The Homeodomain Transcription Factors Antennapedia and POU-M2 Regulate the Transcription of the Steroidogenic Enzyme Gene Phantom in the Silkworm

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Background: The transcriptional regulation of steroidogenic enzymes in the silkworm remains poorly understood.

Results: Antp and POU-M2 are expressed in the PG and regulate the transcription of Phantom.

Conclusion: Antp and POU-M2 coordinate the transcription of Phantom via a protein interaction.

Significance: Our study indicates new roles for homeodomain proteins in regulating insect ecdysteroidogenesis.

The steroid hormone ecdysone, which controls insect molting and metamorphosis, is synthesized in the prothoracic gland (PG), and several steroidogenic enzymes that are expressed specifically in the PG are involved in ecdysteroidogenesis. In this study, we identified new regulators that are involved in the transcriptional control of the silkworm steroidogenic enzyme genes. In silico analysis predicted several potential cis-regulatory elements (CREs) for the homeodomain transcription factors Antennapedia (Antp) and POU-M2 in the proximal promoters of steroidogenic enzyme genes. Antp and POU-M2 are expressed dynamically in the PG during larval development, and their overexpression in silkworm embryo-derived (BmE) cells induced the expression of steroidogenic enzyme genes. Importantly, luciferase reporter analyses, electrophoretic mobility shift assays, and chromatin immunoprecipitation assays revealed that Antp and POU-M2 promote the transcription of the silkworm steroidogenic enzyme gene Phantom (Phm) by binding directly to specific motifs within overlapping CREs in the Phm promoter. Mutations of these CREs in the Phm promoter suppressed the transcriptional activities of both Antp and POU-M2 in BmE cells and decreased the activities of mutated Phm promoters in the silkworm PG. In addition, pulldown and co-immunoprecipitation assays demonstrated that Antp can interact with POU-M2. Moreover, RNA interference-mediated down-regulation of either Antp or POU-M2 during silkworm wandering not only decreased the ecdysone titer but also led to the failure of metamorphosis. In summary, our results suggest that Antp and POU-M2 coordinate the transcription of the silkworm Phm gene directly, indicating new roles for homeodomain proteins in regulating insect ecdysteroidogenesis.

The steroid hormone ecdysone plays important roles in controlling embryogenesis, larval growth, larval molting, metamorphosis, and reproduction in insects (1–3). Ecdysone titers fluctuate temporally during insect growth and development, and high ecdysone pulses periodically appear before each developmental transition, including larval-larval molting and larval-pupal transformation (4). High ecdysone pulses initiate a conserved signaling cascade triggering various biological processes, including apoptosis and tissue remodeling, during developmental transitions (3).

Ecdysone is synthesized primarily in the insect prothoracic gland (PG) via several enzymatic reactions that use cholesterol as a substrate (1). The steroidogenic enzymes that are sequentially involved in the ecdysteroidogenesis pathway have been identified and include the short-chain dehydrogenase/reductase Shroud (Sro) (5) and four cytochrome P450 monooxygenases encoded by the Halloween genes Spook (Spo), Phantom (Phm), Disembodied (Dib), and Shadow (Sad) (6). Ecdysone produced in the PG is secreted into the hemolymph and then is converted to an active form of 20-hydroxyecdysone by a P450 enzyme, Shade (Shd), in peripheral tissues (7).

Insect ecdysteroidogenesis can be orchestrated via the regulation of several types of factors that affect PG activity (8–10). Notably, the insect brain-derived neuropeptide prothoracicotropic hormone, together with bombbyxins or other insulin-like peptides, acts on the PG and thus determines the timing of ecdysteroidogenesis and stimulates ecdysone secretion (1, 11–14). TGFβ/activin and nitric oxide (NO) signals are also required for ecdysteroidogenesis in the PG (15, 16). In contrast to these stimulatory agents, prothoracicotrophic peptide and

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3 The abbreviations used are: PG, prothoracic gland; CRE, cis-regulatory element; AcMNPV, A. californica multiple nucleopolyhedrovirus; qRT, quantitative RT; co-IP, co-immunoprecipitation.
juvenile hormone inhibit ecdysteroidogenesis in the insect PG (17, 18).

Compared with the effects of the above-mentioned factors on ecdysteroidogenesis in the insect PG, the transcriptional regulation of steroidogenic enzymes remains poorly understood. Recently, increasing numbers of studies have demonstrated that insect PG-specific steroidogenic enzyme genes can be transcriptionally regulated. As expected, prothoracicotropic hormone plays roles in the transcriptional up-regulation of steroidogenic enzyme genes (19, 20). In the fruit fly (Drosophila melanogaster), the ecdysone-responsive factors βFTZ-F1 and Broad Complex (Br-C) also positively regulate the transcription of Phm and Dib, indicating a positive feedback effect by ecdysone on ecdysteroidogenesis (21, 22). Similarly, the transcription factor Ventral veins lacking (Vvl) and the nuclear receptor Knirps (Kni) regulate the transcription of the Phm gene by binding directly to the cis-regulatory elements (CREs) in its promoter (23).

In this study, to identify previously unknown regulators involved in the transcriptional regulation of insect steroidogenic enzyme genes, we performed an in silico analysis and predicted multiple potential CREs for two homeodomain transcription factors, Antennapedia (Antp) and POU-M2, within the proximal promoter regions of steroidogenic enzyme genes in the silkworm (Bombyx mori) (Fig. 1A). These results indicate that Antp and POU-M2 may contribute directly to the regulation of the transcription of these steroidogenic enzyme genes. Our investigation identified the key CREs for the binding of both Antp and POU-M2 in the promoter region of the Phm gene and revealed that Antp and POU-M2 can interact to coordinate the transcription of the Phm gene in the silkworm.

Experimental Procedures

Insects and Cell Lines—The silkworm strain Dazao was maintained at Southwest University (Chongqing, China). Fertilized eggs were incubated at 25 °C in the appropriate humidity for hatching, and the silkworm larvae were reared on fresh mulberry leaves at 25 °C with a photoperiod of 12 h light/12 h dark and 75% relative humidity. The silkworm embryo-derived PGs were dissected in PBS and transferred directly to RNA lysis buffer (Beyotime, Shanghai, China). After the proteins had been incubated for 20 min at 25 °C, 5 μM isopropyl β-D-1-thiogalactopyranoside at 16 °C for 20 h to induce the expression of the recombinant proteins. Purification of the recombinant proteins was achieved using nickel-nitrilotriacetic acid affinity columns (GE Healthcare) according to the manufacturer’s instructions, and the purified proteins were subsequently used in binding assays or injected into New Zealand White rabbits for antibody preparation.

Electrophoretic Mobility Shift Assay (EMSA)—To test the binding of the proteins to regulatory sequences, EMSA experiments were performed as described previously (26, 27). The DNA oligonucleotides containing the consensus binding sites of Antp and POU-M2 were labeled using Cy3 at the 5’-end and annealed to produce double-stranded probes. The DNA-binding reactions were performed in 20 μl of solution containing 1–10 μg of the nucleoproteins extracted from the PG or 0.1–1 μg of the purified recombinant proteins with 4 μl of 5× binding buffer (Beyotime, Shanghai, China). After the proteins had been incubated for 20 min at 25 °C, 5 μM labeled probe was

Antp and POU-M2 Coordinate the Transcription of Phantom genes were cloned into the prokaryotic expression vector pET-28a (Novagen, Darmstadt, Germany) or pCold-SUMO (Haigene, Harbin, China), respectively, resulting in the recombinant expression vectors pET-28a/Antp and pCold-SUMO/POU-M2. The recombinant vectors were transformed into Escherichia coli strain BL21 (DE3) competent cells (TransGen, Beijing, China). The E. coli cells were grown at 37 °C in Luria-Bertani medium containing 20 μg/ml kanamycin or ampicillin and treated with 0.2 mM isopropyl β-D-1-thiogalactopyranoside at 16 °C for 20 h to induce the expression of the recombinant proteins. Purification of the recombinant proteins was achieved using nickel-nitrilotriacetic acid affinity columns (GE Healthcare) according to the manufacturer’s instructions, and the purified proteins were subsequently used in binding assays or injected into New Zealand White rabbits for antibody preparation.

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qRT-PCR examination was performed using a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA) and a SYBR Premix Ex Taq kit (TaKaRa, Otsu, Japan). Each reaction was performed under the following conditions: denaturation at 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The relative mRNA levels of the target genes were calculated using the 2-ΔΔCT method (25), and the silkworm eukaryotic translation initiation factor 4α (eIF-4α) gene was used as the internal control. The primers for qRT-PCR, which were designed using Primer 5 software, are listed in Table 1.

DNA Construction and Transfection—The open reading frames (ORFs) of the silkworm Antp fused with N-terminal FLAG tag and POU-M2 fused with C-terminal Myc tag were cloned into the basic pSLfa1180fa vector, which was modified by inserting the hr3 enhancer into the proximal promoter region of the silkworm Act4 gene for overexpression. The coding region of enhanced green fluorescent protein (EGFP) was also cloned into the same basic vector and used as a control. Six different 5’-truncated fragments and three mutated fragments of the silkworm Phm promoter were created by PCR or inverse PCR using the primers listed in Table 1 and cloned into the pGL3–basic plasmid (Promega, Madison, WI). Transfection of the overexpression and luciferase reporter vectors was performed using X-tremeGENE HP DNA transfection reagent (Roche Applied Science), and luciferase activity was measured using commercially available kits (Promega, Madison, WI) according to the manufacturer’s instructions.

Recombinant Expression and Purification—The ORFs of the silkworm Antp and POU-M2 genes were cloned into the prokaryotic expression vector pET-28a (Novagen, Darmstadt, Germany) or pCold-SUMO (Haigene, Harbin, China), respectively, resulting in the recombinant expression vectors pET-28a/Antp and pCold-SUMO/POU-M2. The recombinant vectors were transformed into Escherichia coli strain BL21 (DE3) competent cells (TransGen, Beijing, China). The E. coli cells were grown at 37 °C in Luria-Bertani medium containing 20 μg/ml kanamycin or ampicillin and treated with 0.2 mM isopropyl β-D-1-thiogalactopyranoside at 16 °C for 20 h to induce the expression of the recombinant proteins. Purification of the recombinant proteins was achieved using nickel-nitrilotriacetic acid affinity columns (GE Healthcare) according to the manufacturer’s instructions, and the purified proteins were subsequently used in binding assays or injected into New Zealand White rabbits for antibody preparation.
Antp and POU-M2 Coordinate the Transcription of Phantom

### Table 1
The primers used in this study

| Purpose                     | Primer name | Sense sequence (5'-3') | Antisense sequence (5'-3') |
|-----------------------------|-------------|------------------------|---------------------------|
| qRT-PCR                     | Antp        | AGACCCAGAATGCCCTCTATG  | ACCCGGCTGTATGCTTGTCGA     |
| qRT-PCR                     | POL-M2      | GACCTGCGACACATCGGTA   | AGCTCCAGGCGTCTTTATC      |
| qRT-PCR                     | Spo         | GCACATCGCTGCTTCTCACT  | TCCCTGACAGGCTGTACAG       |
| qRT-PCR                     | Sro         | TGATAGGTGAGCTCTGAGGCGC | GGCTCTGCGCTTGGTTTCTC     |
| qRT-PCR                     | Phm         | AAAACGGATACGCTCGAGAGTA | TTGTATTTGCAAGAATCGAGTT   |
| qRT-PCR                     | Dib         | TGCTGATGACACATCTGTT   | ATCCGAAACCTGGCTGCTC      |
| qRT-PCR                     | Sud         | TGGTGGAGACTCCGATATACG | AAAGAGAATGCTGACAGG       |
| qRT-PCR                     | Shd         | AAGGGTCTGTATCATTGACCTT | AAACATTCATCATGCGTTA      |
| qRT-PCR                     | ExR         | CGCTGCTGTGAGCTGCGTT   | CGAAGATGTCGGCACAATAC     |
| qRT-PCR                     | E74A        | AGAACGCTACGTCAAGGTTA  | GTTCCCGUACATCAAGGTTG     |
| qRT-PCR                     | βFtz-F1     | GACATGACCTACATGTACG   | CACATGTCACTTCCGATC       |
| qRT-PCR                     | Br-C        | AAGACGGGCACACTGACAG  | TCGAGAATGAGCAGACGCT      |
| qRT-PCR                     | eIF-4a      | TGGTACTGCTCTCTGCTT   | CGAAGGTAGACTCAATTTCCCT   |
| Truncation                  | Phm-2500    | ACAAATGGTTGATACAAAAAA | ATCCCTTTAATTTAAAGATAA    |
| Truncation                  | Phm-2087    | ATACCAAGGGTCTGACGGCAATG | ATCAAGTTAAATTTAAAGATAA |
| Truncation                  | Phm-1718    | GCAGATTAATGCACTACCAAGCAAT | ATCAAGTTAAATTTAAAGATAA |
| Truncation                  | Phm-1325    | TGGAGATCACGCTGCTGCTG  | ATGCGATATTATCCTCCCCTC    |
| Truncation                  | Phm-889     | AAAACACGCAGAAGAATACACT | ATCAAGTTAAATTTAAAGATAA    |
| Truncation                  | Phm-553     | TAAACAAATTTAGAGAAGACTGG | ATCAAGTTAAATTTAAAGATAA    |
| Mutagenesis                 | Pro-M-a     | GCAATTAATGAGGTGAGGTTA | ATGCGATATTATCCTCCCCTC    |
| Mutagenesis                 | Pro-M-b     | AGATGGGTATGAGCTGCTGCTG | ATGCGATATTATCCTCCCCTC    |
| Mutagenesis                 | Pro-M-c     | AAAATACCCCTTAATTTATGCTGAA | ATGCGATATTATCCTCCCCTC    |
| ChIP                        | CRE-specific | TATATGAGTTGATAGTGTCCT | TGGTTGATATGCAAGAATGTTA|
| ChIP                        | Non-specific | GAATATAAAATTTGAGGTTA | ATGCGATATTATCCTCCCCTC    |
| dsRNA synthesis             | Antp        | AGACCTTATGCTACCTTGCTG | GCGTCTGTCTGGACAGCAT     |
| dsRNA synthesis             | POL-M2      | CTCGTGCAGAATGCTGCTCA | GCGTACGUCGGCCACCTCAGC    |
| dsRNA synthesis             | GFP         | AGGCACAGGCAATACATTCTC | TGGATACGCTGCTGCTGCTG    |

added, and the incubation was continued for an additional 20 min. For competition assays, a 5–50-fold molar excess of unlabeled or mutant double-stranded probe was added after the labeled probe. Subsequently, 2 μl of 10× loading buffer (Beyotime, Shanghai, China) was added, and the mixtures were then loaded onto 5% (w/v) polyacrylamide gels and electrophoresed in 1× TBE buffer (45 mM Tris borate and 1 mM EDTA, pH 8.3). Finally, the gels were scanned and imaged using a Typhoon scanner (GE Healthcare).

Chromatin Immunoprecipitation (ChIP)—To confirm the binding of the transcription factors to the CREs, ChIP assays were performed according to the manufacturer’s instructions (Millipore, Billerica, MA). Briefly, BmE cells transfected with the overexpression vectors or the PGs isolated from the wandering larvae were fixed with 37% formaldehyde to cross-link chromatin and then sonicated to shear the cross-linked chromatin into fragments that were 200–1,000 bp in length. Immunoprecipitation assays were performed using anti-FLAG, anti-Myc, anti-Antp, anti-POU-M2, and nonspecific rabbit IgG antibodies. The purified DNA from the immunoprecipitated chromatin was used as a template for PCR amplification. The primers used to amplify the specific region covering the potential CRE for either Antp or POU-M2 and nonspecific regions, excluding these CREs, are listed in Table 1. The PCR products were electrophoresed in 2% agarose gels.

Recombinant Autographa californica Multiple Nucleopolyhedrovirus (AcMNPV) Vector-mediated Promoter Activity Analysis in Silkworm Larvae—Recombinant AcMNPVs were generated as described previously (28, 29). Briefly, the mutant Phm promoters created using inverse PCR were cloned into the plasmid pHNLuc-A3RL. The normal Phm promoter was also cloned into the same plasmid and used as the positive control. The donor plasmids were then transferred into E. coli strain DH10BacE1GT competent cells to generate recombinant baculoviruses (bacmids). The purified bacmids were trans-
gentle rotation at 4 °C. The resin was collected by centrifugation and washed another three times with 1 ml of PBS, pH 8.0. Subsequently, 50 μl of elution buffer (50 mM Tris-HCl and 10 mM glutathione, pH 8.0) was added to isolate the bound proteins. Then the supernatants were examined by Western blotting with an anti-His or anti-GST antibody (Sigma).

**Immunohistochemistry and Co-immunoprecipitation (co-IP)—** Immunohistochemical co-localization of Antp and POU-M2 was performed as described previously (31). The BmE cells transfected with the overexpression vectors were cultured for 48 h on glass coverslips and fixed in 4% (v/v) paraformaldehyde in PBS, pH 7.4, for 10 min at 25 °C, followed by blocking for 30 min in PBS, pH 7.4, containing 0.1% (w/v) BSA and 5% (v/v) goat serum. Then the samples were treated with primary antibody targeting FLAG or Myc (Sigma) at a 1:5000 dilution for 1 h and then with secondary antibody (anti-rabbit IgG FITC or anti-mouse Alexa 555) at a 1:500 dilution for another 1 h at 37 °C. Subsequently, the samples were mounted in PBS, pH 7.4, containing 1% (v/v) Triton X-100 and 0.1% (w/v) 4′,6-diamidino-2-phenylindole (DAPI) for 15 min at room temperature. Finally, the preparations were imaged using a fluorescence microscope (Olympus, Tokyo, Japan).

To confirm the interaction between Antp and POU-M2 in vivo, the BmE cells overexpressing FLAG-tagged Antp and Myc-tagged POU-M2 were cultured for 72 h, and the nucleoproteins were then isolated. An antibody (10 μg) directed against FLAG or Myc was diluted in 400 μl of lysis/washing buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 5% (v/v) glycerol, and 0.25 mM phenylmethylsulfonyl fluoride). Subsequently, 50 μl of 5% (w/v) BSA-blocked Dynabeads (Beyotime, Shanghai, China) was added, and the mixture was incubated with gentle rotation for 2 h at room temperature. After the bead-antibody complexes were centrifuged, they were collected and washed three times. The nucleoproteins (1 mg in 350 μl) were then added, and the mixture was incubated with gentle rotation overnight at 4 °C, followed by centrifugation. After the precipitates were washed five times, they were suspended in 50 μl of SDS loading buffer and denatured by heating. Finally, the samples were separated using SDS-PAGE and analyzed by Western blotting with the indicated antibodies.

**Double-stranded RNA Synthesis and RNA Interference (RNAi)—** The template DNA fragments for silkworm Antp and POU-M2 used for the synthesis of double-stranded RNA...
(dsRNA) were amplified by PCR using the primers listed in Table 1 and then cloned into pEASY-T1 simple vector (TransGen, Beijing, China), from which the dsRNA of the target genes was synthesized using T7 RiboMAX large scale RNA production systems (Promega, Madison, WI) according to the manufacturer’s instructions. The dsRNA of GFP was synthesized by the same method and was used as the negative control.

To perform RNA interference of both Antp and POU-M2, 40 μl of dsRNA per animal was injected into the hemolymph of silkworm larva that had just entered the wandering stage, and experiments were performed on three biological replicates, each of which consisted of 20 larvae. The PG, fat body, and hemolymph were separately collected at 48 h after dsRNA treatment for qRT-PCR analysis or ecdysteroid measurement.

**Ecdysteroid Measurements**—Ecdysone concentrations were measured according to methods described previously with slight modifications (22). Briefly, 0.5 ml of hemolymph was collected in a glass tube, and 0.5 ml of methanol was added. After vigorous mixing and centrifugation, the supernatant was transferred to a new tube. The extraction was repeated twice, and the combined methanol phase (1.5 ml) was evaporated in a dry bath at 70 °C. Then the remainder was dissolved in ELISA buffer (1 M phosphate solution containing 1% BSA, 4 M sodium chloride, 10 mM EDTA), and the ecdysone concentrations were measured using a commercial ELISA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s procedures. A standard curve was generated using 20-hydroxyecdysone (Sigma), and the absorbance was determined at 450 nm on a plate reader (BioTek, Winooski, VT).

**Results**

**Proximal Promoters of Silkworm Steroidogenic Enzyme Genes Contain Potential CREs for Antp and POU-M2**—Given that down-regulation of the homeodomain transcription factor gene POUI-M2 disrupts the larval-pupal transition in the silkworm (32), which mimics the effects of ecdysone-deficient mutations in the fruit fly (33), we questioned whether the silkworm POU-M2 might also be involved in silkworm steroidogenesis. Therefore, using the MatInspector program, we analyzed the 2.5-kb potential promoter regions of five steroidogenic enzyme genes in the silkworm, including Spo, Sro, Phm, Dib, and Shd, and found potential transcription factor-binding sites for POU-M2, with the number varying from 2 to 10 (Fig. 1A). Intriguingly, the potential promoter...
regions of these steroidogenic enzyme genes also contain two to seven potential transcription factor-binding sites for another homeodomain transcription factor, Antp. This observation indicates that the transcription of silkworm steroidogenic enzyme genes may be regulated directly by Antp and POU-M2.

Antp and POU-M2 Are Expressed in the Silkworm PG—To evaluate the possible regulation of the transcription of silkworm steroidogenic enzyme genes by Antp and POU-M2, we first profiled the temporal expression of *Antp* and *POU-M2* in the silkworm PG during larval development, from day 2 of the last larval instar (L5D2) to wandering (W0D), by qRT-PCR. The results showed that the expression levels of *Antp* and *POU-M2* in the PG were relatively low during the early feeding stage of the fifth instar but increased after L5D5 and reached the highest level at W0D (Fig. 1, A and B). Further examination revealed that the expression levels of the five steroidogenic enzyme genes also gradually increased in the silkworm PG during the same period (Fig. 1, D–H). The dynamic expression of *Antp*, *POU-M2*, and the steroidogenic enzyme genes positively correlated with the changes in the ecdysone titer during the final instar of the larval stage in the silkworm (34).

Antp and POU-M2 Promote the Transcription of Silkworm Steroidogenic Enzyme Genes—The results described above led us to investigate the effects of transient overexpression of *Antp* and *POU-M2* on the transcription of silkworm steroidogenic enzyme genes. We generated constitutive overexpression constructs of *Antp* (fused to FLAG) and *POU-M2* (fused to Myc) and separately transfected these constructs into silkworm BmE cells. Western blotting analysis revealed that the expression of *Antp* and *POU-M2* was induced after transfection. Further examination by qRT-PCR demonstrated that *Antp* overexpression significantly increased the transcription of PG-specific steroidogenic enzyme genes, including *Spo*, *Sro*, *Phm*, *Dib*, and *Sad*, but had no effect on the transcription of *Shd*, which is expressed in peripheral tissues (Fig. 2A). Similarly, the transcription of *Phm*, *Dib*, and *Sad* significantly increased after *POU-M2* overexpression (Fig. 2B). These observations strongly supported our hypothesis that Antp and POU-M2 are involved in the transcriptional regulation of silkworm PG-specific steroidogenic enzyme genes. Interestingly, compared with that of the promoters of other steroidogenic enzyme genes, the activity of the *Sad* promoter was induced to the greatest extent following the overexpression of either *Antp* or *POU-M2* in BmE cells. However, *Sad* expression was increased to a lesser degree in the silkworm PG. This may be due to the lack of possible suppressions on the activity of the *Sad* promoter in vitro.

The Silkworm Phm Promoter Is Sensitive to Antp and POU-M2—Given that Phantom is one of the rate-limiting enzymes involved in insect ecdysteroidogenesis (35–37), we next examined the effects of *Antp* and *POU-M2* on the activity of the promoter of the silkworm *Phm* gene by using a Dual-Luciferase reporter assay in BmE cells. First, five 5′-flanking deletion constructs coupled to a luciferase reporter gene were generated from the proximal promoter-containing 2.5-kb sequence upstream of the translational start site of the *Phm* gene (Fig. 3). Six different constructs were separately co-transfected into BmE cells with the overexpression constructs previously generated for *Antp* or *POU-M2*. Compared with the control overexpression construct containing *EGFP*, the four truncated promoter constructs spanning the regions –2500 to

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**FIGURE 3. Effects of Antp and POU-M2 overexpression on the activity of the silkworm Phm promoter.** A, effects of *Antp* overexpression on luciferase expression driven by several 5′-truncated promoters of the *Phm* gene. The construct overexpressing *Antp* was co-transfected into BmE cells together with a series of constructs containing a luciferase reporter under the control of different 5′-truncated promoters of the *Phm* gene. A construct overexpressing the *EGFP* gene was used as the control. The cells were collected for luciferase activity analysis at 48 h after transfection. The experiments were independently repeated three times. The data represent the mean ± S.E. (n = 3); *, p < 0.05; **, p < 0.01, compared with the control. OE, overexpression. B, effects of *POU-M2* overexpression on luciferase expression driven by several 5′-truncated promoters of the *Phm* gene.
significantly increased the transactivation of luciferase in the presence of overexpression of either Antp (Fig. 3A) or POU-M2 (Fig. 3B) in BmE cells. However, the promoter regions at −889 to −553 and less than −553 did not respond to the overexpression of either Antp or POU-M2. These data demonstrate that the Phm promoter region covering −1325 to −889 contains key CREs that are involved in the transcriptional regulation of the silkworm Phm gene by Antp and POU-M2.

Antp and POU-M2 Bind Specifically to the Overlapping CREs in the Silkworm Phm Promoter—Next, we examined whether Antp and POU-M2 could bind directly to specific CREs in the region covering −1325 to −889 of the silkworm Phm promoter by using EMSA. Recombinant His-SUMO-Antp and GST-POU-M2 proteins were expressed in E. coli and purified. An *in silico* analysis predicted four potential binding sites for Antp or POU-M2 in the Phm promoter region from −1325 to −889; thus, we designed oligonucleotide probes for these sites as follows: P1 contained the overlapping CREs for both Antp and POU-M2; P2 and P3 for only POU-M2; and P4 for only Antp (Figs. 4A and 5A). The EMSA results revealed that His-SUMO-Antp and GST-POU-M2 could bind directly to the labeled P1 probe in a dose-dependent manner (Figs. 4C and 5C, lanes 2–5) and that this binding could be competitively suppressed by unlabeled P1 probe. D, unlabeled mutant probe P1-M-a partially suppressed the binding of recombinant His-SUMO-Antp to the labeled P1 probe. E, unlabeled mutant probe P1-M-b could not suppress the binding of recombinant His-SUMO-Antp to the labeled P1 probe. F, recombinant His-SUMO-Antp bound weakly to the labeled P4 probe in the Phm promoter region covering −1325 to −889, which contains key CREs for Antp. G, His-SUMO tag alone could not bind to the labeled P1 probe.
The EMSA results showed that although the unlabeled mutant probe P1-M-a partly suppressed the binding of Antp (Fig. 4D), the unlabeled probe P1-M-b, which contained two mutant motifs, could not competitively suppress Antp binding (Fig. 4E). Similarly, the unlabeled mutant probe P1-M-c could not competitively suppress the binding of POU-M2 (Fig. 5D). These findings, together with the observation that neither the His-SUMO tag nor the GST tag alone could bind to the labeled P1 probe (Figs. 4G and 5G), demonstrate that Antp and POU-M2 bind specifically to different motifs within overlapping CREs in the silkworm Phm promoter.

To determine the binding activity of Antp and POU-M2 to the overlapping CREs in the silkworm Phm promoter, we isolated the nucleoproteins from the PGs of silkworm larvae at the wandering stage, when ecdysone is synthesized, to perform EMSA. As shown in Fig. 6, A and B, the silkworm PG nucleoproteins bound to the labeled P1 probe in a dose-dependent manner and that this binding was competitively suppressed by the unlabeled P1 probe. Moreover, incubating the silkworm PG nucleoproteins with a polyclonal antibody against either Antp or POU-M2 disrupted their binding, whereas the incubation of the PG nucleoproteins with preimmune sera had no effect on their binding (Fig. 6, C and D). These results indicate that Antp and POU-M2 can bind specifically to overlapping CREs in the Phm promoter in the PG of silkworm larvae.

Overlapping CREs of Antp and POU-M2 in the Phm Promoter Are Required for the Transcription of Phm in the Silkworm PG—To clarify the roles of the overlapping CREs of Antp and POU-M2 on Phm transcription in vivo, we first performed ChIP assays following the overexpression of either FLAG-tagged Antp or Myc-tagged POU-M2 in BmE cells. The results revealed that in comparison with the results from nonspecific IgG, used as a negative control, and nonspecific primers for amplifying the Phm promoter region that did not cover the overlapping CREs (Fig. 7, B and D), the antibody against either FLAG or Myc could specifically immunoprecipitate the overlapping CREs in the Phm promoter, as demonstrated by the presence of a positive signal that was similar in size to that of the amplified products generated with input DNA (Fig. 7, A and C). Further ChIP assays of the PGs of
silkworm larvae in the wandering stage also demonstrated that the positive DNA region covering the overlapping CREs was detectable in the products precipitated with polyclonal antibodies against Antp and POU-M2 by using CRE-specific primers (Fig. 7E) but not by using nonspecific primers (Fig. 7F). These results further confirm the binding activity between Antp or POU-M2 and the overlapping CREs in the silkworm Phm promoter in vivo.

To determine whether the overlapping CREs for Antp and POU-M2 are necessary for Phm transcription, we next performed Dual-Luciferase assays to examine the activities of three mutated Phm promoters, Pro-M-a, Pro-M-b, and Pro-M-c, which were mutated in different motifs of the overlapping CREs corresponding to P1-M-a, P1-M-b, and P1-M-c, respectively, as described above for the EMSAs, in the presence of overexpression of either Antp or POU-M2. The results in BmE cells showed that Antp overexpression increased the activity of the mutated Pro-M-c promoter (Fig. 8C) but had no effect on the activity of either Pro-M-a or Pro-M-b (Fig. 8A and B), whereas POU-M2 overexpression increased the activity of both Pro-M-a and Pro-M-b (Fig. 8, A and B) but had no effect on Pro-M-c activity (Fig. 8C). Moreover, we used an AcMNPV expression system-mediated luciferase assay in silkworm larvae and found that, at 96 h after injection of the recombinant AcMNPVs harboring either normal or mutated Phm promoters, the activity of each of three mutated Phm promoters with mutations in the overlapping CREs for Antp and POU-M2, compared with the normal promoter, which was used as the positive control, was significantly decreased in larval PGs (Fig. 8D). Together, the evidence from these in vivo experiments supported the hypothesis that Antp and POU-M2 regulate the transcription of the silkworm Phm gene by binding to their overlapping CREs in the Phm promoter in the larval PG.

Antp Interacts with POU-M2—Given that Antp and POU-M2 bind directly and separately to adjacent motifs in overlapping CREs in the silkworm Phm promoter, and given the ability of different homeodomain proteins to interact with each other to regulate gene transcription (32, 38), we questioned...
whether Antp interacts with POU-M2 to regulate the transcription of silkworm Phm. To test this hypothesis, we first performed pulldown assays and EMSAs in vitro. Recombinant GST-POU-M2 was incubated with His-SUMO-Antp and subsequently isolated by GST tag affinity chromatography. Intriguingly, Antp was specifically detected by Western blotting assay with the antibody against the His tag (Fig. 9A, lane 5). Moreover, EMSA results showed that co-incubating the recombinant proteins of both His-SUMO-Antp and GST-POU-M2 with the labeled P1 probe retarded the mobility of the band (Fig. 9B), indicating that Antp and POU-M2 can interact to form a complex to bind to the overlapping CREs in the silkworm Phm promoter. Based on these results, we subsequently performed a co-IP assay to examine the interaction between Antp and POU-M2 in vivo. After overexpressing both FLAG-tagged Antp and Myc-tagged POU-M2 in BmE cells, we found that these two proteins co-localize in nuclei, as determined by immunostaining analysis (Fig. 9C) and by Western blot analysis (Fig. 9D). The co-IP results showed that the POU-M2 protein was present in anti-Antp immunoprecipitates and that Antp was present in anti-POU-M2 immunoprecipitates (Fig. 9E). These data strongly support our hypothesis that Antp interacts with POU-M2.

Next, we examined the effects of co-overexpression of Antp and POU-M2 on the activity of the silkworm Phm promoter. As shown in Fig. 9F, compared with the separate overexpression of either Antp or POU-M2, the simultaneous overexpression of both Antp and POU-M2 significantly increased the activity of the Phm promoter, which drove the expression of a luciferase reporter gene. This result indicates that Antp and POU-M2 may coordinate the transcription of the silkworm Phm gene via an interaction between them. However, determining the role of the interaction between Antp and POU-M2 in the transcriptional regulation of the silkworm Phm gene requires further studies.

RNAi for Either Antp or POU-M2 Results in a Decrease in Ecdysone Titer and the Failure of Metamorphosis—To investigate the functions of Antp and POU-M2 in the regulation of silkworm ecldysteroidogenesis in vivo, we next used dsRNA-mediated RNAi for either Antp or POU-M2 in silkworm larvae at the initiation of the wandering stage. qRT-PCR analyses revealed that the expression of either Antp or POU-M2 was...
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FIGURE 8. Activity changes of the mutated silkworm Phm promoters in BmE cells overexpressing either Antp or POU-M2 and in the PG of the silkworm larvae. A–C, effects of either Antp or POU-M2 overexpression on luciferase expression driven by the mutated Phm promoters. A construct overexpressing either Antp or POU-M2 was co-transfected into BmE cells with the construct containing a luciferase reporter under the control of the mutated Phm promoter Pro-M-a, Pro-M-b, or Pro-M-c. A construct overexpressing the EGFP gene was used as the control. The cells were collected for luciferase activity analysis at 48 h after transfection. The experiments were independently repeated three times. The data represent the mean ± S.E. (n = 3). *p < 0.05; **p < 0.01, compared with the control. OE, overexpression. D, recombinant AcMNPV system-mediated analysis of luciferase expression driven by the mutated Phm promoters in PGs of silkworm larvae. The silkworm larvae were infected with recombinant AcMNPVs carrying the luciferase reporter gene driven by either a normal or mutated Phm promoter (Pro-M-a, Pro-M-b, or Pro-M-c) at day 5 of the fifth instar. The PGs were collected 96 h after infection for luciferase analyses.

efficiently decreased in the PG at 48 h after dsRNA treatment (Fig. 10, A and B). As expected, the transcription of the silkworm Phm gene was significantly down-regulated following exposure to RNAi of either Antp or POU-M2 (Fig. 10C). Moreover, the expression of four steroidogenic enzyme genes, Spo, Sro, Dib, and Sad, was also decreased in the PG after application of RNAi of either Antp or POU-M2 (Fig. 10, D–G).

We then measured the effects of RNAi of either Antp or POU-M2 on ecdysone titer, ecdysone signaling, and ecdysone-mediated metamorphosis. As expected, following the use of an RNAi of either Antp or POU-M2, the ecdysone titer in the hemolymph was significantly decreased (Fig. 10H). Similarly, the expressions of four ecdysone response genes, Ecr, E74A, βFtz-F1, and Br-C, were also down-regulated in the fat body, a target tissue of ecdysone action (Fig. 10, I–L). In addition, at 60 h after RNAi treatment, silkworm larvae failed to complete the larval-pupal transition (Fig. 10M). Together, our data demonstrate that Antp and POU-M2 are involved in silkworm ecdysteroidogenesis by regulating the transcription of Phm and other steroidogenic enzyme genes.

Discussion

Insect endocrine systems play crucial roles in various biological processes by secreting several key hormones, including ecdysone and juvenile hormone (3). Although extensive studies have focused on the signaling network of endocrine hormones (3, 39), the transcriptional regulation of hormone biosynthesis remains poorly understood. This study shows that the home-domain transcription factors Antp and POU-M2 are expressed in the silkworm PG, where ecdysone is synthesized, and that both factors promote the transcription of PG-specific steroidogenic enzyme genes. We also demonstrate that Antp and POU-M2 are involved in the transcriptional regulation of the steroidogenic enzyme gene Phm by binding directly to specific motifs within overlapping CREs in its promoter. Previous studies have shown that homeodomain transcription factors are involved primarily in the control of body segmentation, organ development, and cell-specific gene transcription throughout the life cycle of insects (40–42). Our findings provide new insight into the roles of homeodomain transcription factors in insect ecdysteroidogenesis.

Antp is a member of the insect antennapedia complex (ANT-C), which belongs to the Hox subclass of the homeodomain transcription factor family and is composed of five genes as follows: Antp, Sex Combs Reduced (Scr), Deformed (Dfd), Proboscidipedia (Pb), and Labial (Lab) (42, 43). Previous studies in insects have reported that Antp is functionally required for thorax segmentation in the fruit fly (44–46), silkworm (47, 48), and red flour beetle (Tribolium castanenum) (49). In addition, Antp has been shown to positively or negatively mediate the spatial expression of its target genes in organs located within the insect head and thorax, including sericin-1 (Ser1) in the middle silk gland of the silkworm (27) as well as Lab in the brain, Homothorax (Hth) in the antenna, Eyeless (Ey) in the eye, Centromomin (Cnn) in the central nervous system, and Teashirt (Tsh)
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**FIGURE 9. Interaction between Antp and POU-M2.** A, GST pulldown assay to determine the interaction between Antp and POU-M2. Recombinant His-SUMO-Antp was incubated with recombinant GST-POU-M2 and then detected by Western blotting using a monoclonal antibody against the His tag. B, EMSA showed that recombinant His-SUMO-Antp and GST-POU-M2 formed a complex that bound to overlapping CREs in the silkworm Phm promoter. C, immunohistochemical analysis of the co-localization of the recombinant FLAG-tagged Antp and Myc-tagged POU-M2 in BmE cells by using monoclonal antibodies against the FLAG and Myc tags. Scale bar, 20 μm. D, Western blot analysis of the nucleoproteins isolated from silkworm BmE cells overexpressing FLAG-tagged Antp and Myc-tagged POU-M2 by using monoclonal antibodies against the FLAG and Myc tags. E, co-IP assays of the interaction between Antp and POU-M2. The nucleoproteins isolated from the BmE cells overexpressing FLAG-tagged Antp and Myc-tagged POU-M2 were immunoprecipitated with a monoclonal antibody against the FLAG tag or the Myc tag followed by Western blot analysis using a monoclonal antibody against the Myc tag or the FLAG tag. F, effects of Antp and POU-M2 co-expression on the activity of the silkworm Phm promoter in BmE cells. Constructs overexpressing Antp, POU-M2, or both were separately co-transfected into BmE cells with a construct containing the luciferase gene driven by the Phm promoter. A construct overexpressing the EGFP gene was used as the control. The cells were collected for luciferase activity analysis at 48 h after transfection. The experiments were independently repeated three times, and the data represent the mean ± S.E. (n = 3). ***, p < 0.001, compared with the control. A, Antp overexpression; P, POU-M2 overexpression; C, EGFP overexpression. G, proposed model for the transcriptional regulation of the silkworm Phm gene. The homeodomain transcription factors Antp and POU-M2 form a heterodimer via a protein interaction, and this complex subsequently binds specifically to different motifs in the overlapping CREs for Antp and POU-M2 in the Phm promoter to regulate the transcription of the Phm gene.

in the anterior midgut of the fruit fly (50–54). The PG, which is responsible for producing ecdysone, is located within the larval thorax region of insect species, including the silkworm (55). Our study of the silkworm shows that Antp is expressed in the PG and is directly involved in regulating the transcription of the steroidogenic enzyme gene Phm. These novel findings indicate that Antp is likely required for PG specification and for the PG-specific expression of its downstream targets, including steroidogenic enzyme genes.

POU-M2 has been characterized in the silkworm and belongs to the POU homeobox subfamily of homeodomain transcription factors (56). The POU homeobox genes, which contain a homeodomain and a POU-specific domain, have been shown to be involved in various biological processes in both vertebrates and invertebrates (57–59). Recent reports have shown that POU homeobox genes play key roles in the development of neuroendocrine systems and in the synthesis of certain neuropeptides and endocrine hormones. For example, the POU protein Pit-1 regulates the expression of genes encoding the growth hormones prolactin and thyrotropin in mammals (60). POU-3F1 affects the activity of the gonadotropin-releasing hormone promoter (60, 61). In insects, Har-POU regulates the biosynthesis of diapause hormone in the cotton bollworm (*Helicoverpa armigera*) (62), and Vvl mediates the production of both juvenile hormone and ecdysone in the fruit fly and the red flour beetle (23, 63). Drifter plays an essential role during the development and differentiation of the neuroendocrine system in the fruit fly (64). Silkworm POU-M2 has been shown to regulate the expression of the *DH-PBAN* gene, which encodes diapause hormone and pheromone biosynthesis-activating neuropeptide (56). Our data reveal that silkworm *POU-M2* expression can be detected at the early stage of the fifth larval instar before the appearance of ecdysone pulse, and it displays a developmental change in expression that is positively synchronous with the altered expression of steroidogenic enzyme genes in the PG. These results, together with our observation that POU-M2 directly regulates the transcription of the *Phm* gene, suggest that POU-M2 may control the timing of silkworm ecysteroiogenesis.

Our results, together with a previous finding that silkworm *POU-M2* exhibits a transcriptional response to ecdysone (32), indicate that POU-M2 may also be involved in feedback regulation during silkworm ecysteroiogenesis. βFtz-F1 and Br-C, two secondary ecdysone response genes that are transcriptionally regulated by ecdysone and that mediate ecdysone signaling (65), have been shown to act via feedback regulation on steroido-
ogenic activity in the fruit fly ring gland (22). This feedback regulation ensures the rapid amplification of ecdysone pulses, which trigger metamorphosis at the onset, and the timely self-limiting decline of ecdysone levels at the end (22). Interestingly, our data confirmed that POU-M2 RNAi arrests the larval-pupal transition in the silkworm (Fig. 10 M). This is different from a previous report showing that knockdown of Br-C expression in the fruit fly results in only a delay of pupariation (22). Taken together with the developmental expression of the POU-M2 gene, we conclude that compared with the feedback regulation of Br-C, POU-M2 may play dual roles in the regulation of silkworm ecdysteroidogenesis, in both initiation and feedback.

A striking finding of our study is that Antp can interact with POU-M2 in vitro and in vivo. Homeodomain factors have been shown to form heterodimers or homodimers via protein interactions and to thereby regulate the transcription of target genes (66–68). For instance, Pbx and Meis, which belong to the TALE family of homeodomain proteins, can cooperate with Hox proteins in vertebrates and insects (69, 70). Dimerization is required for the function and specificity of the homeodomain proteins Scr and Fushi tarazu (Ftz) in the fruit fly (71, 72). Previous studies have reported that Antp and POU-M2 also have the capacity to interact with other partners, including Ey, TATA box-binding protein, and Bric-à-brac interacting protein 2 for Antp in the fruit fly (45, 46, 73) and Abdominal-A for POU-M2 in the silkworm (32). Given our findings in the silkworm and previously presented evidence, we propose a model, which is shown in Fig. 9 G, in which Antp and POU-M2 positively regulate the transcription of the silkworm Phm gene and participate in ecdysteroidogenesis by binding directly to adjacent motifs within overlapping CREs in its promoter, through which they determine the spatial and temporal specificity of Phm transcription. A protein interaction between Antp and POU-M2 may be involved in their regulation of silkworm Phm.

**FIGURE 10.** RNAi of either Antp or POU-M2 decreases ecdysone titer and results in failure to complete metamorphosis. A and B, expression changes of Antp and POU-M2 in the PG following treatment with RNAi of either Antp or POU-M2. A total of 40 μg of dsRNA per animal was injected into silkworm larvae at the initiation of wandering. The PGs were collected 48 h after dsRNA treatment for qRT-PCR analysis. The experiments were independently repeated three times, and the data represent the mean ± S.E. (n = 3), *p < 0.05; **p < 0.01, compared with the control. C–G, expression levels of Phm, Spo, Sro, Dib, and Sad in the PG were decreased following treatment with RNAi of either Antp or POU-M2. H–L, ecdysone titer in hemolymph and the expression of the ecdysone response genes (EcR, E74A, βFtz-F1, and Br-C) in fat bodies were decreased following treatment with RNAi of either Antp or POU-M2. The hemolymph and fat bodies were collected 48 h after treatment with dsRNA against either Antp or POU-M2 to perform ecdysone measurements or qRT-PCR analyses. M, effects of treatment with RNAi of either Antp or POU-M2 on silkworm metamorphosis.
transcription; however, the mechanism underlying the interaction between Antp and POU-M2 and the regulation of the transcription of the silkworm Phm gene needs to be explored in more detail in future studies.

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