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Hox transcription factor Antp regulates *sericin-1* gene expression in the terminal differentiated silk gland of *Bombyx mori*.

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Running title: Regulation of *sericin-1* gene expression by Antp.

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Abstract

Hox genes are well-known master regulators in developmental morphogenesis along the anteroposterior axis of animals. However, the molecular mechanisms by which Hox proteins regulate their target genes and determine cell fates are not fully understood. The silk gland of *Bombyx mori* is a tubular tissue divided into several subparts along the anteroposterior axis, and the silk genes are expressed with specific patterns. The *sericin-1* gene (*ser1*) is expressed in the middle silk gland (MSG) with sublocal specificity. Here we show that the Hox protein Antp is a component of the middle silk gland-specific complex, MIC (MSG-intermolt-specific complex), binds to the essential promoter element of *ser1*, and activates its expression. Ectopic expression of *Antp* in transgenic silkworms induced the expression of *ser1* in the posterior silk gland (PSG), but not in the anterior part of MSG (MSG-A). Correspondingly, a MIC-like complex was formed by the addition of recombinant Antp in extracts from PSG with its cofactors Exd and Hth, but not in extracts from MSG-A. Splicing patterns of *ser1* mRNA induced by the ectopic expression of *Antp* in PSG were almost the same as those in MSG at the fifth instar and altered depending on the induction timing of Antp. Other Hox genes were expressed with sublocal specificity in the silk gland. The *Bombyx* silk gland might provide a useful system for understanding how Hox proteins select and regulate their target genes.

Introduction

The silkworm *Bombyx mori* has a pair of silk glands extending from the spinneret on the head to the seventh abdominal segment. The silk gland can be divided into several subparts along the anteroposterior axis on the basis of its morphology and function (Suzuki et al., 1990; and see Fig. 1A). The posterior silk gland (PSG) secretes fibroins, core fibers of silk, which are delivered forward in the lumen and coated by a mixture of sericins secreted from the middle silk gland (MSG). MSG is further separated into three compartments according to the type of sericin (ser1, 2 and 3 and their isoforms) produced (Couble et al., 1987; Takasu et al., 2010). The cuticle-lined anterior silk gland (ASG) provides an outlet for the gland.

The silk gland produces a mass of silk proteins at the last instar to make cocoons, but a certain amount is constantly synthesized throughout all larval instars, except each molting stage. Correspondingly, transcription of the silk genes is active during intermolts, and reaches maximum levels at the last instar (Couble et al., 1983; Ishikawa and Suzuki, 1985; Maekawa and Suzuki, 1980; Suzuki and Suzuki, 1974). To elucidate regulation mechanisms of the silk genes, the first *in vitro* transcription system using tissue extract of the silk glands was developed (Tsuda and Suzuki, 1981), and promoter elements of the *H-fibroin* and *ser1* genes and their binding factors have been analyzed. Then, an *in vivo* transient assay system using a gene gun further elucidated enhancer elements of these genes (Shimizu et al., 2007; Takiya et al., 2011).

Expression of the *ser1* gene is restricted to the middle and posterior parts of MSG in the last instar (Matsunami et al., 1998; Takasu et al., 2010). Several promoter elements of *ser1* have been identified *in vitro* (Matsuno et al., 1989, 1990). The SA site (-103/-85) is recognized by SGF-1/Fork head (Fkh), which is considered to play a role in silk gland development and transcription of the silk genes (14, 15). The SB (-149/-135) and SC (-204/-183) sites are occupied by POU-M1, but *POU-M1* is expressed efficiently in the anterior region next to the *ser1* expression region, and is thought to be a repressor of *ser1* (Fukuta et al., 1993; Kimoto et al., 2012). Recently, we identified that the -70 element of *ser1* is essential for its promoter activity *in*

vivo. A complex that binds to this site with the same spatiotemporal specificity as *ser1* expression was detected and designated MSG-intermolt-specific complex (MIC), suggesting that MIC is an activator of *ser1* (Takiya et al., 2011).

MIC binds to several ATTA-core AT-rich elements in *ser1* promoter. Such ATTA motifs are known to be recognized by homeodomain proteins, but MIC composition has not been determined. Several homeodomain-encoding genes are expressed with specific patterns in the silk gland. Two segmentation genes, *engrailed* (*en*) and the related *invected* (*in*), are coexpressed in MSG but not in PSG (Hui et al., 1992). *POU-M1* is expressed in ASG and the anterior part of MSG (Kimoto et al., 2012; Matsunami et al., 1998). Territory-specific expression of some Hox genes in the silk gland was also reported; *Antennapedia* (*Antp*) is expressed specifically in MSG, whereas *Ultrabithorax* (*Ubx*) is expressed in PSG (Dhawan and Gopinathan, 2003; Nagata et al., 1996).

Hox proteins act as master regulators of developmental morphogenesis along the anteroposterior axis in vertebrates and invertebrates. Although it has been assumed that Hox proteins function by regulating a set of downstream genes, knowledge of the genetic pathways, including the direct or indirect targets regulated by Hox proteins, are extremely limited (Hueber and Lohmann, 2008). In addition, all Hox proteins bind to a similar set of AT-rich sites, raising the question of how specificity is achieved *in vivo*. This problem was partially solved by the finding that the cooperation of Hox proteins with cofactors such as Extradenticle (Exd) and Homothorax (Hth) increases their DNA-binding specificities (Mann et al., 2009; Moens and Selleri, 2006). However, one Hox-cofactor complex can still recognize divergent sequences, leading to difficulty in predicting target genes regulated by Hox proteins (Ebner et al., 2005; Uhl et al., 2010).

Here we show that *Bombyx* Antp and its cofactors Exd and Hth are components of MIC, which binds to the -70 element essential for *ser1* expression. Misexpression of *Antp* in transgenic silkworms induced ectopic expression of *ser1* in PSG. The results demonstrated that Antp regulates *ser1* expression in the differentiated silk gland of the silkworm *Bombyx mori*.

Materials and Methods

Animals

Kinshu x Showa (Figs. 1-4) and White-C (Figs. 5-6) were used as wild-type strains of *B. mori*. Kinshu x Showa eggs were purchased from Ueda Sanshu (Ueda, Japan). Larvae were reared at 25°C under LD 16:8 on an artificial diet obtained from Nippon Nosan-Kogyo (Yokohama, Japan) and staged as described previously (Kiguchi and Agui, 1981; Suzuki et al., 1990).

Cloning of Exd and Hth

We identified *Bombyx Exd* and *Hth* *in silico* based on its similarity to *Drosophila* homologues. The ORF of *Bombyx Exd* was cloned by PCR using cDNA of MSG of day 2 fifth instars with primers 5'-atggacgateccgacc-3' and 5'-tcattgtcgtcaacatatg-3' (GenBank accession no. AB841084). The ORF of *Bombyx Hth* was cloned using the same cDNA with primers 5'-atggctcagcctaggtac-3' and 5'-ttagaggtggtgtccgtaatac-3' (GenBank accession no. AB841083)

Transgenic silkworms and heat-shock treatment

Antp ORF was amplified with primers 5'-aaccctaggcaacaattgacagctatg-3' and 5'-aaccctaggccgactaagctaaggtttac-3', and was inserted into the *BlnI* site of pBacUASMCS vector (Sakudoh et al., 2007) with [3xP3-AmCyan] screening marker. The plasmid was injected into *w1-pnd* embryos to obtain UAS-*Antp* strains. The established strains were crossed with *hsp70-GAL4* strain for misexpression (Uchino et al., 2006). Larvae were placed in 100-ml flasks plugged with cotton. Flasks were then submersed into a water bath and heated for 2 hours at 42°C (Uhlirva et al., 2002). Following heat shock, larvae were returned to food.

RNA extraction and RT-PCR

Total RNA was extracted using the Illustra RNAspin MINI RNA Isolation kit (GE Healthcare) and cDNA was synthesized using the PrimeScript RT-PCR kit (Takara) as described previously (Kimoto et al., 2012). PCR amplification was performed using ExTaqHS polymerase (Takara) with primers and appropriate cycles listed in Table S1. PCR products were analyzed on 5% polyacrylamide gels for splicing variants of *ser1* and 1% agarose gels for other genes.

qRT-PCR

Real-time RT-PCR was performed using the ABI 7300 PCR system and Power SYBR Green PCR Master mix (Applied Biosystems) as described previously (Kimoto et al., 2012). The *ser1* was amplified with primers 5'-tcgttctgtgctgcactttg-3' and 5'-tcactgctagctgcattgtac-3'. *Exd* was amplified with primers 5'-cacgctgaactgcatagaa-3' and 5'-ccctccgcaatcaacata-3'. *Hth* was amplified with primers 5'-cgaaggtcgccacaaatc-3' and 5'-cctacgtctcgcgtttatga-3'. *GAPDH* was amplified with primers 5'-tgtgctccgtgcttctatt-3' and 5'-gccatgggtggaatcatact-3'. Data were normalized separately with *GAPDH*.

Whole mount *in situ* hybridization.

Digoxigenin (DIG)-labeled RNA probes for *ser1* (nucleotide sequence 106-775, J01040) and *H-fibroin* (nucleotide sequence 62425-62479 and 63451-63830, AF226688) were synthesized using a DIG RNA Labeling Kit (Roche). Silk glands were isolated from wild-type worms (+/+) or heat-treated transgenic worms (hs-Gal4/UAS-Antp of day 2 fourth instars) and sequentially fixed with 100% methanol, 100% ethanol and 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; 137mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.76 mM KH₂PO₄, pH 7.4). After washing with PBS, tissues were digested with 0.2 N hydrochloric acid in PBS for 20

min, washed with PBS, treated with 10 µg/ml Proteinase K (Wako) in PBS for 20 min, washed with PBS, refixed in 4% PFA in PBS and washed with PBS. Tissues were then prehybridized in 5x SSC (1x SSC is 150 mM NaCl and 15 mM sodium citrate), 50% formamide, 50 µg/ml heparin and 50 µg/ml salmon sperm DNA at 50°C for 1 hr. Hybridization was performed using probes at 100-250 ng/ml concentration in the same solution at 50°C for 24 hr. Hybridized tissues were washed with 2x SSC/0.1% Tween 20 at 50°C for 20 min, followed by washing twice with 0.2x SSC/0.1% Tween 20 at room temperature for 20 min. After incubation with 1% blocking reagent (Roche) in PBS for 1 hr, the tissues were incubated with a 1:4000 dilution of anti-DIG AP conjugated polyclonal antibodies (Roche) for 1 hr at 37°C. After washing three times with DIG Buffer 1 (100 mM maleic acid, 150 mM NaCl and 0.01% TritonX-100, pH 7.5), the tissues were equilibrated with freshly prepared DIG buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5). Coloring reactions were performed using Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) tablet (Roche Molecular Biochemicals). Tissues were then counterstained with a graded series of ethanol.

Preparation of recombinant proteins.

To obtain rAntp-FL, rAntp-N, rAntp-C, rAntp-C^{YPWM}, rEn-C, rExd-N and rHth-N proteins, the entire ORF (amino acid positions 1-259), N-terminal (1-176), C-terminal (168-259), YPWM-less C-terminal region (173-259) of Antp, C-terminal region of En (268-372), N-terminal region of Exd (1-225) and N-terminal region of Hth (1-243) were cloned into pGEX-6P-1, respectively. GST-fusion proteins were expressed in *E.coli* BL21 cells and purified on glutathione-Sepharose beads, followed by removal of GST using PreScission protease as described previously (Kimoto et al., 2012; Takiya et al., 2005). Recombinant proteins were characterized for size and purity by SDS-PAGE and protein was measured by Bradford's method with Protein Assay reagent (BioRad).

Electrophoretic mobility shift assay (EMSA)

Tissue extracts from each portion of the silk gland were prepared as described (Takiya et al., 1990; Tsuda and Suzuki, 1981). The electrophoretic mobility shift assay (EMSA) was carried out as reported previously (Takiya et al., 1997, 2005) using the oligonucleotides shown in Figure 1 as the probe and competitors. Protein-probe complexes were separated on 7% polyacrylamide gels. For competition experiments, 12.5 pmol unlabeled oligonucleotides shown in Fig. 1A were added to the reaction. To prepare antibodies, rAntp-N, rAntp-C, rExd-N and rHth-N proteins or C-terminal peptide of POU-M1 (amino acid position 340-352) were used to immunize rabbits. Monoclonal anti-*Drosophila* Antp antibody (4C3) was purchased from the Developmental Studies Hybridoma Bank.

Western blot

Each silk gland extract (25 µg protein) was separated by SDS-PAGE and transferred to a PVDF membrane. Filters were probed with antibodies and visualized with the Amersham ECL Prime Western Blotting Detection Kit (GE Healthcare) according to the manufacturer's instructions. Anti-alpha tubulin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Results

Antp Shows Similar DNA-binding Specificity to MIC

Nucleotide sequence around the -70 region of *ser1* promoter (the transcription start site is denoted as +1) is indispensable for MSG-specific *ser1* expression (Fig. 1; see also Takiya et al., 2011). Promoter activity is lost almost completely by the introduction of mutations, such as M2 sequence, into the 1.6-kb *ser1* promoter, while the promoter containing M4 sequence mutations is as active as the wild-type construct (Fig. 1B). MIC was first detected as a factor binding to the -70 element in extracts from the posterior region of MSG (MSG-P). MIC binding to the -70 element was competed with the M4 oligonucleotide as well as the wild-type -70 element itself but not with M2 oligonucleotide (Fig. 1C). Moreover, MIC has identical spatiotemporal specificity to *ser1* expression. It is detectable in MSG of the fifth instar larvae, but not in PSG or the fourth molting stage (Kimoto et al., 2012; Takiya et al., 2011).

The -70 and other MIC-binding elements contain an AT-rich sequence with an ATTA core motif (Takiya et al., 2011), known to be recognized by homeodomain proteins. We investigated the expression of several genes encoding a homeodomain protein in the silk gland on day 2 of the fifth instar (Fig. 2A). Some Hox and homeobox genes exhibited characteristic expression patterns along the anteroposterior axis of the silk gland. Anterior Hox genes such as *labial (lab)* and *Sex combs reduced (Scr)* were expressed specifically in ASG, but central Hox gene expression was not observed in ASG. *Antp* was expressed in MSG, *abdominal-A (abd-A)* in the entire MSG and PSG, and the expression levels of *Ubx* were very low in every subpart at this stage. The posterior Hox gene *Abdominal-B (Abd-B)* was expressed efficiently in the posterior of PSG (PSG-P) and in ASG. The maternal and zygotic homeobox gene, *caudal (cad)*, was expressed specifically in ASG. *en* and its paralogue *in* were expressed in ASG and posterior region of MSG. Taken together, *Antp* and *en/in* were expressed strongly and/or specifically in MSG.

DNA-binding specificity of *Antp* and *En* was examined in EMSA using

recombinant proteins. Both C-terminal region proteins containing each homeodomain of Antp or En (rAntp-C and rEn-C) bound to the -70 element, but with different sequence specificities (Fig. 2B). The rEn-C bound more strongly to the M2 than the M4 sequence, while rAntp-C preferred M4 over M2, displaying similar DNA-binding specificity to that of MIC (see Fig. 1C). Recombinant protein containing the entire ORF of Antp (rAntp-FL) showed the same sequence specificity as rAntp-C (Fig. 2C). Furthermore, a MIC-like complex appeared when rAntp-FL was added to MSG-P extracts, suggesting that rAntp-FL interacts with other proteins to generate the MIC-like complex (Fig. 2C).

MIC Includes Antp and its Cofactors, Exd and Hth

To examine whether MIC contains Antp, a supershift assay in EMSA was carried out using anti-Antp antibodies. Co-incubation of polyclonal antibody against the N-terminal or C-terminal region of *Bombyx* Antp in EMSAs abolished the formation of MIC in MSG-P extracts (Fig. 3A). Addition of monoclonal anti-*Drosophila* Antp antibody resulted in a supershift of the MIC band, whereas preimmune sera and antiserum against POU-M1 did not interfere with the formation of MIC (Fig. 3A and Fig. S1).

The M2 oligonucleotide, which has mutations rendering *sericin-1* promoter inactive, competed partially for the binding of rAntp-FL to the -70 element, while MIC and MIC-like complex were not competed by the M2 oligonucleotide (Fig. 2C and Fig. S2). Interaction of Hox proteins with cofactors is known to increase their DNA-binding specificities. The two best-characterized cofactors of Hox proteins in *Drosophila* are Exd and Hth. To know whether MIC contains Exd and Hth, specific antisera against *Bombyx* Exd and Hth were added into EMSA reactions. Both antisera abolished the formation of MIC completely (Fig. 3A), indicating that MIC contains Exd and Hth together with Antp. The MIC-like complex formed in MSG-P extracts by the addition of rAntp-FL also reacted with these antibodies (Fig. 3B). The mobility of the MIC-like complex and MIC differed. It is possible that MIC involves components other than Antp, Exd and Hth.

We sometimes observed two bands for MIC-like complex (Fig. 3B). Two types of MIC-like complex might be formed by different interactions of those components with rAntp.

It is well known that most Hox proteins interact with cofactors via the YPWM motif existing upstream of the homeodomain (Passner et al., 1999; Prince et al., 2008). The C-terminal region of Antp containing the homeodomain and YPWM motif (rAntp-C) interacted with Exd and Hth in MSG extracts and formed a MIC-like complex (Fig. 3C, lane1-4). YPWM motif-less rAntp-C (rAntp-C^{YPWM-}) bound to the -70 element with the same specificity as rAntp-C (Fig. 3C, lane9-12), but MIC-like complex was not formed with rAntp-C^{YPWM-} in MSG-P extracts (Fig. 3C, lane5 and 7). The results indicate that *Bombyx* Antp interacts with Exd and Hth via its YPWM motif, as do other Hox proteins.

Localization of Antp, Exd and Hth Proteins in the Silk Gland

To know the localization of Antp and its cofactors in the silk gland, quantitative RT-PCR (qRT-PCR) assay and Western blot analyses were performed with total RNA or tissue extracts from each part of the silk gland. qRT-PCR showed that *Antp* mRNA was abundant in MSG while *Exd* mRNA was present in all regions of the silk gland (Fig. 4A). Transcripts of *Hth* were detectable also in all the regions, but the level in MSG-A was very low. By Western blotting, Antp protein was detected specifically in MSG-M and -P. The level of *Antp* mRNA in MSG-A was estimated to be about one third of that in MSG-P, but Antp protein was scarcely detectable in MSG-A. Localization of Exd and Hth proteins was in good agreement with the results of qRT-PCR (Fig. 4B). Accordingly, MIC was not observed in PSG extracts, but the MIC-like complex was formed by the addition of rAntp-FL (Fig. 4C). The MIC-like complex in PSG extracts reacted with antibodies against Exd and Hth (not shown), and showed the same DNA-binding specificity as MIC (Fig. S2).

Antp Induces Ectopic Expression of *Sericin-1* in PSG

The observations that cofactors of Hox proteins, Exd and Hth, were present in PSG prompted us to examine whether *ser1* is induced by the enforced expression of *Antp* in PSG. We generated transgenic silk worms that possess a *hs-GAL4* transgene in which *Gal4* was controlled under a heat-inducible promoter, and a *UAS-Antp* transgene, in which *Bombyx Antp* was controlled under the UAS promoter. Transgenic worms were heated at 42°C for two hours on day 1 of the fifth instar, and then gene expression was analyzed in PSG. *Gal4* was expressed immediately and transiently after heat treatment, followed by *Antp* induction (Fig. 5A). One day after heat treatment, ectopic expression of *ser1* was observed in PSG. Although expression levels varied in individual worms (Fig. 5B), *ser1* was always induced by misexpression of *Antp* in PSG, and results were confirmed also with qRT-PCR (Fig. 5C). To assess the position effects of the transgene, we repeated the experiments using another UAS-*Antp* transgenic line and obtained similar results (Fig. S3).

To compare *ser1* mRNA distribution in the silk gland of normal and transgenic silkworms, whole-mount *in situ* hybridization was performed. Because the silk glands at the fifth instar are too large for whole-mount *in situ* hybridization, we used the silk glands from the fourth instar. At this stage, the *ser1* gene is expressed in MSG-P alone in normal worms (Fig. 5D, E) while, as in the RT-PCR findings, *ser1* mRNA was detected in PSG of heat-treated transgenic worms (Fig. 5F, G). The expression pattern of *H-fibroin* was not affected by this heat treatment and misexpression of *Antp* (Fig. S4 and Fig. S5). Expression levels of other Hox genes, *Ubx*, *abd-A* and *Abd-B*, and putative repressor of the *ser1* gene, *POU-M1*, were not changed in PSG after heat treatment (Fig. S5).

Misexpression of *Antp* is Insufficient to Induce *Sericin-1* Expression in MSG-A and Other Tissues

We next investigated whether ectopic expression of *ser1* occurs in the anterior of the silk gland or in other tissues of transgenic worms. *Antp*

overexpression was observed throughout the silk gland after heat treatment, but *ser1* expression was not detected in ASG and MSG-A by RT-PCR (Fig. 6A). *Antp* was expressed normally in the CNS, salivary glands and trachea in addition to MSG of wild-type fifth instar larvae. After heat treatment of transgenic worms, *Antp* overexpression was observed in all tissues examined, but ectopic expression of *ser1* was not detected in tissues other than PSG (Fig. 6A). Thus, *Antp* overexpression was not sufficient to induce *ser1* in most tissues.

As described above, Antp bound to the target DNA cooperatively with cofactors Exd and Hth. Fig. 6B shows that *Exd* was expressed in all normal tissues, but *Hth* was not expressed in the fat body, gonads and MSG-A. The absence of cofactor Hth in these tissues was thought to explain the failure of *ser1* induction by the misexpression of *Antp* and for the inability to form MIC-like complex in MSG-A extracts with rAntp addition. Furthermore, strong expression of *POU-M1* was found in the CNS, malpighian tubule and trachea as well as ASG and MSG-A (Fig. 6B). POU-M1 can bind to several AT-rich sequences of *ser1* promoter, including the -70 element, and suppresses the activity of *ser1* promoter (Kimoto et al., 2012).

Splicing Patterns of *Sericin-1* mRNA Vary in Tissue- and Developmental Stage-dependent Manners

The *ser1* gene gives rise to several distinct mRNAs by alternative splicing, which is dependent on stages of larval development and on topological locations within MSG (Couble et al., 1987; Garel et al., 1997). This tissue- and developmentally regulated maturation of *ser1* mRNA is considered to contribute to the physicochemical properties of silk threads. We compared splicing patterns of *ser1* in normal and transgenic worms. During the fourth instar, *ser1* was transcribed only in MSG-P cells and an isoform produced there was devoid of a central 6.6-kb-long alternative exon (Fig. 7, referred as *ser1B* in Garel et al., 1997). After the fourth molt, the region of *ser1* expression expanded to MSG-M and these cells began to produce the largest mRNA containing all exons (*ser1C*). As development proceeded, exon 4 was

spliced out from the isoforms in MSG-M (lower bands of *ser1B* and *ser1C*), whereas the smallest mRNA (*ser1A*), which does not contain exons 3 to 6, arose in MSG-P. *Ser1D* lacking exons 3 and 4 was generated in late stages of the fifth instar.

When transgenic silkworms were heat-treated on 1 day of the fifth instar, *ser1* activated in PSG produced *ser1C* and *ser1B* isoforms corresponding to those observed in MSG cells of early stages of normal fifth instar larvae (Fig. 7). The same transcripts were detected even if ectopic expression was induced at the fourth instar. By contrast, *ser1D* isoform arose in PSG when transgenic worms were heated at late stages of the fifth instar, and the same developmental transition of splicing patterns was seen in MSG-M of wild-type worms.

Discussion

MIC was a putative transcriptional activator of *ser1*, detected specifically in MSG, and binds to regulatory elements containing the ATTA-core sequence of *ser1* promoter (Takiya et al., 2011). Such sequences are the typical recognition motif of homeodomain proteins. To find homeodomain protein candidates for MIC, we investigated expression patterns of homeodomain coding genes including Hox genes, and found that *Antp* was expressed specifically in MSG. Recombinant Antp protein could bind to the -70 element of *ser1* promoter with similar specificity to MIC. Antp protein generated a MIC-like complex when the recombinant protein was added to MSG extracts as well as PSG extracts. In addition, antisera against Antp abolished the formation of MIC, indicating that Antp is a component of MIC. Well-known cofactors for Hox proteins, Exd and Hth, were also suggested to be included in the complex. Ectopic expression of *Antp* in the silk gland resulted in *ser1* induction in PSG. The results demonstrate that Antp forms MIC together with cofactors Exd and Hth, and activates *ser1* expression in the larval silk gland.

Sericin-1 is a Downstream Target Gene of Antp in *Bombyx*

It is well known that the Hox genes specify segmental identity along the anteroposterior axis of animals (Lewis, 1978; Mann and Morata, 2000), and act also in organogenesis within the segment, such as in the development of the posterior spiracle, salivary gland and midgut of *Drosophila* (Hombria and Lovegrove, 2003). In addition, Hox genes are expressed in some adult organs, suggesting their necessity to maintain organ architecture and to regulate tissue-specific functions (Morgan, 2006). However, only a few target genes of Hox proteins have been identified in many cases. In this study, we showed that the Hox protein Antp regulates *ser1* expression positively in the terminal differentiated larval silk gland. Since Antp binds to the essential promoter element of *ser1* gene as a component of MIC *in vitro*, *ser1* seems to be a direct target of Antp. The Nc mutant lacking the 3'-terminal region of

Antp results in severe growth suppression of the silk gland, besides compression of the thoracic segments (Nagata et al., 1996). Therefore, *Bombyx* *Antp* activity is necessary for *ser1* expression throughout post-embryogenesis as well as for silk gland development during embryogenesis.

Expression of *Antp* and Cofactors and Ability of Complex Formation Differ in Subparts of the Silk Gland

Hox proteins are known to interact with cofactors that help with DNA-binding site selection (Gebelein et al., 2002; Mann et al., 2009). We showed that MIC contains cofactors Exd and Hth in addition to *Antp*. These proteins were present in MSG-M and -P, where MIC was detected and *ser1* is expressed. In contrast, *Antp* was absent from PSG and MIC was not observed. Interestingly, a MIC-like complex was formed by the addition of *Antp* protein to PSG extracts, in which both Exd and Hth were present. Correspondingly, *ser1* was expressed ectopically in PSG by misexpression of *Antp* in transgenic worms.

Antp protein was not detected in MSG-A in normal worms, despite the detectable levels of *Antp* mRNA, implying that translational repression or post-translational regulation of *Antp* might occur in MSG-A. Moreover, MIC-like complex did not appear even when *Antp* protein was added to MSG-A extracts. This inability to form MIC-like complex may in part be because the expression level of Hth was very low in MSG-A. In line with these observations, misexpression of *Antp* could not induce *ser1* expression in MSG-A of transgenic worms.

The silk gland is considered as an evolutionary homologue of *Drosophila* salivary gland, both arising from the labial segment, and *Bombyx* salivary glands arise from the mandibular segment. *Bombyx* silk glands and salivary glands might share some common features including gene expression profiles (Parthasarathy and Gopinathan, 2005). However, *ser1* was suppressed in the salivary gland, even though *Antp* and its cofactors are expressed and the expression level of *POU-M1* was very low. It was

suggested that MIC contains other components than Antp, Exd and Hth. Expression of such components may be different between the silk gland and salivary gland, leading the suppression of *ser1* in *Bombyx* salivary glands.

The splicing pattern of ectopic *ser1* transcripts in PSG was the same as that of endogenous *ser1* in MSG-M during the fifth instar. On the other hand, the longest isoform, which arises in MSG of wild-type worms at the early fifth instar, was found in PSG of transgenic worms at the fourth instar. Regulatory factors affecting splicing patterns might differ between MSG and PSG in the fourth instar, or the splicing machinery in PSG matures earlier than MSG in the fourth instar.

Some Hox Genes are Expressed in Territory-specific Manners in the Silk Gland

The order of Hox genes along the chromosome generally reflects their order of action along the anteroposterior axis in the body or organs (Kmita and Duboule, 2003; Lewis, 1978). We found that some Hox genes are expressed with characteristic patterns along the anteroposterior axis in the silk gland. For example, *lab* and *Scr* were expressed in ASG and *Abd-B* expression was restricted to the posterior portion of PSG. It is interesting whether these Hox genes play roles in region-specific functions in the silk gland, such as regulation of territory-specific expression of silk genes. There are MIC-binding sites in *ser1* promoter besides the -70 element (Takiya et al., 2011), and putative homeodomain recognition elements are found also in promoter of *H-* and *L-fibroin* genes (Hui and Suzuki, 1990). Hox proteins bind similar AT-rich DNA elements *in vitro*, and it has long been debated how these proteins recognize appropriate target genes to achieve their specific functions (Sorge et al., 2012).

The silk gland is large enough to prepare extracts from each subpart, and a gene gun system was successful in the silk gland to examine promoter activities transiently (Shimizu et al., 2007; Takiya et al., 2011). In addition, the present study showed that transgenic silkworms carrying a heat-inducible transgene are useful to examine the function of transcription

factors in the silk gland during larval stages. Thus, the silk gland could be a good model system to understand the roles of Hox genes in terminal differentiated tissues and to reveal how they select their target genes.

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Figure legends

Fig. 1. Correlation of MIC-binding activity of the -70 element with promoter activity of the *sericin-1* gene. (A) Representation of the silk gland of *B. mori*. MSG-A, -M, -P are anterior, middle and posterior parts of the middle silk gland (MSG). PSG-A and -P are the anterior and posterior halves of the posterior silk gland (PSG). (B) Wild-type and mutant nucleotide sequences of the -70 element used as the probe and competitors in EMSA are shown below the map of *ser1* promoter. Relationships between MIC binding to the -70 element and promoter activity of *ser1* (-1602 to +42) are summarized on the right of the nucleotide sequences. Promoter activities were assessed by luciferase assay using a gene gun and tissue transplantation (Takiya et al., 2011). (C) EMSA was carried out using extracts from the posterior region of MSG (MSG-P) on day 2 of the fifth instar with the -70 probe in the presence or absence of competitors (comp.).

Fig. 2. Gene expression and DNA-binding activity of homeodomain transcription factors. (A) Expression patterns of genes encoding homeodomain transcription factors were analyzed by RT-PCR using silk glands on day 2 of the fifth instar. The *ribosomal protein 49* (*Rp49*) gene was used as a control. (B) EMSA was performed using 1.6 pmol recombinant proteins of the C-terminal region of En (rEn-C) or C-terminal region of Antp (rAntp-C) and the -70 probe. Wild-type (W) -70 oligonucleotide or M2 and M4 oligonucleotides were used as competitors. (C) Recombinant protein of entire Antp (rAntp-FL) formed a MIC-like complex (arrow) in MSG-P extracts and showed the same DNA-binding specificity as MIC. EMSA was performed using 5 pmol rAntp-FL (left panel) or 0.5 pmol rAntp-FL and 10 µg MSG-P extract (right panel).

Fig. 3. Effects of antibodies specific to Antp and the cofactors on MIC formation in EMSA. (A) MSG-P extract (10 µg) was incubated with or without antisera. Each antiserum added to EMSA reactions is shown at the top of the panel. Preimmune sera (PI) from the same rabbit in which

α Antp.N was generated was used as a control. The regions used for immunization are as follows: α Antp.N: 1-176 of *Bombyx* Antp, α Antp.C: 168-259 of *Bombyx* Antp, α Exd: 1-225 of *Bombyx* Exd, and α Hth: 1-243 of *Bombyx* Hth. α DmAntp is the monoclonal antibody against *Drosophila* Antp (4C3, Reuter and Scott, 1990). (B) MSG-P extract (2.5 μ g) was incubated with rAntp-FL (5 pmol) and specific antisera. Left-side lane shows the EMSA pattern with MSG-P extract (10 μ g) alone. Arrow indicates a MIC-like complex. (C) EMSA was performed using 5 μ g MSG-P extract and 1 pmol recombinant proteins. Antisera specific to Antp (α Antp), Exd (α Exd) or Hth (α Hth), shown at the top of the panel, were added. Open arrowhead indicates complexes of the -70 probe and rAntp-C alone with or without YPWM motif.

Fig. 4. Localization of Antp and cofactors in the silk gland. (A) Real-time RT-PCR analyses of *Antp*, *Exd* and *Hth* in each part of the silk gland from day 2 fifth instars. Each mRNA level was normalized to *GAPDH* and is shown as the fold difference calculated relative to the level in ASG. Error bars indicate SD (n = 3). (B) Western blot analyses of Antp and cofactors. Tissue extracts (25 μ g protein) from each part of the silk gland from day 2 fifth instars were blotted with antisera specific to Antp, Exd and Hth, or alpha-tubulin (Tub) as a control. (C) EMSA was carried out using extracts (5 μ g each) from different parts of the silk gland with or without rAntp-FL (5 pmol).

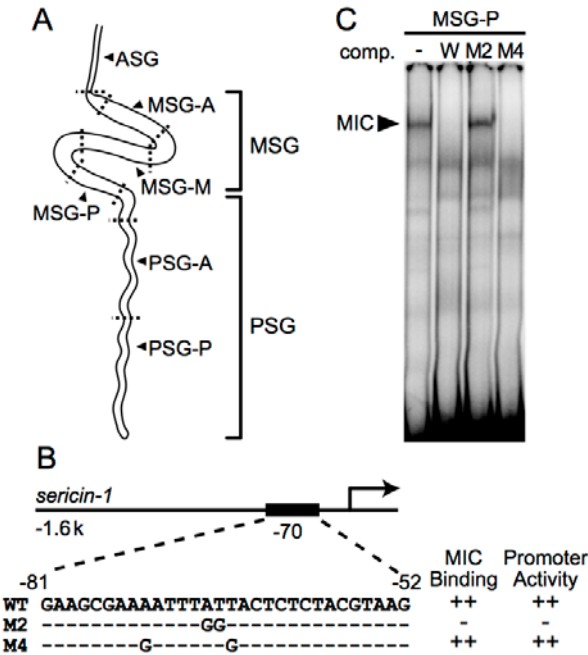
Fig. 5. Ectopic expression of Antp induced *sericin-1* expression in PSG. (A) RT-PCR was performed with total RNA from PSG of transgenic silkworms carrying hs-Gal4 alone or hs-Gal4/UAS-Antp after heat treatment at 42 °C. The dissection time after heat treatment is shown at the top of panels. (B) RT-PCR was performed with total RNA from MSG (M) and PSG (P) of the wild type (+/+) or RNA from individual PSG of 5 worms each of hs-Gal4/+ or hs-Gal4/UAS-Antp strain. (C) Expression levels of *ser1* were analyzed by real-time RT-PCR on MSG and PSG. Total RNA was extracted from silk glands of heat-treated hs-Gal4/+ (-) or hs-Gal4/UAS-Antp (+) day 2 fifth instar. The mRNA levels were normalized to *GAPDH* and are shown as the

fold difference relative to the level in PSG of *hs-Gal4/+*. Error bars indicate SD ($n = 5$). (D-G) *In situ* hybridization of *ser1* mRNA in the silk gland. Silk glands were collected from day 1 fourth instar larvae of heat-treated *hs-Gal4/+* (D, E) or *hs-Gal4/UAS-Antp* (F, G) strains. (D, F) Low magnification views of the entire silk gland. (E, G) High magnification views showing regions around the boundary of MSG and PSG. Arrowheads point to examples of expression in MSG and arrows point to examples of expression in PSG. Bar represents 0.5 mm.

Fig. 6. Effects of ectopic expression of *Antp* in other portions of the silk gland and various tissues. (A) Expression of *Antp* and *ser1* in various tissues were analyzed by RT-PCR. Tissues were obtained from heat-treated *hs-Gal4/+* (upper panels) or *hs-Gal4/UAS-Antp* worms (lower panels) 24 hr after heat treatment of day 1 fifth instars. (B) Expression analysis of *Antp*, *Exd*, *Hth* and *POU-M1* in various tissues of wild-type day 2 fifth instars.

Fig. 7. Isoform-specific expression patterns of *sericin-1*. Schematic structure of the *sericin-1* gene and isoforms of mRNA are shown on the left. Exons are represented as boxes with exon numbers above, and splicing patterns are indicated with V-shaped lines above each isoform. RT-PCR was performed with total RNA from each part of the silk gland of wild type (W) or heat-treated *hs-Gal4/UAS-Antp* transgenic (T) worms, using primers indicated in the schema and shown in Table S1. Light-shaded boxes are exons predicted from all known isoforms sharing exons 1, 2, 7, 8 and 9 (Garel et al., 1997). IV2 means the day 2 fourth instar and V0, V1, V2, V3, V4 and V5 means the day 0, 1, 2, 3, 4 and 5 of the fifth instar, respectively.

Fig. 1



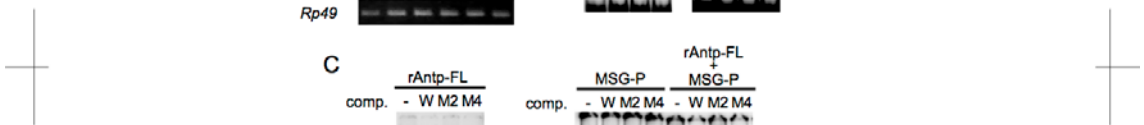




Fig. 4

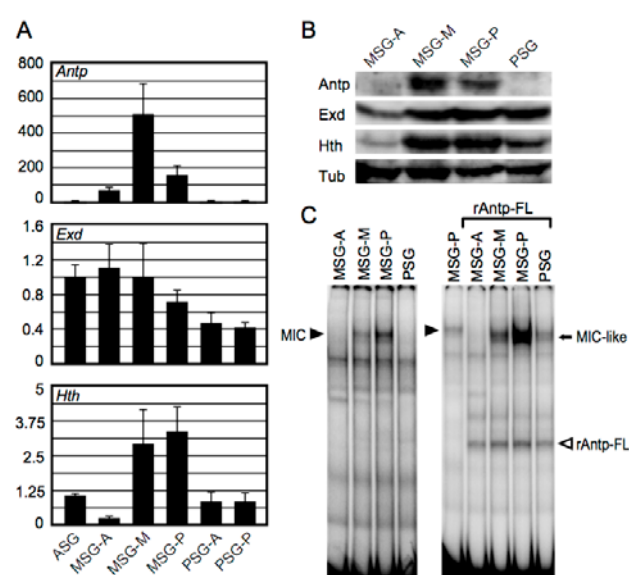


Fig. 5

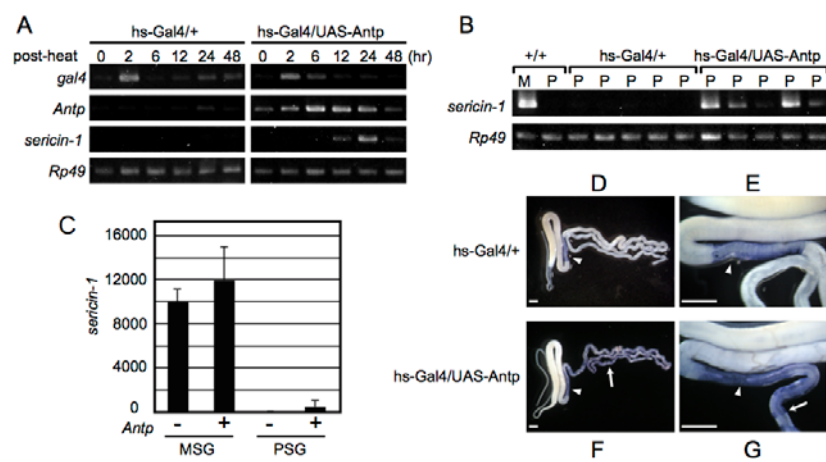


Fig. 6

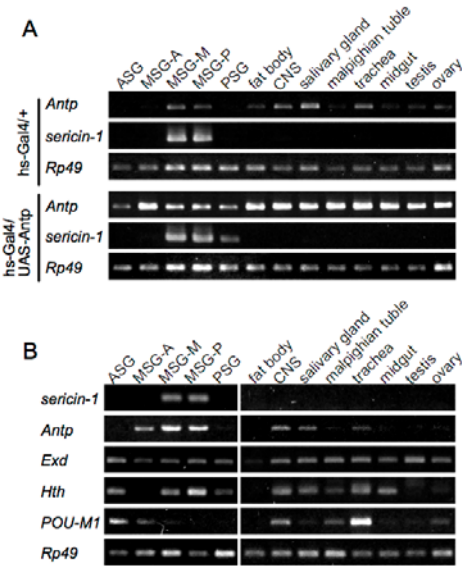


Fig. 7

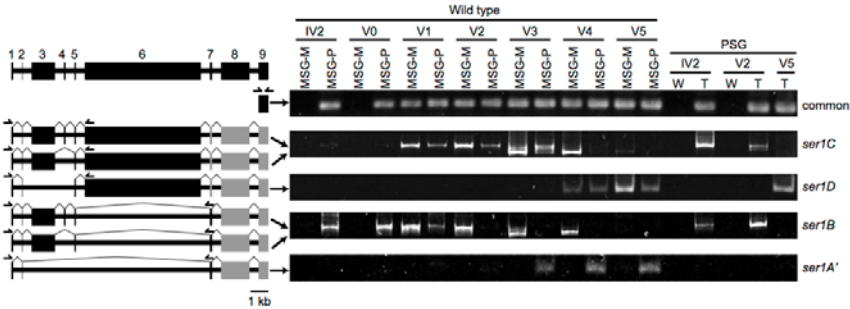


Fig. S1. Specificity of antibodies used in this study. Supershift assay was carried out using silk gland extracts, the -70 probe and each anti-serum. Anti-POU-M1 antibody reacted with the complex observed in MSG-A extract (POU), but not with MIC. Conversely, anti-Antp, -Exd or Hth did not affect POU-M1 in the MSG-A extract.

Fig. S2. MIC-like complexes showed the same DNA-binding specificity as MIC. EMSA was performed by incubation of 10 µg silk gland extracts (MSG-P or PSG) and 0.5 pmol recombinant proteins (rAntp-FL or rAntp-C) with the -70 probe and competitors (comp.) Arrowhead shows MIC-like complex.

Fig. S3. Induction of *ser1* gene expression by *Antp* was confirmed using another transgenic line with the UAS-Antp gene at different locus. RT-PCR was performed with total RNA from MSG (M) and PSG (P) of transgenic silkworms carrying hs-Gal4 alone or hs-Gal4/UAS-Antp after heat treatment at 42°C.

Fig. S4. *In situ* hybridization of *H-fibroin* mRNA in the silk gland. Silk glands were collected from day 1 fourth instar larvae of wild-type (+/+) (A, B) or heat-treated hs-Gal4/UAS-Antp (C, D) strains. (A, C) Low magnification views of the entire silk gland; *H-fibroin* mRNA was detected in PSG of transgenic and wild-type silk glands. (B, D) High magnification views showing regions around the boundary of MSG and PSG. Arrowheads point to the boundary between MSG and PSG and arrows point to examples of expression in PSG. Bar represents 0.5 mm.

Fig. S5. Effects of ectopic expression of *Antp* on gene expression patterns in the silk gland. RT-PCR was performed with total RNAs from each part of the silk gland of hs-Gal4/UAS-Antp worms with or without heat treatment of day 2 fifth instar.

Fig. S1

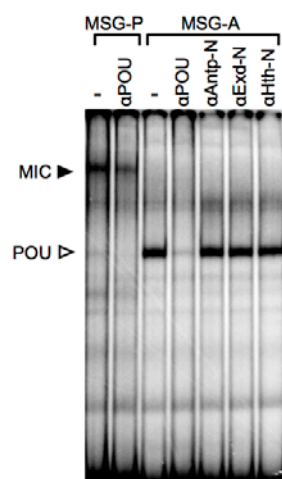


Fig. S2

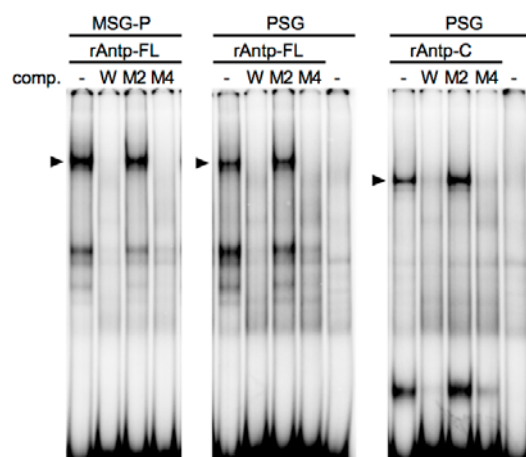


Fig. S3

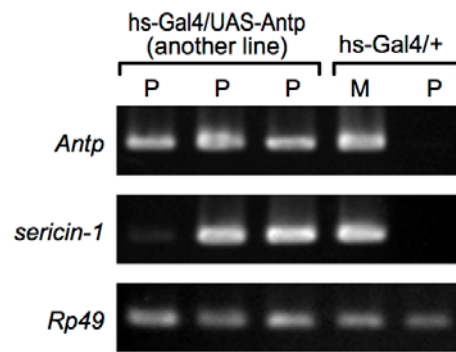


Fig. S4

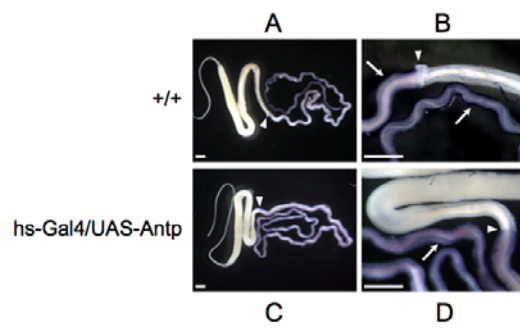


Fig. S5

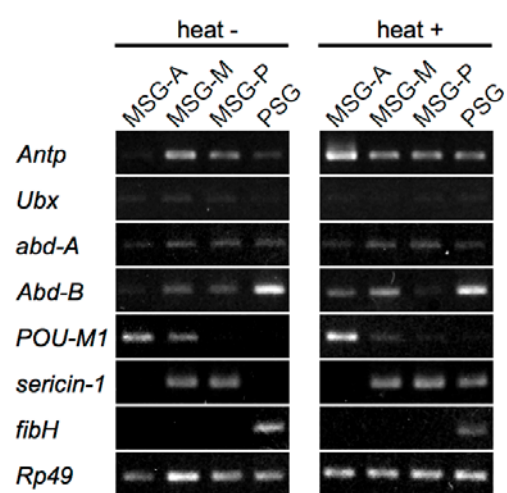


Table S1. Primers and PCR cycles for each gene.

Gene		Primers	Accession no.	Cycles
<i>sericin-1</i> ^{*2}	F (ex1) ^{*1}	5'-tgcgtttcgttctgtgctgcactttg-3'	Z48892, J01034	*3
	F (ex9)	5'-tctgaagacggtttctggtg-3'		
	R (ex3)	5'-taactccgtaagaaccgtcagcgttc-3'		
	R (ex6)	5'-aagcagccctagagctcgaggatgag-3'		
	R (ex7)	5'-cacctcggaagagtcttcacttagtg-3'		
	R (ex9)	5'-ttcttacggagatggtaagg-3'		
<i>H-fibroin</i>	F	5'-tccaactctcaagatgag-3'	AF226688	20
	R	5'-tatecttgatgagtgtg-3'		
<i>lab</i>	F	5'-acttcaccaacaacagctc-3'	AB120761	35
	R	5'-tgatccgtttcttctgcttc-3'		
<i>Scr</i>	F	5'-tctaccagttcgtcaactc-3'	D83533	35
	R	5'-atgacgtcctctgacgtttc-3'		
<i>Antp</i>	F	5'-atggatggctgcatcagca-3'	D16684	30
	R	5'-taaggtttactgtggcgaggt-3'		
<i>Ubx</i>	F	5'-atgaactcttacttcgagc-3'	AB505052	35
	R	5'-gttcaaagtgttttaagtgcg-3'		
<i>abd-A</i>	F	5'-tctacctcgtgtctagttc-3'	AB461860	37
	R	5'-tgctgctgcttcattctgtc-3'		
<i>Abd-B</i>	F	5'-caaccagcagtggtgaatac-3'	AB461858	35
	R	5'-ttgttggtctgggcagcttg-3'		
<i>cad</i>	F	5'-tcaatccgctcgccatgtac-3'	D16683	35
	R	5'-tgctgttcgagagaccaag-3'		
<i>en</i>	F	5'-atggccttcgaggaccgt-3'	M64335	35
	R	5'-ttacgtaacattgatctcctct-3'		
<i>in</i>	F	5'-gtgaagacgagatacagg-3'	M64336	35
	R	5'-ttaacatctattcttcagtct-3'		
<i>POU-M1</i>	F	5'-ggagcgggatcagcccgaggag-3'	AY334012	33
	R	5'-gtggcgtcctcctctctcttt-3'		
<i>Exd</i>	F	5'-atggacgatccgaacc-3'	AB841084	30
	R	5'-ttaacgtgagcgcagaatcatcac-3'		
<i>Hth</i>	F	5'-ttagaggtggtgtcc-3'	AB841083	35
	R	5'-agtaacgccagcatcg-3'		
<i>Rp49</i>	F	5'-catacaagatggctataagacc-3'	AY769302	20
	R	5'-gtgagctgctgggctctttc-3'		

*1 F: Forward primer, R: Reverse primer, ex: exon.

*2 The forward primer located in exon 1 and reverse primer in exon 3 were used for detection of *sericin-1* in all experiments except for Figure 7.

*3 For amplification of *sericin-1*, 20 and 25 cycles of PCR were performed with cDNA obtained from all parts of MSG and PSG at the fourth instar, respectively, and 30 cycles for cDNA from PSG at the fifth instar.