Identification of a *cis*-regulatory element that directs prothoracicotropic hormone gene expression in the silkworm *Bombyx mori*

Kazunori Ohtsuka, Tsutomu Atsumi, Yoshihisa Fukushima, and Kunihiro Shiomi[‡]

[1]Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan.

[‡]Address correspondence to: K. Shiomi, Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan
Tel: +81-268-21-5338; Fax: +81-268-21-5331; E-mail: shiomi@shinshu-u.ac.jp

Abbreviations: PTTH, prothoracicotropic hormone; BmPitx, *Bombyx mori* pituitary homeobox; BmMEF2, *Bombyx mori* myocyte enhancer factor 2; DH-PBAN, diapause hormone-pheromone biosynthesis activating neuropeptide; EGFP, enhanced green fluorescence protein; AcNPV, *Autographa californica* nucleopolyhedrovirus; PTPCs, PTTH-producing neurosecretory cells.

Running title: [*Cis*-regulatory element directing *PTTH* expression of *Bombyx*]

ABSTRACT

In the silkworm *Bombyx mori* and other insects, prothoracicotropic hormone (PTTH) plays a central role in controlling molting and metamorphosis by stimulating the prothoracic glands to synthesize and release the molting hormone ecdysone. Using an AcNPV (*Autographa californica* nucleopolyhedrovirus)-mediated transient gene transfer system, we identified a *cis*-regulatory element that participates in the decision to switch expression of *PTTH* on or off in PTTH-producing neurosecretory cells (PTPCs). The nucleotide sequence of this *cis*-regulatory element is similar to a *cis*-regulatory element that participates in direction of expression of diapause hormone-pheromone biosynthesis activating neuropeptide gene (*DH-PBAN*) (Shiomi et al., 2007). Furthermore, we found that *Bombyx mori* Pitx (BmPitx), a bicoid-like homeobox transcription factor, binds the element and activates *PTTH* expression. Therefore, we propose that the cell-specific expression of two neuropeptide hormone genes, *PTTH* and *DH-PBAN*, is activated by the Pitx transcription factor, which may act as a pan-activator in the insect neuroendocrine system and in vertebrate pituitary cells.

Key words: neurosecretory cell, *cis*-regulatory element, *PTTH*, *DH-PBAN*, Pitx, insect

1. Introduction

Neuropeptide hormones are preferentially synthesized in specific neurosecretory cells, which have a defined morphology and histology, to regulate the different and specific physiological and behavioral process under control of these hormones. Prothoracicotropic hormone (PTTH) plays an essential role in regulating the production and/or release of ecdysone, the steroid necessary for molting, metamorphosis, and the termination of pupal diapause by stimulating the prothoracic glands in many insects (Bollenbacher and Granger, 1985). In *Bombyx mori*, PTTH is produced exclusively in two pairs of lateral PTTH-producing neurosecretory cells (PTPCs) in the brain (Mizoguchi et al., 1990). From there, PTTH is transported via axons that arise from the somata and run to the neurohemal organ, the corpora allata (CA), and then it is released into the hemolymph. The stringent regulation of neuropeptide hormone secretion seems to be essential for the neuropeptide to exert its own physiological function, However, how expression of *PTTH* is limited to a specific set of neurons is not well understood, although presumably *PTTH* expression is under the control of integrated regulation machinery involving *cis*-regulatory elements and/or cellular *trans*-activating factors.

To help reveal the molecular mechanism of *PTTH* expression in PTPCs, we have characterized *PTTH* promoter elements. Previously, we found that a *cis*-regulatory element located at nucleotides -170 to -161 upstream of the transcription initiation site

of the *PTTH* gene participates in enhancing *PTTH* expression. Furthermore, we showed that *Bombyx* myocyte enhancer factor 2 (BmMEF2) binds to this element using a high-throughput, baculovirus-based reporter gene system, the AcNPV gene transfer system (Shiomi et al., 2003; Shiomi et al., 2005). Based on the results of serial deletion experiments, we made the preliminary prediction that the 5'-upstream region from nucleotides -119 to -105 of *PTTH* are involved in direction of *PTTH* expression in PTPCs (Shiomi et al., 2005). We could not perform further characterization of the *cis*-regulatory region at that time because the fluorescence read-out we were using was observed only faintly when BmMEF2 binding sequences were not present on the reporter constructs. In previous work, we also identified a *cis*-regulatory element located at nucleotides -1123 to -1103 of the *DH-PBAN* gene that regulates expression in diapause hormone-pheromone biosynthesis-activating neuropeptide (DH-PBAN)-producing neurosecretory cells (DHPCs), helping to reveal the mechanisms of regulation of cell-specific *DH-PBAN* expression in *Bombyx* (Shiomi et al., 2007). *Bombyx mori* Pitx (BmPitx), a bicoid-like homeobox transcription factor, binds this element and activates *DH-PBAN* expression.

In *Drosophila melanogaster*, PTTH-producing neurons directly innervate the prothoracic gland, instead of the CA, to regulate ecdysone production, which indirectly controls by the gap gene *giant* (McBrayer et al., 2007; Ghosh et al., 2010). This difference suggests that analysis of the regulation of PTTH secretion may be an excellent model for understanding conservation and diversification in the

neuroendocrine system during insect evolution.

In this study, we identified a *cis*-regulatory element in the core promoter region that directs *PTTH* expression. Furthermore, we found evidence that BmPitx may bind this element and activate *PTTH* expression as well as *DH-PBAN* expression. Therefore, we propose that the cell-specific expression of two neuropeptide hormone genes, *PTTH* and *DH-PBAN*, is activated by Pitx transcription factor, which may act as a pan-activator in insect neuroendocrine systems as well as in vertebrate pituitary cells.

2. Materials and Methods

2.1. Insects

The polyvoltine (N4) strain of *Bombyx mori* was used in these experiments. Eggs were incubated at 25°C under continuous darkness. Larvae were reared on an artificial diet (Kuwano-hana, JA Zennoh Gunma, Gunma, Japan) and at the fifth instar stage, larvae were given fresh mulberry leaves. During larval stages, silkworms were reared at 25 to 27°C under a 12 h light, 12 h dark cycle. Pupae were kept at 25°C to allow adult development. Injection of recombinant AcNPV was performed according to Shiomi *et al*. (2005, 2007).

2.2. Preparation of recombinant AcNPVs

Recombinant AcNPVs were prepared using the Bac-to-bac baculovirus system (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions and as described by Shiomi *et al*. (2003). Recombinant AcNPV containing the promoter region of *PTTH* from -180 to +52 fused to *EGFP* was prepared as described previously (Shiomi et al., 2005) and is referred to as the *Wt* virus in this study. Nine recombinant AcNPV transfer vectors were constructed, comprising deletions of promoter region of *PTTH* from -119 to -29 (*M1*), from -119 to -90 (*M2*), from -89 to -29 (*M3*), from -89 to -59 (*M4*), from -71 to -57 (*M5*), from -62 to -48 (*M6*), from -56 to -42 (*M7*), from -49 to -36 (*M8*), and from -45 to -29 (*M9*) (see Fig. 1B). The mutant

promoter regions were constructed using PCR amplification as described by Habib and Hasnain (2000) with appropriate modifications. In the final PCR amplification step, the forward primers included a *Sal*I site and the reverse primer included a *Nco*I site. The PCR products were digested with *Sal*I and *Nco*I and then inserted into pPT/EGFP (Shiomi et al., 2003) lacking the promoter region of *PTTH*. We used a PCR-based method to generate mutant forms of the AcNPV reporters (designated *Ma*, *Mb*, *Mc* and *DC1*) containing mutant sequences in the region -59 to -30 of the *PTTH* promoter (Fig. 3A). Each recombinant plasmid was sequenced and the recombinant AcNPVs were prepared. Recombinant AcNPVs were prepared via transposition of the expression cassettes from the recombinant plasmids according to the manufacturer's instructions (Invitrogen). The titers of budded virions were determined using the BD BacPAK Baculovirus Rapid Titer Kit (BD Biosciences, Palo Alto, CA, USA).

2.3. Reporter gene analysis and immunohistochemistry

Budded virions (20 µl/pupa; 2×10^4 pfu) were injected into pupae 0-6 hr after pupation as described by Shiomi *et al*. (2003, 2005). Four days after injection with recombinant AcNPV, the brain-subesophageal ganglion (SG) complexes of pupae were dissected in phosphate-buffered saline (PBS) and subjected to immunohistochemistry using an anti-PTTH antibody (3E5mAb) (Mizoguchi et al., 1990) as described by Shiomi *et al*. (2005). EGFP fluorescence and anti-PTTH immunofluorescence were observed using a Radiance 2000 confocal microscope (Bio-Rad, Hercules, CA, USA).

Confocal scanning was first adjusted to optimize detection of EGFP fluorescence and anti-PTTH immunofluorescence (Cy3) using appropriate control samples, then the scanning conditions were kept the same. Images were adjusted and assembled in Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, USA). The relative fluorescence intensities of the PTPCs somata are indicated by (+++), (++), (+), and (-) in Fig. 1B.

2.4. Gel-mobility shift assay

Cell extracts were prepared from a mixture of brain-SG complexes from day 1, 3, and 5 pupae of the N4 strain according to Ueda and Hirose (1990). The gel-mobility shift assays were performed as described by Ueda and Hirose (1991) and Shiomi *et al*. (2005). As appropriate, a rabbit anti-Ptx3/PTX3 affinity purified polyclonal antibody (Cemines, LLC, Evergreen, CO, USA), which recognizes 15 amino acids in the homeodomain (helix III) of Pitx protein (see Fig. 4A), was added to the cell extracts and incubated for 1 hr on ice prior to addition of the probe.

2.5. Preparation of recombinant BmPitx using a baculovirus expression system

The open reading frame of a full-length *BmPitx* cDNA (from +296 to +1459 of the nucleotide sequence with NCBI Accession No. AB162107) (Shiomi et al., 2007) was PCR amplified. The forward primer included a *Nco*I site and the reverse primer included a *Sal*I site. The PCR product was sequenced and digested with *Nco*I and *Sal*I, and then inserted into pFASTBacHTb (Invitrogen). Recombinant BmPitx protein fused

with an N-terminal His tag was expressed according to the manufacturer's instructions (Invitrogen). Cell extracts from SF9 cells were suspended with extraction buffer [100 mM NaH_2PO_4, 10 mM Tris-HCl containing 8 M urea (pH 8.0)] followed by sonication. After centrifugation, the supernatant was dialyzed overnight against dialysis buffer [20 mM HEPES (pH 7.9), 50 mM NaCl, 0.25 mM EDTA, 0.5 mM dithiothretiol and 20% Glycerol], and then subjected to immunoblot analysis.

2.6. Immunoblot analysis

Ten brain-SG complexes were dissected from pupae 4 days after pupation and homogenized in 1x Laemmli sample buffer. After separation by 12% SDS-PAGE, proteins were transferred to a Immobilon-P PVDF membrane (Millipore, Billerica, MA, USA), which was then incubated with anti-Ptx3/PTX3 polyclonal antibody (Cemines) or Penta-His antibody (Qiagen, Hilden, Germany) at 1:2500 and 1:10000, respectively, in PBST (PBS containing 0.2% Tween-20) with 2% skimmed milk at 4°C overnight. The signals were detected using horseradish peroxidase-linked goat anti-rabbit IgG (Invitrogen) and ECL-Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK).

3. Results and Discussion

3.1. Identification of a cis*-regulatory element directing PTTH expression*

Our first goal was to identify a region(s) of the *PTTH* promoter that may be necessary for specific control of *PTTH* expression. To do this, we first confirmed that, when under control of nucleotides -180 to +52 of the *PTTH* promoter, EGFP expression is observed in the somata and neurites of PTPCs using AcNPV-mediated reporter gene analysis (Shiomi et al., 2005). In virus containing the wild-type promoter (*Wt)*, the intense fluorescence signals were localized within two pairs of lateral cells in the protocerebrum, and the axons emanating from the somata of the two pairs of lateral cells extend towards the pars intercerebralis with some arborization (Figs. 2A and A'). Comparing PTTH as visualized with anti-PTTH antibodies with the EGFP signal, we found that the same cells within the somata are positive for both markers (Fig. 2A). Thus, the region of the *PTTH* promoter present in the reporter construct (*i.e.*, nucleotides -180 to +52) appears to contain the *cis*-regulatory element(s) that direct expression of *PTTH* specifically in PTPCs. Next, we constructed nine recombinant AcNPVs with various truncated *PTTH* promoter sequences, and performed reporter gene analysis. Recombinant AcNPV in which nucleotides -119 to -29 have been deleted (*i.e.*, the *M1* virus) resulted in no detectable EGFP signal in PTPCs (n = 10; Figs. 2B and B'), although faint signal can still be detected in small, non-PTPC cells in the lateral brain (Fig. 2B, arrowhead). In contrast, the signal observed for the *M2* virus in PTPCs

(n = 10) was indistinguishable for that observed with the *Wt* virus (Figs. 2C and C'). Furthermore, EGFP fluorescence was observed in PTPCs when *M4* (n = 15), *M5* (n = 15), *M6* (n = 10), or *M7* (n = 10) virus was injected (Figs. 2E-H and E'-H'), although the fluorescence signals were slightly lower for the *M4* and *M5* viruses than for the others. With recombinant AcNPVs *M3* (n = 20), *M8* (n = 20), or *M9* (n = 20), no EGFP signals were observed in PTPCs (Figs. 2D, I and J). These results suggest that there are two *cis*-regulatory elements in nucleotides -180 to +52 of *PTTH*. The 5'-upstream region of *PTTH* (nucleotides -89 to -62) may contain a *cis*-regulatory element with some influence on *PTTH* expression and, furthermore, that sequences in the region from -42 to -36 of the *PTTH* promoter contain a *cis*-regulatory element involved in direction of *PTTH* expression. Previously, Wei *et al*. (2010) and we tried to identify *cis*-elements participating *PTTH* expression in *Bombyx* but had not succeeded in identifying such an element. The results of the present study clearly demonstrate the presence of a *cis*-regulatory element in the core promoter region of *PTTH*. Our data are currently insufficient, however, to fully explain the relationship between this element and *cis*-regulatory elements contained in the -180 to +52 bp region of *PTTH*.

3.2. Consensus binding sequences for Pitx homeodomain transcription factor are important for PTTH expression

To identify the *trans*-activating factor(s) that direct *PTTH* expression, we first performed a gel mobility shift assay using four double-stranded oligonucleotides

encoding nucleotides -67 to -38, -59 to -30, -44 to -15, and -36 to -7 of the *PTTH* promoter as probes (Fig. 3B, lanes 1-4). Intense shifted bands were detected using a probe encoding nucleotides -59 to -30 of the *PTTH* promoter (Fig. 3B, lanes 2). The shifted bands disappeared when unlabeled nucleotides were added at 100-fold excess as a competitor (Fig. 3B, lanes 5 and 6). We further synthesized three double-stranded oligonucleotides as competitors to analyze the sequence specificity of the protein bound to *Wt.* These oligonucleotides, designated *Ma*, *Mb*, and *Mc*, replace nucleotides -59 to -48, -46 to 30, or -51 to -35, respectively, with GT repeats (Fig. 3A). When unlabeled *Ma* was added, shifted bands were eliminated (Fig. 3B, lane 7). However, even at 100-fold excess, *Mb* was unable to compete for binding to *Wt* (Fig. 3B, lane 8). With *Mc,* we observed no change in the intensity of the bands (Fig. 3B, lane 9).

We also performed reporter gene analysis using recombinant AcNPVs with nucleotides -180 ~ +52 of *PTTH* promoter in which nucleotides -59 ~ -30 were replaced with the *Ma*, *Mb*, and *Mc* replacements, respectively (Figs. 3C-E). Based on fluorescence intensity, the *Ma* virus was as effective at mediating EGFP expression as the *Wt* virus (Figs. 3C and C'). The *Mb* virus resulted in an abrupt relative decrease in expression of EGFP in PTPCs (Figs. 3D and D'). No EGFP expression was observed with the *Mc* virus, which disrupts from nucleotides -51 to -35 of the *PTTH* promoter (Figs. 3E and E'). Taken together, these results suggest that the protein(s) detected as shifted band(s) in the gel-mobility shift assays contributes to directing *PTTH* expression in PTPCs.

Interestingly, we found that the 15-bp sequence from nucleotide -50 to -36 of the *PTTH* promoter is similar to the *DC1* sequence, a sequence in the *DH-PBAN* gene region that is bound by the BmPitx transcriptional factor (Fig. 3A) (Shiomi et al., 2007). Therefore, we next asked if the *DC1* sequence affects *EGFP* expression in PTPCs by replacing nucleotides -59 to -30 of the *Wt* virus with the *DC1* BmPitx binding sequence. We found that with this reporter construct, intense EGFP signals are observed in PTPCs (Figs. 3F and F'). Furthermore, unlabeled *DC1* competes for binding to nucleotides -59 ~ -30 of *PTTH* promoter in a gel-mobility shift assay (Fig. 3B lane 10). In addition, the shifted band was less intense in the presence of anti-Ptx3/PTX3 IgG (Fig. 3B, lane 12), which recognizes the vertebrate Pitx homeodomain. By contrast, normal rabbit IgG had no effect (Fig. 3B, lane 11), suggesting that the effect observed with anti-Ptx3/PTX3 is specific. It seems likely that the presence of anti-Ptx3/PTX3 resulted in reduced binding of the protein-DNA complex (*i.e.* shifted bands) because antibody binding to the Pitx-immunoreactive protein in the extract blocked binding to *Wt* double-stranded oligonucleotides. These results suggest that Pitx or a Pitx-immunoreactive protein functions as a *trans*-activating factor to direct *PTTH* expression. We speculate that the higher shifted band (Fig. 3B, arrow) and the lower shifted band (Fig. 3B, asterisk) in the gel-mobility shift assay represent a Pitx protein-DNA complex and a complex between the DNA and another binding factor, perhaps a homeodomain protein (see below). Consistent with this idea, the higher shifted band is more sensitive than the lower shifted band to the addition of *DC1* sequence and to the addition of anti-Ptx3/PTX3

antibody.

Next, we performed immunoblot analysis using anti-Ptx3/PTX3 antibody to identify the protein bound to *Wt* and *DC1* double-strand oligonucleotides in gel-mobility shift assay. His-tagged BmPitx expressed using a baculovirus expression system was recognized by both anti-Penta-His and anti-Ptx3/PTX3 antibodies at about 44 kDa (Fig. 4, lanes 1 and 2), which is similar to the predicted molecular weight (BmPitx, 41052.09 Da; His tag, 3114.20 Da). Likewise, a 41-kDa protein band was predominantly detected in *Bombyx* brain extracts using anti-Ptx3/PTX3 antibody (Fig. 4, lane 3), suggesting that the 41-kDa immunoreactive protein is attributable to BmPitx. The *Bombyx* genome encodes only one member of Pitx family (Chai et al., 2008; Shiomi et al., 2007) and the other intense bands we observed were too small to be homeodomain proteins. Thus, we conclude that BmPitx binds nucleotides from -59 to -30 of the *PTTH* promoter, such that it functions as a *trans*-activating factor to direct *PTTH* expression.

The canonical core sequence of the Pitx of bicoid-like transcription factors is TAAGCT; however, this sequence is not conserved in the *PTTH* promoter. It has been proposed that the DNA-binding specificity of homeodomain-containing proteins in the endogenous setting is determined not by a single consensus but instead by a combinational molecular code among the DNA-contacting residues (Damante et al., 1996). For example, the binding specificity of the *Drosophila* Hox-Exd homeodomain protein complex is markedly different from that of the individual monomers (Joshi et al.,

2007), raising the possibility that the binding preferences of monomers assayed *in vitro* may not always be relevant to targeting *in vivo*. A conserved DNA sequence predicted to be bound by a homeodomain transcription factor was found downstream of Pitx binding sequence (nucleotides -43 to -37; CAATCAA) by using the software tool MATINSPECTOR (http://www.genomatix.de/) (Berger et al., 2008). Thus, since the *cis*-regulatory element directing the *PTTH* expression does not contain a conserved consensus sequence for Pitx binding, sequences adjacent to the core Pitx binding sequence may be necessary for BmPitx binding and expression of *PTTH*.

3.3. Putative function of the Pitx homeodomain protein in the Bombyx *neuroendocrine system*

In mammals, Pitx1 is present in all pituitary lineages and is a strong activator of several pituitary-specific promoters; thus, Pitx1 can be considered a pan-pituitary transcriptional activator (Tremblay et al., 1998). In addition, mammalian Pitx1 acts to control expression at promoters of hormone-encoding genes, including the promoters of growth hormone (GH), prolactin (PRL), pro-opiomelanocortin (POMC), luteinizing hormone β-subunit (LHβ), glycoprotein hormone α-subunit, follicle-stimulating hormone β, and thyroid-stimulating hormone (Tremblay et al., 1998). In this report, we have provided evidence that the BmPitx transcription factor is involved in expression of the *PTTH* neuropeptide gene in PTPCs, similar to its role in *DH-PBAN* expression in DHPCs (Shiomi et al., 2007). Thus, it appears that Pitx function contributes to

neuropeptide hormone gene expression in neurosecretory cells not only in the vertebrate pituitary but also in the insect central nervous system. BmPitx may act as a pan-activator like mammalian Pitx1. Moreover, BmPitx may form heterodimers with unknown protein(s) to regulate expression of *DH-PBAN* and *PTTH*.

In *Drosophila*, the differentiation of neurosecretory cells and the neuropeptide hormone secretory pathway have been investigated in detail using genetic, anatomical, and molecular approaches. DIMMED, a bHLH transcription factor, is a master regulator that promotes a specific neurosecretory phenotype in *Drosophila* (Park and Taghert, 2009). Interestingly, PTTH-positive cells are the principal example of DIMMED-negative neuroendocrine cells. Thus, our results might shed light on evolutionary diversification and conservation in insect neuroendocrine systems as well as specifically helping us to understand a general mechanism for regulation of non-DIMMED cells in insects.

Acknowledgements

We thank Dr. Yutaka Banno of Kyushu University and Dr. Hideaki Maekawa of the Ryukyus for kindly providing the fresh mulberry leaves during winter seasons, and Dr. Akira Mizoguchi for the gift of the anti-PTTH antibody. This research was supported by Grants-in-Aid for Scientific Research (17658027) from the Japan Society for the Promotion of Science. We are also indebted to the Division of Gene Research, Research Center for Human and Environmental Sciences, Shinshu University, for providing facilities for these studies.

FIGURE LEGENDS

Figure 1. Construction of recombinant AcNPVs for *PTTH* reporter gene analysis. (A) 5'-upstream region of *PTTH* from nucleotides -180 to +52. A MEF2 binding consensus (-170 ~ -161) is shaded. The transcriptional initiation site (+1) is shown in bold letters. The translational initiation site is boxed. In our reporter construct, the start codon Met and subsequent coding sequence is from an *EGFP* cDNA. A putative TATA box is underlined. (B) Schematic representation of the promoter regions of wild-type and mutant recombinant AcNPV reporter constructs. Ten constructs (*Wt* and *M1* through *M9*) were generated using a PCR-based approach. The promoter regions indicated with lines were deleted. A MEF2 binding consensus is shaded. Relative fluorescence intensities of PTPCs somata in the reporter gene assay are represented in decreasing order of intensity as follows: (+++), (++), (+), (-).

Figure 2. AcNPV-mediated reporter gene analysis detects a *cis*-regulatory element that directs *PTTH* expression in *Bombyx mori* PTPCs. Fluorescence microscopy was used to visualize EGFP expression in the brains of pupae injected with recombinant AcNPVs. Representative images are shown. The axon emanating from the somata (asterisks and enlarged in A'-J') runs towards the midline (blue circle) of the brain with some arborization (arrow). In addition, faint signals on small cells (which are not PTPCs) are also detected in the lateral brain (arrowhead). In A'-J', the PTPCs in a recombinant

AcNPV injected pupae were examined by immunohistochemistry with an anti-PTTH mAb (magenta). EGFP fluorescence is shown in green. Scale bar = 100 μm.

Figure 3. Mutational analysis of the *c*is-regulatory element participating in direction in PTPCs. Properties of the wild-type and mutant promoter regions were assayed by gel mobility shift assay (B) and reporter gene analysis (C-F). (A) Mutant constructs used in this study. The Pitx consensus binding sequence (MatInspector, Lamonerie et al., 1996) is shown in green letters. The 6-bp Pitx core binding sequence is boxed. In the *Wt* and *DC1* sequences, the sequences corresponding to the Pitx consensus binding sequence are shown in green letters. In *Ma* ~ *Mc*, mutated nucleotides are shown in capital letters. (B) In the gel mobility shift assay, double-stranded oligonucleotides encoding from -67 to -38, -59 to -30 (*Wt*), -44 to -15, or -36 to -7 of the *PTTH* gene were used as probes. Double-stranded oligonucleotides *Ma*, *Mb*, *Mc*, and *DC1* were used as competitor DNA. Normal rabbit IgG (NRI) and rabbit anti-Ptx3/PTX3 (aPx) were added to the cell extracts and incubated prior to addition of the probe. The shifted bands are indicated by an arrow and an asterisk. (C-F) Reporter gene analysis, as performed using recombinant AcNPVs containing nucleotides -180 to +52 of the *PTTH* promoter with the *M1*, *M2*, *M3* or *DC1* mutant sequence changes. Representative images are shown. The somata of PTPCs are indicated by asterisks. In C'-F', the PTPCs in a recombinant AcNPV-injected pupae were examined by immunohistochemistry with an anti-PTTH mAb (magenta). EGFP fluorescence is shown in green. Scale bar = 100 μm.

Figure 4. Immunoblot analysis of anti-Ptx3/PTX3 immunoreactive materials associated with *cis*-regulatory element binding. (A) Amino acids sequences of both the antigenic peptide recognized by anti-Ptx3/PTX3 and the internal sequence of homeodomain helix III in BmPitx (NCBI Genbank Accession No. AB162107). The corresponding sequences are shaded. (B) Protein extract from cells expressing BmPitx fused to a His tag (HP) was tested with both anti-Penta-His (aHis) and anti-Ptx3/PTX3 (aPx) antibodies. Pupal brain extract in an amount equivalent to ten brain-SG complexes was also reacted to anti-Ptx3/PTX3 (aPx). BmPitx is indicated with an asterisk.

References

Berger, M.F., Badis, G., Gehrke, A.R., Talukder, S., Philippakis, A.A., Pena-Castillo, L., Alleyne, T.M., Mnaimneh, S., Botvinnik, O.B., Chan, E.T., Khalid, F., Zhang, W., Newburger, D., Jaeger, S.A., Morris, Q.D., Bulyk, M.L., Hughes, T.R., 2008. Variation in homeodomain DNA binding revealed by high-resolution analysis of sequence preferences. Cell. 133**,** 1266-76.

Bollenbacher, W.E., Granger, N.A., 1985. Endocrinology of the prothoracicotropic hormone. In: Kerkut, G.A, Gilbert, L.I. (Eds), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 7, Pergamon, Oxford, pp. 109-151.

Chai, C.L., Zhang, Z., Huang, F.F., Wang, X.Y., Yu, Q.Y., Liu, B.B., Tian, T., Xia, Q.Y., Lu, C., Xiang, Z.H., 2008. A genomewide survey of homeobox genes and identification of novel structure of the Hox cluster in the silkworm, Bombyx mori. Insect Biochem Mol Biol. 38**,** 1111-20.

Damante, G., Pellizzari, L., Esposito, G., Fogolari, F., Viglino, P., Fabbro, D., Tell, G., Formisano, S., Di Lauro, R., 1996. A molecular code dictates sequence-specific DNA recognition by homeodomains. EMBO J. 15**,** 4992-5000.

Ghosh, A., McBrayer, Z., O'Connor, M.B., 2010. The Drosophila gap gene giant regulates ecdysone production through specification of the PTTH-producing neurons. Dev Biol. 347**,** 271-8.

Habib, S., Hasnain, S.E., 2000. Differential activity of two non-hr origins during replication of the baculovirus Autographa californica nuclear polyhedrosis virus genome. J Virol. 74**,** 5182-9.

Joshi, R., Passner, J.M., Rohs, R., Jain, R., Sosinsky, A., Crickmore, M.A., Jacob, V., Aggarwal, A.K., Honig, B., Mann, R.S., 2007. Functional specificity of a Hox protein mediated by the recognition of minor groove structure. Cell. 131**,** 530-43.

Lamonerie, T., Tremblay, J.J., Lanctot, C., Therrien, M., Gauthier, Y., Drouin, J., 1996. Ptx1, a bicoid-related homeo box transcription factor involved in transcription of the pro-opiomelanocortin gene. Genes Dev. 10**,** 1284-95.

McBrayer, Z., Ono, H., Shimell, M., Parvy, J.P., Beckstead, R.B., Warren, J.T., Thummel, C.S., Dauphin-Villemant, C., Gilbert, L.I., O'Connor, M.B., 2007. Prothoracicotropic hormone regulates developmental timing and body size in Drosophila. Dev Cell. 13**,** 857-71.

Mizoguchi, A., Oka, T., Kataoka, H., Nagasawa, H., Suzuki, A., Ishizaki, H., 1990. Immunohistochemical localization of prothoracicotropic hormone-producing neurosecretory cells in the brain of *Bombyx mori*. Dev Growth Differ. 32**,** 591-598.

Park, D., Taghert, P.H., 2009. Peptidergic neurosecretory cells in insects: organization and control by the bHLH protein DIMMED. Gen Comp Endocrinol. 162**,** 2-7.

Shiomi, K., Kajiura, Z., Nakagaki, M., Yamashita, O., 2003. Baculovirus-mediated efficient gene transfer into the central nervous system of the silkworm, *Bombyx mori*. J Insect Biotechnol Sericol. 72**,** 149-55.

Shiomi, K., Fujiwara, Y., Atsumi, T., Kajiura, Z., Nakagaki, M., Tanaka, Y., Mizoguchi, A., Yaginuma, T., Yamashita, O., 2005. Myocyte enhancer factor 2 (MEF2) is a key modulator of the expression of the prothoracicotropic hormone gene in the silkworm, Bombyx mori. Febs J. 272**,**

3853-62.

Shiomi, K., Fujiwara, Y., Yasukochi, Y., Kajiura, Z., Nakagaki, M., Yaginuma, T., 2007. The Pitx homeobox gene in Bombyx mori: regulation of DH-PBAN neuropeptide hormone gene expression. Mol Cell Neurosci. 34**,** 209-18.

Tremblay, J.J., Lanctot, C., Drouin, J., 1998. The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit1 and is an upstream regulator of the Lim-homeodomain gene Lim3/Lhx3. Mol Endocrinol. 12**,** 428-41.

Ueda, H., Hirose, S., 1990. Identification and purification of a Bombyx mori homologue of FTZ-F1. Nucleic Acids Res. 18**,** 7229-34.

Ueda, H., Hirose, S., 1991. Defining the sequence recognized with BmFTZ-F1, a sequence specific DNA binding factor in the silkworm, Bombyx mori, as revealed by direct sequencing of bound oligonucleotides and gel mobility shift competition analysis. Nucleic Acids Res. 19**,** 3689-93.

Wei, Z.J., Yu, M., Tang, S.M., Yi, Y.Z., Hong, G.Y., Jiang, S.T., 2010. Transcriptional regulation of the gene for prothoracicotropic hormone in the silkworm, Bombyx mori. Mol Biol Rep. (Online First).

A

```
-180 CACAATGGTT CTATTTTAAG GATTTATCAC AAAGCCGAAA ACAATAGCTG GACACAGAAA AAGGTATTGT -111
-110 ATCGCCATAT TTATCATCTG ACACCCTCAT AAGGATTTCG TTTCTAATGG ATCTTTTCTT TACCCATCAA  -41
 -40 TCAATATCCC CTATATAAGG GAGAAAATAT TTCATCCCTT ATCATTCCTT TGAGAAGCAT CGTTCAGTTG  +30
 +31 AGTTATCCAG CATTCCCAAT CCATG                                                    +55
```

B

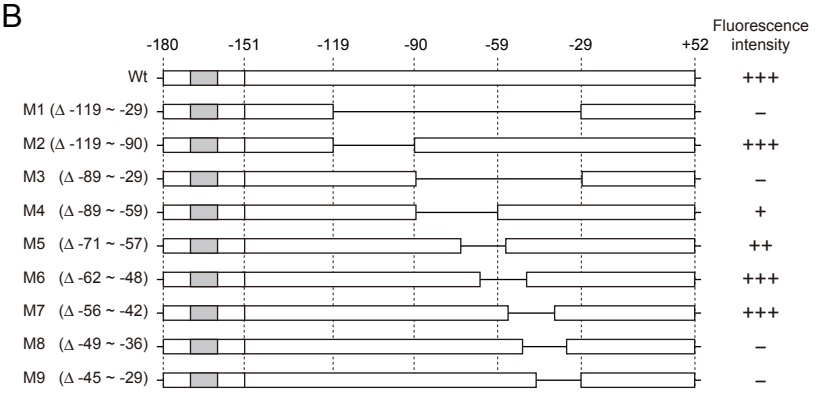

Fig. 1. (Ohtsuka et al.)

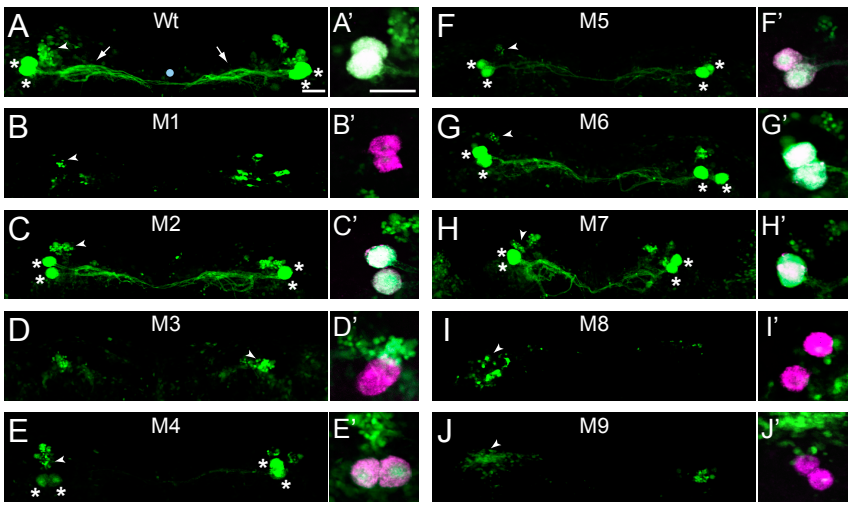

Fig. 2. (Ohtsuka et al.)

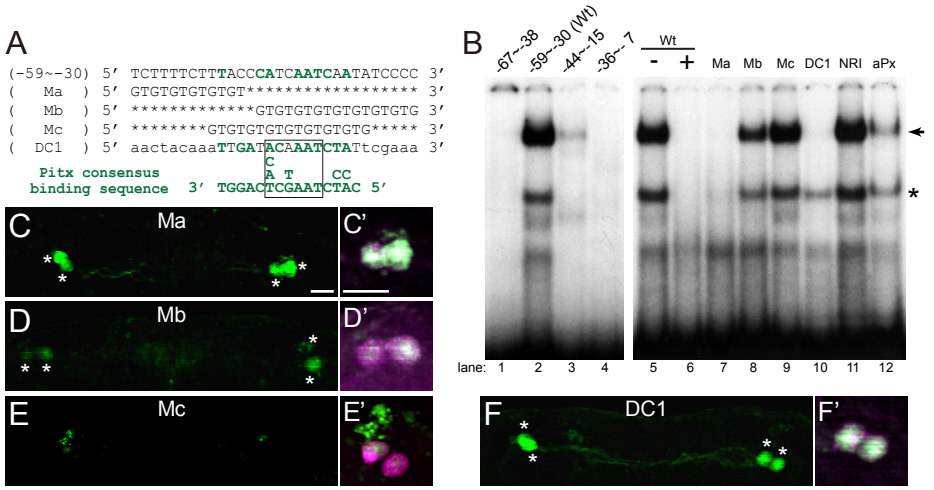

Fig. 3. (Ohtsuka et al.)

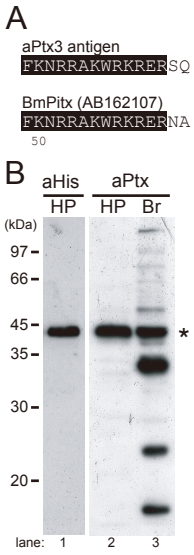

Fig. 4. (Ohtsuka et al.)