μg of pure PTTH was obtained from a batch of 5×10^5 *Bombyx* heads. One tenth ng of the pure PTTH was able to induce adult development when injected into a *Bombyx* brainless pupa. Since one *Bombyx* brain (fifth larval instar to pupa) contains ca. 10 *Bombyx* units of PTTH activity (Ishizaki *et al.*, 1983b), one brain was calculated to contain ca. 1 ng of PTTH. When the purified PTTH was subjected to SDS-PAGE after reduction by the 2-mercaptoethanol treatment, two bands of 16-17 kDa appeared, though intact PTTH was obtained as a single 30-kDa band. Since the sequence analysis of the peptides generated by reductive alkylation of PTTH after separation by an HPLC showed the same results regarding the N-terminal sequence as intact PTTH, we concluded that PTTH was a dimeric protein composed of two identical or nearly identical subunits which were linked by a disulfide bond(s).

PTTH comprises heterogeneous molecules, as in the case of bombyxin. Unlike bombyxin, however, even by the HPLC of the last step of purification, each molecular species of PTTH was not resolved as a single, well-defined peak. Figure 6 depicts the chromatographic profiles of the 15th and 16th (final) steps of purification. In the TSK gel SP-5PW ion-exchange HPLC (15th step, Fig. 6 left), PTTH activity scattered over the four fractions whose OD profile showed four peaks that overlapped one another. When each of the four fractions was further subjected to another HPLC, the Hi-Pore RP-304 reversed phase HPLC (step 16, the final step, Fig. 6, right), PTTH activity was recovered again from widely scattered fractions, whose OD profile showed several overlapping peaks. An important fact was noticed that the specific activity of each fraction of these preparations was not raised any more from step 15 to 16. Furthermore, the amino acid compositions of these fractions were about the same, and almost identical peptide mapping patterns were obtained after the V8 protease digestion of these fractions. When several fractions of the 16th-step HPLC were subjected to the Edman degradation, none of the fractions yielded a single clean sequence, but instead, a mixture of three kinds of phenylthiohydantoin amino acids was released at each step of degradation. After careful inspection and quantitative analysis of the data, we came to the conclusion that each fraction contained three components having the sequences that differed

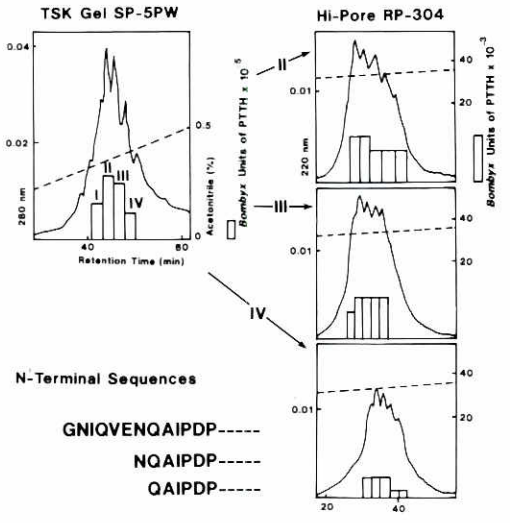

Fig. 6. Chromatographic profiles of the last two steps of PTTH purification. *TSK-Gel SP-5PW HPLC (left, the 15th step of the purification scheme) and Hi-Pore RP-304 HPLC [right, the last (16th) step]. Each of fraction II, III and IV in the left HPLC was subjected to the right HPLC. Histograms, PTTH activity. Sequencing data of the active fractions were analyzed to reach the conclusion that all fractions contained three heterogeneous PTTH molecules differing only at their N-termini in length, as shown at bottom left.*

only by the deletion of short portions at the N-terminus, as shown in lower left of Fig. 6.

Based on the above data, we concluded that the elimination of substances other than PTTH had been completed at step 16, and the seemingly heterogeneous OD profiles with overlapping peaks were due to a microheterogeneity of PTTH resulting from minor variations at the N-terminal lengths. Circumstantial evidence suggested that similar deletions may be present at the C-terminus also. We gathered the fragmentary sequencing data obtained for each fraction of step 16 to construct the sequence of the longest (having the full N-terminus) PTTH subunit up to the 104th residue from the N-terminus (Kataoka *et al.*, 1991), without having reached the C-terminus. The limited amount of pure PTTH did not allow us to sequence PTTH any more. The 41st residue was undetermined since no phenylthiohydantoin amino acid was detected at this cycle of degradation.

Bombyx PTTH cDNA and deduced amino acid sequence: determination of the entire PTTH subunit sequence

Using a mouse antiserum recognizing *Bombyx* PTTH which was raised against a synthetic peptide corresponding to the N-terminal 15-amino acid sequence of the PTTH subunit, we screened a cDNA expression library prepared from mRNA of *Bombyx* larval brains to clone and characterize PTTH cDNA (Kawakami *et al.*, 1990). Two cDNAs were obtained which coded for a putative PTTH subunit consisting of 109 amino acids, the N-terminal 104 amino acids which matched precisely those clarified by sequencing the purified natural PTTH (Fig. 7A). Thus, the chemical analysis of the purified peptide had left only five C-terminal amino acids undetermined. Each subunit contains seven Cys. Positions 41-43 represent Asn, Lys, and Thr, indicating that a carbohydrate moiety is attached to this site. Besides the PTTH subunit, the cDNA encodes the signal peptide (29 amino acids), a 2-kDa peptide (p2K, 21 amino acids), and a 6-kDa peptide (p6K, 57 amino acids). The presence of two or three basic amino acids flanking these component peptides suggests that the posttranslational proteolytic cleavage occurs at these sites. Thus, we presumed that a large precursor protein, prepro-PTTH-subunit, consisting of 224 amino acids, is first synthesized, and then the PTTH subunit is liberated, before or after disulfide bond formation, by proteolytic cleavage. The two peptide components, p2K and p6K, are thought to be liberated along with the PTTH subunit, though their functions are unknown. As far as could be ascertained by data base search, the coding region of the PTTH cDNAs showed no sequence similarity with any known protein. When introduced into *Escherichia coli*, a portion of the cDNA encoding the PTTH subunit directed the expression of an active peptide that was functionally indistinguishable from natural PTTH, indicating that the carbohydrate moiety is not essential for biological activity.

Location of disulfide bonds in PTTH

The inter- and intra-chain disulfide bonds in PTTH have been located using *Escherichia coli* recombinant PTTH (Ishibashi *et al.*, 1994). The following account outlines briefly the procedures. Based on the hypothesis that interchain disulfide bonds may be more susceptible to the reduction than intrachain disulfide bonds, we first treated the recombinant PTTH with a moderate reducing reagent, tributylphosphine, in the presence of an *S*-alkylating reagent, 4-vinylpyridine. We expected that, under certain appropri-

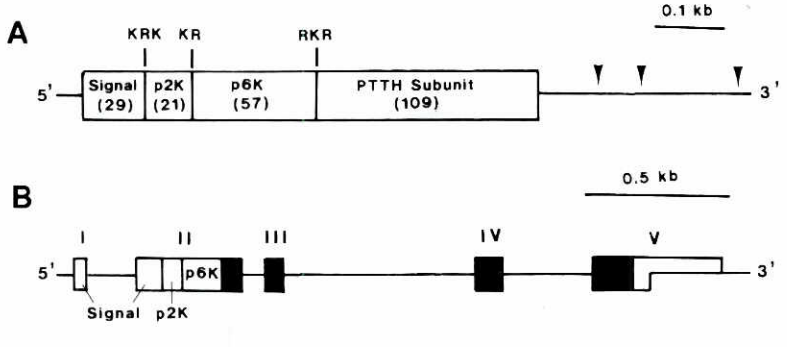

Fig. 7. Schematic representation of the cDNA and gene encoding prepro-PTTH-subunit. (A) *cDNA. The PTTH subunit (109 amino acids) is encoded by the 3'-end portion of the coding region. The signal peptide (29 amino acids), p2K (21 amino acids), and p6K (57 amino acids), which are bounded by proteolytic cleavage signals (KRK, KR, and RKR), and the PTTH subunit form a 224 amino acid prepro-PTTH-subunit. Arrow heads, polyadenylation sites.* **(B)** *Gene. The gene consists of five exons (boxes numbered I to V). Solid boxes code for the PTTH subunit.*

ate conditions, this reduction may cause dissociation of dimeric PTTH into a monomeric form, keeping intrachain disulfide bonds intact. We expected also that 4-vinylpyridine protects the freed sulfhydryl groups immediately after having been formed, hindering a possible disulfide bond exchange which may lead to erroneous judgement for disulfide bond locations. After titrating the conditions for such a partial reduction, we successfully obtained partially reduced, monomeric PTTH. This monomeric PTTH was completely reduced with dithiothreitol and *S*-carboxymethylated with sodium iodoacetate and digested with lysil endopeptidase. The generated peptides were isolated by an HPLC, and subjected to sequence analysis to determine *S*-pyridylethylated and *S*-carboxymethylated Cys's. The results showed that only Cys 15 was *S*-pyridylethylated, leading to the conclusion that Cys 15 was participated in the interchain disulfide bond. Next, the partially reduced and *S*-pyridylethylated PTTH monomer was digested with lysil endopeptidase and thermolysin, and the generated peptides were separated by HPLCs and subjected to sequence and mass analyses. The results indicated that there were three intrachain disulfide bonds, Cys 17-Cys 54, Cys 40-Cys 96, and Cys 48-Cys 98.

Figure 8 shows the entire primary structure of dimeric PTTH thus determined.

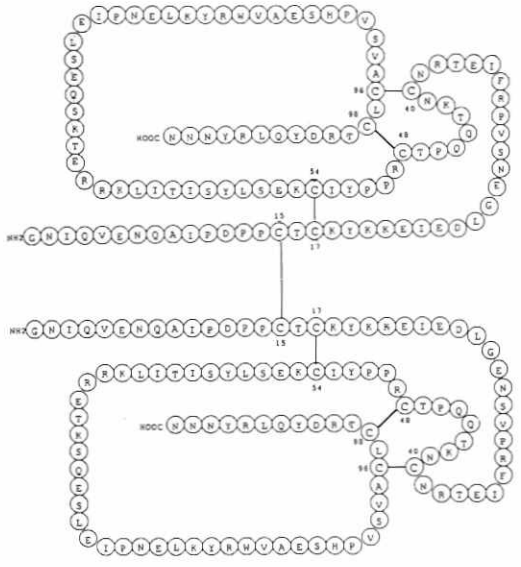

Fig. 8. Schematic drawing of the *Bombyx* PTTH primary structure. *C-C represents the disulfide bond. Parallel orientation of the two subunits is hypothetical.*

Genes coding for prepro-PTTH-subunit

We cloned two genes coding for prepro-PTTH-subunit (signal peptide/p2K/p6K/PTTH subunit) from a *Bombyx* genomic DNA library using the PTTH cDNA as a probe (Adachi-Yamada *et al.*, 1994). As shown diagrammatically in Fig. 7B, these PTTH genes consist of five exons which are intervened by four introns. The 3'-terminal portion of the second exon and the third to fifth exons encode the PTTH subunit. The nucleotide sequences of the two genes differ slightly from each other and from those of the two PTTH cDNAs described in the previous section, but the amino acid sequences deduced from the coding regions of the two genes and the two cDNAs are identical, except for the 44th position of the prepro-PTTH-subunit (Ser or Arg). Southern analysis of *Bombyx* genomic DNA revealed that the PTTH gene exists as a single copy per *Bombyx* haploid genome and we concluded that the two PTTH genes isolated and the genes from which the two cDNAs have been derived are allelic in nature. Thus at least four allelic genes have been proved to exist. The PTTH subunits deduced from the genes and cDNAs all contained 109 amino acids. The microheterogeneity in the primary structure of purified natural PTTH found by chemical analysis, in terms of the shortened N- and C-termini, is therefore likely to have derived from posttranslational processing or denaturation during purification, rather than from the genetic variation. The possibility exists, however, that genes with the coding region for shortened PTTH subunits remain undetected.

Data base search revealed that a part of the 5'-noncoding region was highly similar to the first intron of the *Bombyx* fibroin gene (Tsujimoto and Suzuki, 1970). Another portion in the third intron shows similarity with the repetitive gene of *Bombyx*, Bm-1 (Adams *et al.*, 1986).

The PTTH gene is expressed only in the brain, as far as examined by Northern analysis of total RNA prepared from various tissues, in accordance with the historical view that PTTH is a neurohormone specific to brain. The analysis using polymerase chain reaction, however, revealed that PTTH mRNA was contained in the gut, epidermis, and silk gland at low concentrations (Adachi-Yamada *et al.*, 1994). The presence of PTTH mRNA in the gut is not unexpected, because the proteins having PTTH bioactivity have been found in proctodaea of the European corn borer *Ostrinia nubilalis* and the gypsy moth *Lymantria dispar* (Gelman *et al.*, 1993). Similarly, the brain insulin-related peptide of *Locusta migratoria* has been shown to be expressed in the epidermis and fat body (Lagueux, cited from Hoffmann and Joosse, 1991). Expression of peptides originally found in nervous tissues have often been shown to be expressed in gut, and *vice versa*, both in invertebrates and vertebrates (Endo, Y. *et al.*, 1990).

Using a *Bombyx* PTTH cDNA as a probe, Adachi-Yamada *et al.* (1992; oral communication) cloned a cDNA encoding a putative prepro-PTTH-subunit from a *Samia* brain cDNA library. As shown in Fig. 9, the *Samia* PTTH subunit contains 125 amino acids which are 46% similar to the *Bombyx* PTTH subunit. Seven Cys residues are completely conserved. A large deviation from the *Bombyx* PTTH sequence is seen in the C-terminal 22 amino acids. Two or three basic amino acids intervening the signal peptide, p2K, and p6K, which are found in the *Bombyx* prepro-PTTH-subunit, are not observed at the corresponding sites of the *Samia* prepro-PTTH-subunits, suggesting the difference in the posttranslational processing of the prepro-PTTH-subunit between the two species. *Escherichia coli* recombinant *Samia* PTTH exhibited the prothoracicotropic activity when tested with *Samia* brainless pupae (Ishibashi *et al.*, in preparation).

PTTH-producing cells

A monoclonal antibody recognizing PTTH was raised against a synthetic peptide corresponding to the N-terminal 1-15 amino acid sequence of the *Bombyx* PTTH subunit (Mizoguchi, 1990; Mizoguchi *et al.*, 1990). This antibody immunostained two pairs of dorsolateral neurosecretory cells of *Bombyx* brain (Fig. 4C). The immunoreactive material was also detected in the axons of these neurosecretory cells which run across the brain midline to reach the contralateral corpora allata, suggesting that PTTH may be liberated into

Fig. 9. Alignment of the amino acid sequences of the *Bombyx* PTTH and *Samia* PTTH subunits. *Gaps are introduced for maximum alignment. Identical residues are shaded. The region C-terminal to RKR in bracket, a putative proteolytic cleavage signal, represents the PTTH subunit. The Samia sequence is from Adachi-Yamada et al. (1992). The nucleotide sequences of the Samia PTTH cDNAs have been submitted to Genbank with accession number L25668.*

hemolymph from the corpora allata. The corpora allata have previously been shown to be the neurohemal organ for PTTH release in the tobacco hornworm, *Manduca sexta* (Agui *et al.*, 1980). The axon terminals containing the PTTH immunoreactivity penetrated the corpora allata (Fig. 4D), contrasting with the axons containing bombyxin, which are preferentially localized in the surface region of the allata (Fig. 4B). By *in situ* hybridization for PTTH mRNA using the *Bombyx* PTTH cDNA as a probe, the same two dorsolateral neurosecretory cells of *Bombyx* brain were radiolabeled (Fig. 4F), confirming that PTTH mRNA is actually synthesized in these cells (Kawakami *et al.*, 1990).

Two pairs of dorsolateral brain neurosecretory cells of *Samia* brain, at the position similar to the *Bombyx* PTTH cells, were immunostained with a monoclonal antibody raised against a synthetic peptide corresponding to the N-terminal 1-17-amino acid sequence of the *Samia* PTTH subunit that was deduced from the *Samia* PTTH cDNA (Yagi *et al.*, in preparation). The same immunohistochemical results for PTTH cells as those obtained for *Bombyx* and *Samia*, in terms of the site and number of PTTH cells and the PTTH pathway through axons to the corpora allata, have been previously described for *Manduca sexta* (O'Brien *et al.*, 1988).

Concluding remarks

We were able to elucidate the outlines of the molecular features of *Bombyx* PTTH and the functionally PTTH-like peptide, bombyxin. The nature of PTTH and bombyxin homologs of *Samia* were also clarified to a certain extent. Gene technology has been a powerful tool for accomplishing these works, but we wish to stress that enough background for molecular features, established by tedious purification and amino acid sequencing studies in which we had to invest tremendous energy for more than 20 years, made it possible to apply the gene approach successfully. In fact, we have sent the cDNA and gene probes to several laboratories upon request, but as yet we have heard no information of success in cloning PTTH genes from other insect species, including such important insects as *Drosophila* and the tobacco hawkmoth *Manduca sexta*. Failure in cloning PTTH genes simply by using the *Bombyx* cDNA or gene probes seems not surprising, in view of the 46% amino acid sequence similarity between the *Bombyx* PTTH and *Samia* PTTH, which is unexpectedly low considering the relatively close phylogenetic relatedness of the two species. The PTTH gene structure may vary largely within Insecta. We are reminded of a story of cloning the *Drosophila* gene for cadherin, a cell-cell adhesion molecule (Mahoney *et al.*, 1991). Intense trials to clone the *Drosophila* cadherin gene by using probes representing the cytoplasmic domain, which is highly conserved among different vertebrate cadherins turned out negative, and successful results were obtained by a polymerase chain reaction strategy applied to the cytoplasmic domain sequence which shows very low similarity in vertebrate cadherins. We hope sophisticated strategies and tenacious efforts may clarify sooner or later the PTTH gene structure of other insects.

The most intriguing, and as yet unsolved, problem relates to an evolutionary aspect of insect PTTH, i.e., the "big PTTH" vs "small PTTH" (or small PTTH-like molecule) issue. As described in the present paper, the *Bombyx* brain produces 30-kDa genuine PTTH and 5-kDa bombyxin, which is totally different from PTTH in structure but exhibits PTTH activity when assayed with the heterologous moth *Samia*. Bombyxin has been shown to activate the prothoracic gland of *Bombyx in vitro* to enhance ecdysone release, but only at an unphysiologically high concentration (Kiriishi *et al.*, 1992). On the other hand, our unpublished experiments have shown that the *Samia* brain contains ~5-kDa and ~30-kDa PTTHs, both of which can provoke adult development when injected into *Samia* brainless pupae. A recombinant 30-kDa *Samia* PTTH homolog, produced by using *Samia* PTTH cDNA homologs to *Bombyx* PTTH cDNA, exhibited PTTH activity when injected into *Samia* brainless pupae (Ishibashi *et al.*, in preparation). A monoclonal antibody recognizing this recombinant *Samia* PTTH homolog immunostained two pairs of dorsolateral neurosecretory cells of *Samia* brain localizing at the same place as *Bombyx* PTTH cells (Yagi *et al.*, in preparation). SBRPs, the bombyxin homologs of *Samia*, which were chemically synthesized based on the amino acid sequences deduced from the SBRP genes, have also been shown to possess PTTH activity when injected into *Samia* brainless pupae (Nagata *et al.*, in preparation). The SBRP antibodies immunostained 16 pairs of dorsomedial neurosecretory cells of *Samia* brain, at the position equivalent to that of the bombyxin cells of *Bombyx* brain (Yagi *et al.*, in preparation). From these results, it seems highly probable that the 5-kDa and 30-kDa PTTHs detected in *Samia* brain by bioassay represent the homologs of 30-kDa *Bombyx* PTTH and 5-kDa bombyxin, respectively. In the tobacco hornworm *Manduca sexta*, Bollenbacher *et al.* (1984) demonstrated the presence of big PTTH (22-28 kDa) and small PTTH (4-7 kDa), and this molecular differentiation of the two forms of PTTH has been implicated in their stage-specific differential functions. They used the *in vitro* assay method for PTTH (Bollenbacher *et al.*, 1979), which measures the increase in ecdysone release from the prothoracic gland in culture upon addition of PTTH. Later, Watson *et al.* (1989) have shown that the small PTTH of *Manduca* is several orders of magnitude less potent than the big PTTH, when assayed by the *in vivo* larval assay (Gibbs and Riddiford, 1977) which

monitors the induction of moulting in the neck-ligated penultimate-instar larvae. The fact that the purification studies for *Manduca* PTTH using the *in vivo* larval assay detected only big PTTH (Gibbs and Riddiford, 1977; Kingan, 1981) may be reconciled by the far less potent *in vivo* activity of small PTTH. Similar big and small PTTH forms have been documented for the Asian comma butterfly *Polygonia c-aureum* L. (Endo, K. *et al.*, 1990), the gypsy moth *Lymantria dispar* (Kelly *et al.*, 1991), and *Drosophila* (Pak *et al.*, 1992), all of which used only the *in vitro* assay. From the above survey of the PTTH molecules thus far reported for various insects, it is clear that all the insects possess two, big and small, molecular forms of the peptide manifesting prothoracicotropic activity. Historically, PTTH was first defined as a brain hormonal factor that elicits the resumption of development when introduced into insects deprived of their brain and caused to enter developmental arrest (e.g., Williams, 1947). Among the PTTH molecules so far studied, only the Bombyx 30-kDa PTTH and the *Manduca* big (22-28 kDa) PTTH have been proved to fulfill this criterion of the *in vivo* effect. Regarding the small PTTHs described for many insect species, on the other hand, they certainly exhibited prothoracicotropic activity in terms of an *in vitro* enhancement of ecdysone release by the prothoracic gland, but they have been proved to be inactive or far less active *in vivo*, or the *in vivo* activity has not, in most cases, been examined. In the case of SBRP in *Samia*, synthetic SBRPs displayed a clear-cut *in vivo* PTTH activity, but it is not certain whether native SBRP is actually involved in the physiological activation of the prothoracic glands. Thus, it is an enigma whether small PTTHs play the physiological role in the prothoracic gland activation and for what purpose they had to be evolved in insects. Elucidation of the primary structure of small PTTHs and, more hopefully, understanding of receptor molecules and signal transduction pathway will answer this question. Recent progress in the molecular study of insect neuropeptides has been very rapid; as many as 80 insect neuropeptides have been sequenced and more than 10 genes characterized (Kelly *et al.*, 1994). Studies on receptors and signal transduction of insect neuropeptides are rather scanty, however, compared to the advances made for their molecules, and the next era of insect neuroendocrinology will focus on these fields.

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