LIM-homeodomain transcription factor Awh is a key component activating all three fibroin genes, fibH, fibL and fhx, in the silk gland of the silkworm, Bombyx mori.
LIM-homeodomain transcription factor Awh is a key component activating all three fibroin genes, \textit{fibH}, \textit{fibL} and \textit{fhx}, in the silk gland of the silkworm, \textit{Bombyx mori}.

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Abstract

In the silkworm *Bombyx mori*, three fibroin genes, *fibroin-heavy-chain (fibH)*, *fibroin-light-chain (fibL)* and *fibrohexamerin (fhx)*, are coexpressed only in the posterior silk gland (PSG) cells, while the sericin genes encoding silk glue proteins are expressed in the middle silk gland (MSG) cells. Silk gland factor-2 (SGF-2) is a PSG-specific activator complex of *fibH*, composed of a LIM-homeodomain protein, Awh, and its cofactors, Ldb and Lcaf. We investigated whether SGF-2 can activate other fibroin genes using transgenic silkworms. The genes for Ldb and Lcaf were expressed ubiquitously in various tissues, while the gene for Awh was expressed strictly specific in PSG of the wild type silkworms. Misexpression of *Awh* in transgenic silkworms induced ectopic expression of *fibL* and *fhx* as well as *fibH* in MSG. Coincidently with the induction of *fibL* and *fhx* by Awh, binding of SGF-2 to the promoter of *fibL* and *fhx* was detected *in vitro*, and SGF-2 binds directly to the *fhx* core promoter. Ectopic expression of the fibroin genes was observed at high levels in the middle part of MSG. Moreover, *fibL* and *fhx* were induced in the anterior silk gland (ASG) of the transgenic silkworms, but *fibH* was not. These results indicate that Awh is a key activator of all three fibroin genes, and the activity is probably regulated in conjunction with additional factors.
Graphical abstract

POU-M1

ser1, (fibH)

Exd Hth

Antp

SGF-2

Ldb

Lcaf

Awh

ser1

fibH, fibL, fhx

larval silk gland

homeodomain proteins

silk genes
Highlights

- Awh encoding LIM-homeodomain protein, which constitutes SGF-2, is expressed specifically in the posterior silk gland (PSG).
- Genes for other components of SGF-2, Ldb and Lcaf, are expressed in most tissues.
- Misexpression of Awh induces expression of the fibH, fibL and fhx genes in the middle silk gland (MSG).
- SGF-2 can activate fhx through binding to its core promoter.
- Ectopic expression of the fibroin genes was detected only in the silk gland with sublocal specificity in Awh transgenic silkworms.

Abbreviations: ASG, anterior silk gland; Awh, Arrowhead; EMSA, electrophoretic mobility shift assay; FMBP-1, fibroin-modulator-binding protein-1; Lcaf, LIM-homeodomain and Ldb complex-associated factor; Ldb, LIM domain-binding protein; MSG, middle silk gland; PSG, posterior silk gland; SGF-2, silk gland factor-2; ser1, sericin-1
1. Introduction

The silkworm *B. mori* spins a small amount of silk threads during all larval instars except for each molting stage, and produces massive amount of silk proteins to make a cocoon at the end of the last instar. The silk fibroin synthesized by *B. mori* consists of a fibroin-heavy-chain (FibH), fibroin-light-chain (FibL) and fibrohexamerin (Fhx) proteins, with a 6:6:1 ratio (Inoue et al., 2000). FibH and FibL are linked by a disulfide bond and this linkage is essential for secretion of these proteins from silk gland cells. Fhx associates with the FibH-FibL complex through non-covalent interactions and is thought to play a role in maintaining the integrity of the fiber. The core silk fibers are coated by sericins, a family of glue proteins that bind the fibroin fibers together. These silk proteins are synthesized in the silk gland with strict regional specificity regulated at the transcriptional level. The sericin genes (*ser1*, 2 and 3) are expressed in the middle silk gland (MSG), which is further divided into three subparts along the anteroposterior axis based on expression patterns of the sericin genes (Couble et al., 1987; Takasu et al., 2010). Three fibroin genes (*fibH*, *fibL* and *fhx*) are expressed in a similar expression pattern specifically in the posterior silk gland (PSG), suggesting that their expression is coordinately regulated, though these genes are located on different chromosomes (Bello et al., 1994; Inoue et al., 2005).
The promoter of fibH has been analyzed in detail. Cell-free transcription assays using silk gland extracts revealed that a 200 base-pair upstream region of the promoter is essential for its transcriptional activity (Tsuda and Suzuki, 1983). Sequence -30 to +6 acts as the core promoter and the proximal region from -94 to -32 is occupied by SGF-1/BmFkh, which is proposed to play important roles in silk gland development and silk gland-specific expression of the silk genes (Tsuda and Suzuki, 1981; Takiya et al., 1990; Hui et al., 1990; Julien et al., 2002; Mach et al., 1995; Takiya et al., 1997). Recently, it was reported that Bmsage, a basic helix-loop-helix transcription factor expressed specifically in the silk gland, stimulates expression of fibH through interaction with Fkh, and is probably responsible for the massive production of the silk proteins at the last instar (Zhao et al. 2014). The distal part from -238 to -73 of fibH promoter contains a cluster of homeodomain-binding sites and is required for PSG-specific transcriptional enhancement (Suzuki et al., 1986; Hui et al., 1990). These homeodomain binding sites are bound by SGF-2, SGF-3/POU-M1 and FMBP-1, though FMBP-1 is not a homeodomain protein (Takiya et al., 1997, 2005; Hui et al., 1990; Fukuta et al., 1993).

The SGF-2 complex is detected specifically in PSG (Hui et al. 1990), and mainly consists of a LIM-homeodomain transcription factor Arrowhead (Awh), LIM domain-binding protein (Ldb) and Lcaf belonging to a sequence-specific single-stranded DNA-binding protein (SSDP) family (Ohno...
et al., 2013). Ldb proteins are known as cofactor for LIM-homeodomain proteins (Matthews and Visvader, 2003), and SSDP proteins are presumed to play a role in the stabilization of Ldb (Chen et al., 2002; Xu et al., 2007; Wang et al., 2010). Furthermore, Lcaf would mediate long-range enhancer-promoter communication on chromatin by its self-oligomerization activity (Ohno et al., 2013).

Enforced expression of Awh in transgenic silkworms induces ectopic expression of fibH in MSG, but, it has not been tested whether misexpression of Awh activates two other fibroin genes fibL and fhx, and whether is sufficient for activation of fibH in other tissues. In this study, we show that Awh is expressed in PSG specifically in wild type silkworms, while other components of SGF-2 are expressed ubiquitously in most tissues. Overexpression of Awh using transgenic silkworms resulted in ectopic expression of the fibH, fibL and fhx genes in the silk gland with subregional specificities. However, tissues other than the silk gland remained unaffected.
2. **Materials and Methods**

2.1. Animals

This study used w-c as a wild-type strain of *B. mori*. The transgenic strains used in this study are also maintained in w-c genetic background. Larvae were reared at 25°C under light–dark (LD) exposure of 16:8 hours on an artificial diet obtained from Nippon Nosan-Kogyo (Yokohama, Japan) and staged as described previously (Kiguchi and Agui, 1981; Suzuki *et al.*, 1990). Tissue extracts were prepared using the Kinshu x Showa strain purchased from Ueda Sanshu (Ueda, Japan).

2.2. Transgenic silkworms and heat-shock treatment

Awh transgenic silkworms were prepared as described in Ohno *et al.* (2013). The ORF was amplified by PCR, and 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. The established UAS-Awh strains were crossed with *hsp70*·GAL4 strain (Uchino *et al.*, 2006). Larvae were placed in 100-ml flasks plugged with cotton. Flasks were then submersed in a water bath and heated at 42°C for 2 hours (Uhlirova *et al.*, 2006).
2002; Kimoto et al., 2014). Following the heat shock, larvae were returned to food.

2.3. RNA extraction and RT-PCR

Total RNA was extracted using Illustra RNAspin MINI RNA Isolation kit (GE Healthcare), and cDNA was synthesized using 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 1% agarose gels.

2.4. Cloning of the Lcaf gene

For RT-PCR, the region from 61 to 669 of Lcaf (Genbank accession number: AB687556) was amplified with the primer sets indicated in Table S1. The sequence obtained differed partly from the previously reported Lcaf transcript (Ohno et al., 2013), and we registered the novel sequence in Genbank (AB910676). The sequence from 520 to 623 of AB687556 was replaced by the sequence from 460 to 515 of AB910676. This difference might be attributed to the distinct silkworm strains, Kinshu x Showa and w-c.
2.5. Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as described previously (Takiya et al. 1997, 2005; Ohno et al. 2013) with several modifications. Tissue extracts were prepared from entire MSG on day 2 of the fifth instar and PSGs on the fourth molting stage or day 2 of the fifth instar. Double-stranded oligonucleotides (50 nucleotide long) listed in Table S2 were labeled with Klenow polymerase and used as probes. The probes were incubated in each tissue extract (10 µg protein) on ice for 20 min., and analyzed by electrophoresis on 5% polyacrylamide gels in 50 mM Tris borate (pH8.3) and 1 mM EDTA buffer at 4°C. Glycerol was omitted from the gels. Anti-Awh antiserum was prepared by the immunization of mice with the C-terminal region (208 to 274) of recombinant Awh protein.

2.6. Whole mount in situ hybridization

Digoxigenin (DIG)-labeled RNA probes for fibH (nucleotide sequence 62425-62479 and 63451-63830, AF226688) were synthesized using a DIG RNA Labeling kit (Roche). Silk glands were isolated from wild-type (+/+) or heat-treated transgenic silkworms (hs-Gal4/UAS-Awh of day 2 fifth instars) and in situ hybridization was performed as described previously (Kimoto et al., 2014). Coloring reactions were performed using Nitroblue
tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) tablet (Roche). Tissues were then cleared with a graded series of ethanol.
3. Results

3.1. Genes for Ldb and Lcaf are expressed ubiquitously, but Awh is expressed specifically in PSG

Ohno et al. (2013) showed PSG-specific expression of Awh in the silk gland, but the expression in other tissues has not been examined. To explore gene expression patterns of the components of SGF-2 in B. mori, we performed RT-PCR with total RNA from each part of the silk gland or other tissues of day 2 fifth instar larvae. Of these genes, Ldb and Lcaf were expressed in all tissues examined, in addition to their expression throughout the silk gland, whereas Awh was expressed strictly in PSG alone (Fig. 1). PSG-specific expression of all three fibroin genes, fibH, fibL and fhx, were confirmed as reported previously (Bello et al., 1994).

3.2. Awh induces ectopic expression of the fibroin genes in MSG

The results that Ldb and Lcaf were expressed in most tissues and PSG-specific expression of Awh suggest that the localization of Awh restricts the region where a functional SGF-2 complex is formed, leading PSG-specific expression of fibH. We showed previously that misexpression of Awh induces ectopic expression of fibH in MSG at the fourth instar using transgenic
silkworms that allow heat-inducible expression of Awh. In this study, the transgenic silkworms were treated at 42°C for two hours on day 1 of the fifth instar, and gene expression was analyzed 1 day after the heat treatment. Expression of fibH was observed in MSG at the fifth instar as same as the fourth instar (Fig. 2A). We further investigated time course of the induction of Awh and fibH after heat treatment. Gal4 was expressed both immediately and transiently after heat treatment, followed by Awh induction in MSG within 6 hours (Fig. 2B). The ectopic expression of fibH was then observed after another 6 hours and lasted at least 2 days. Induction of two other PSG-specific fibroin genes, fibL and fhx, was observed in MSG with almost the same time course in the transgenic silkworms (Fig. 2B). The data suggests that Awh acts as a common activator of these fibroin genes.

3.3 SGF-2 binds to the promoter of fibL and fhx

To investigate whether SGF-2 can bind to the promoter region of fibL and fhx, we first compared nucleotide sequence of fibH, fibL and fhx promoters. Although paired binding elements existing in the E and C sites of fibH promoter was not observed around the promoter of fibL and fhx, half elements like the -1620 region in the fibH upstream enhancer were found at the -230 and -50 regions of fibL promoter as well as the +100 region of fhx intron (Fig. 3).
Binding of SGF-2 to these elements was examined in EMSAs (Fig. 4A, B, C). A specific band was detected with the fibH-200 probe corresponding to the E site using PSG extracts on day 2 of the fifth instar, but not on PSG extracts of the fourth molting stage or MSG extracts on day 2 of the fifth instar (Fig 4A). The band was supershifted by the addition of anti-Awh antiserum into EMSA reactions, and was competed with fibL-50 and fhx+100 oligonucleotides as well as fibH-200 and fibH-120 oligonucleotides, suggesting that the fibL-50 and fhx+100 regions contain SGF-2-binding elements. Using these oligonucleotides as probes, EMSAs were performed, and SGF-2-like complexes were detected, though the background signals were often high under the conditions used here, and mobility of the band with the fibL-50 probe was delayed (Fig. 4B).

3.4 The fhx core promoter is a binding site for SGF-2

Horard et al. (1997) showed that PSGF site (-45 to -39) is a key element for PSG-specific expression of fhx, and combination of SGFB site (-74 to -56) with the PSGF site is sufficient for the PSG-specific expression. The SGFB site contains binding elements for Fkh and FMBP-1 (Fig. 3), but the sequences of the SGFB and PSGF sites do not coincide with the typical sequence (CAATTA) for SGF-2 binding. However, we found that the nucleotide sequence of the PSGF site and TATA box (TATAATTA) of fhx
(designated fhx-30 region) is similar to the paired SGF-2-binding element in the E site of fibH (Fig. 4D), and investigated whether SGF-2 can bind to the fhx-30 region. As shown in Fig. 4A, fhx-30 oligonucleotide competed the binding of SGF-2 to the fibH-200 probe, and gave shifted band as a probe in EMSA at similar position with the fibL-50 probe (Fig. 4B and C). On the other hand, fhx-30M oligonucleotide containing the canonical TATA box sequence (TATAAAAA) did not compete the SGF-2 binding as a competitor nor did it give a shifted band as a probe. The band with the fhx-30 probe was competed with fibH-200 and fibL-50 oligonucleotides as well as the fhx-30 oligonucleotides itself but not with the fhx-30M oligonucleotide (Fig. 4C). Furthermore, the band was supershifted with anti-Awh antiserum, but not with anti-POU-M1 antiserum as a control. These results indicate that the fhx-30 region is a target site of SGF-2 binding.

3.5 Subregional localization of ectopic expression of the fibroin genes in the silk gland

MSG is further divided into three distinct parts both anatomically and physiologically, referred to as the anterior, middle and posterior parts of MSG (MSG-A, MSG-M and MSG-P). We analyzed expression levels of fibH induced by the misexpression of Awh in each region of MSG. With RT-PCR, expression of fibH was detected clearly in MSG-M, but the levels in MSG-A...
and MSG-P were extremely low in spite of the expression of Awh in all parts of MSG (Fig. 5A). To confirm the expression pattern of fibH in the silk gland of Awh transgenic silkworms, whole-mount in situ hybridization of fibH was performed using silk glands of heat-treated fifth instar larvae. In addition to the normal expression of fibH in PSG, ectopic accumulation of fibH mRNA was detected in MSG-M, as in the RT-PCR findings (Fig. 5B). Similarly, expression levels of the fibL and fhx genes were higher in MSG-M than in MSG-A and MSG-P of the transgenic silkworms.

We further investigated whether ectopic expression of the fibroin genes is induced in the anterior silk gland (ASG) or other tissues. Although Awh overexpression occurred throughout the body, expression of the fibroin genes was restricted to the silk gland (Fig. 5A). In ASG, where no silk genes are expressed in the wild-type silkworms, fibL and fhx were induced by the Awh misexpression, whereas the expression of fibH was not detected.
4. Discussion

SGF-2 was identified as a complex that binds to the E (-214/-180) and C (-133/-96) sites in the distal upstream element of fibH promoter and is detected specifically in PSG extracts (Hui et al., 1990). Ohno et al. (2013) revealed that SGF-2 contains Awh, Ldb, and Lcaf proteins, and that misexpression of Awh induces ectopic expression of fibH in MSG. Since Awh is expressed only in PSG in the wild type silkworms, in contrast to the ubiquitous expression of Ldb and Lcaf, Awh could be a key factor in the PSG-specific expression of fibH.

Two other fibroin genes, fibL and fhx, were also induced in MSG by the misexpression of Awh, indicating that all three fibroin genes share the same transcriptional regulation mechanism for their PSG-specific expression (Figs. 2 and 5). SGF-2-binding sites were found in the immediate 5' flanking region or intron near the promoter of fibL and fhx (Fig. 3). These SGF-2-binding sites contain a half element (CAATTA) of the E or C site in fibH promoter, and SGF-2 can bind these half elements (Fig. 3). A key element around at -1620 in the far upstream enhancer of fibH also contains the half element of the E and C sites (Shimizu et al., 2007).

Promoter activity of the fibroin genes was analyzed using transgenic Drosophila carrying the promoter of fibH (-1500/+66) or fhx (-1451/+76) fused to the beta-galactosidase gene, with expression of both transgenes being
observed in the salivary gland (Bello et al., 1994; Nony et al., 1995). It is notable that both promoters of *fibH* and *fhx* were activated in a restricted territory of the salivary gland, located between the glue-protein-secreting cells and the imarginal cells, which resemble the expression domain of the *Drosophila Awh* gene (Curtiss and Heiling, 1995, 1997). These observations support our results that the transcription of *fibH* and *fhx* is under the control of Awh.

Horard et al. (1997) showed that the combination of the SGFB site and PSGF site is sufficient to drive PSG-specific expression of *fhx* introduced into the silk gland with the use of a particle gun. The SGFB site contains binding-elements for Fkh and FMBP-1 (Fig. 3), and the PSGF site has been demonstrated to be a part of SGF-2-binding element in this study. The fibL-50 probe used covers TATA box of *fibL*. This may be a reason as to why the fib-50L probe gave a retarded band that was similar with the fhx-30 probe in EMSA. Moreover, the TATA box (TATATAATA) of *fibL* contains suitable binding-sequences for Fkh and FMBP-1 (Fig. 3). The core promoters themselves of *fibL* and *fhx* might regulate their tissue-specific expression.

The ectopic expression of *fibH* was restricted only to MSG cells, particularly in the middle part, suggesting existence of additional regulators that reduce the occurrence of inappropriate expression of *fibH* in other tissues (Fig. 5). Fkh is known to serve in the regulation of *fibH* and *fhx* as well as *ser1* (Mach et al. 1995; Takiya et al. 1997; Julien et al. 2002), and a
silk gland-specific factor, sage, activates \textit{fibH} expression through interaction with Fkh (Zhao \textit{et al.} 2014). Another factor capable of restricting \textit{fibH} gene expression is a POU-homeodomain protein POU-M1 expressed highly in ASG and MSG-A (Kokubo \textit{et al.} 1997; Matsunami \textit{et al.} 1998; Kimoto \textit{et al.} 2012). Our previous analysis showed that POU-M1 represses \textit{ser1} expression through competition with the binding sites of an activator complex MIC for \textit{ser1} (Kimoto \textit{et al.}, 2012). Since a typical and high-affinity POU-M1-binding sequence exists within the C site of \textit{fibH} promoter (Fig. 6, Hui \textit{et al.}, 1990), it is possible that POU-M1 competes the binding of SGF-2 to the C site and regulates \textit{fibH} negatively. On the other hand, no typical POU-M1-binding sequence is found near the SGF-2-binding sites around promoter of \textit{fibL} and \textit{fhx}, which were expressed ectopically in ASG by the misexpression of \textit{Awh} (Figs. 3 and 5).

Expression levels of the fibroin genes were generally low in MSG-P under the conditions of \textit{Awh} overexpression. The homeobox gene \textit{inected} (\textit{in}), a homolog of \textit{engrailed} (\textit{en}) (Simmonds \textit{et al.}, 1995; Gustavson \textit{et al.}, 1996), was expressed highly in the MSG-P (Kimoto \textit{et al.}, 2014). The engrailed-family proteins are known to recruit the co-repressor groucho and repress target genes through the use of chromatin modifications (Tolkunova \textit{et al.}, 1998; Peel \textit{et al.}, 2006). These homeodomain proteins might be involved in regulation of the fibroin genes. Inhibition of mRNA accumulation of the fibroin genes by a territory-specific digestion in MSG-P cells or
region-specific translational regulation of Awh mRNA is also possible in the
region-specific induction of the fibroin genes in the transgenic silkworms,
though such a mechanism is not yet known.

We have demonstrated that LIM-homeodomain protein Awh activates all
fibroin genes in the silk gland. Expression of ser1 is activated by the Hox
protein Antp, and repressed by the POU-homeodomain protein POU-M1,
(Kimoto et al., 2012, 2014; Takiya et al., 2011). Thus,
homeodomain-containing transcription factors appear to play roles in
region-specific expressions of the silk genes in the larval silk gland in
conjunction with other non-homeodomain transcription factors expressed in
the silk gland such as Fkh, sage and FMBP-1.
Acknowledgments

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Figure legends

Fig. 1. Gene expression of the components of SGF-2 and three fibroin proteins in the silk gland and various tissues. RT-PCR was performed with total RNA from each part of the silk gland and other tissues of wild-type day 2 fifth instars. Awh, Ldb and Lcaf are SGF-2 components. The ribosomal protein 49 (Rp49) gene was used as a control. MSG-A, -M and -P are the anterior, middle and posterior parts of the middle silk gland (MSG), respectively. CNS: central nervous system.

Fig. 2. Misexpression of Awh induces ectopic expression of fibH in MSG. (A) RT-PCR was performed with total RNA from the MSG or PSG of non-heated wild-type strains (+/+) on day 2 of the fifth instar or +/hs-Gal4 strain carrying hs-Gal4 transgene alone or two hs-Gal4/UAS-Awh strains (Awh TG1, 2) 24 hr after heat treatment on day 1 of the fifth instar. (B) Time course of the gene expression in MSG of transgenic silkworms after heat treatment. RT-PCR was performed with total RNA from MSG of Awh transgenic silkworms (hs-Gal4/UAS-Awh) after heat treatment on day 1 of the fifth instar. The dissection time after heat treatment is shown at the top of the panels. Expression of fhx was detected at 12 hours after heat treatment by RT-PCR with 24 cycles in this figure, but further data for fhx used in this paper was obtained through RT-PCR with 21 cycles.
Fig. 3. Binding sites of transcription factors on the promoter of fibH, fibL and fhx. Nucleotide sequence around the promoter including the first intron of fibH, fibL and fhx is shown (Tsujimoto and Suzuki, 1979; Kikuchi et al., 1992; Couble et al., 1985). Number +1 represents the transcriptional start site. TATA box is marked with a box. Single underlines indicate protein coding regions. Double underlines indicate LIM-homeodomain recognition sequences (fibH) and putative LIM-homeodomain binding sequences (fibL and fhx). Broken lines indicate FMBP-1-binding elements, and arrows indicate Fkh-binding elements. Wavy underline indicates a typical and high-affinity POU-M1 binding sequence. Brackets indicate the E and C sites of fibH promoter.

Fig. 4. Binding of SGF-2 to the promoter of fibL and fhx. (A) EMSA was carried out with fibH-200 probe and extracts from entire MSG on day 2 of the fifth instar (V2 MSG), PSG on the fourth molting stage (IVm PSG) or PSG on day 2 of the fifth instar (V2 PSG). For competition assays, 100-fold excess of unlabeled oligonucleotides indicated at the top of panel was added into EMSA reactions. For supershift assay, anti-Awh antiserum (+aAwh) or anti-POU-M1 antiserum (+aPOU) was added. (B) Each double stranded oligonucleotide probe was used for EMSA using IVm PSG extract or V2 PSG extract. Filled circles indicate a shifted band specific to V2 PSG extract. (C)
SGF-2 complex was detected with fhx-30 probe in EMSA. (D) Nucleotide sequences around SGF-2-binding element of fhx-30, fhx-30M and fibH-200 are compared. The TATA box of fhx-30 region is changed to the canonical TATA box sequence in fhx-30M oligonucleotide. xx indicates mutations from the typical SGF-2-binding element.

Fig. 5. Effects of ectopic expression of Awh in each part of the silk gland and various tissues. (A) Expressions of Awh and fibroin genes were analyzed by RT-PCR. Tissues were obtained from Awh transgenic silkworms (hs·Gal4/UAS·Antp) 24 hr after heat treatment of day 1 fifth instar. (B) Localization of fibH mRNA in the silk gland was analyzed using in situ hybridization. Silk glands were collected from day 2 fifth instar larvae of wild type (+/+) or a heat-treated Awh transgenic strain (hs·Gal4/UAS·Antp). Arrows point to the boundary between fibH-positive and -negative cells. Bar: 2 mm.
Fig. 2

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B

hs-Gal4/UAS-Awh (MSG)

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**Fig. 3**

**fibH**
-240 AAAATTCTTTAAAAATATTAAAAGTAAGAACAATAAGATCAATTAATCATATAATACAC  
-180 ATGTGTCATGATCCAATTTAATTACTCTCATACGTGGTATTGTTATTGAAAATAAAAAAG  
-120 ATTATTTCTATGTAATTCTGTACAAACAATGAATGTAGATTATATTCTATCGAAA  
-60 GTAATACGTCAAAACTCGAAAAATTTCCAGTTATATAAAAGGTTCAACTTTTTCAATCAGC  
+1 ATCAGTTCCGTTCCAACCTCAGTGAAGGTCAAAAACCTTTGTGATCTTTGTGCTTGCGCT

**fibL**
-240 GGAATATTAGATCCTTTTCTTTTGCACACACACTTTAGTTAACTAGAGGCCTACACCTCA  
-180 AGAAATCATTCCTTCATTGAACAAACTAAAACCTAAAAATCGCAATAATATAAACGATAAGTT  
-120 TAACGTAAATGCAAAGGTCTTTTGAAGCTTAGTGCTGTCAGCGGTTCGTTGGTACAGTGTG  
-60 TTGATATTATTATTTTATACGTCTTTTATATAAATAGTGGAACATTTAATCAGGAATCC  
+1 TGTATAGTATATACCGATTTGGTCACATAACAGACCACTAAAATGAAGCCTATATTTTTGG

**fnx**
-120 CGCGCGCGCAACAAATAAAGAACATTTATTCGTGCAATTTTTCACGCAGCTATTATTATTAC  
-60 GTTATTCTGTTGAGGAAAATACTTTTTATAATTATTTGATCAGGCTTAACCAGACGC  
+1 AGTTGGTTTATTATTCGGCAACATGCTGCCGGTGCTCTACTGTAAGCCGCTGCTTGCAGT  
+61 TTTGGCTTTCTGACAGTAAAGTGCGATGTTTCTTGATTAGTGCTAATATAACTACACGTAAAC  
+121 GCTTTTGATTAATCGGGAAGACTTTTGATATTATATTATTTCCGCAACAGGGGGTATATAT
Fig. 4

A

B

C

D

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<tr>
<th>Probe</th>
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<td>V2</td>
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V2 PSG

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<td>* d* A* W* U*</td>
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SGF-2

D

fhx-30  GGAACAATTATTTGTATAATTAATGTTG

fhx-30M  GGAACAATTATTTGTATAAAAATGTTG

fibH-200 AGATCAAATTAATCATAATTAATCACA
**A**

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<th>MSG-P</th>
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<th>ASG</th>
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<th>CNS</th>
<th>salivary gland</th>
<th>malpighian tubule</th>
<th>trachea</th>
<th>midgut</th>
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**B**

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<th></th>
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<tr>
<td>Image 2</td>
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Scale bar: 1 mm
Table S1. Primers and PCR cycles for each gene.

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<th>Primers</th>
<th>Accession no.</th>
<th>Cycles</th>
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<td>R 5'-TATCCCTGATGAGTGCTG-3'</td>
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<td>fibL</td>
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<td>R 5'-TTCAGCTGAACCTGGCTG-3'</td>
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<td>fhx</td>
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<td>R 5'-TCAGTGTGGCAACCCAAGTC-3'</td>
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<td>R 5'-TGTTGATAGCGCCCTCCAGTC-3'</td>
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*1 F: Forward primer, R: Reverse primer

*2 Expression of fhx was analyzed by RT-PCR with 24 cycles in the Fig. 3, but other data for fhx in this paper were obtained by RT-PCR with 21 cycles.
Table S2. Probes and competitors used in EMSA.

<table>
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<th>Probe</th>
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<td>fibH-200</td>
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<td>B 5’-aattGTGATCTGAACATGTATATTATGATTTAATTGATC-3’</td>
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<td>fibH-120</td>
<td>5’-aattATGGTTAAAATAGTTATTTCTATAGTTATCTGACAT-3’</td>
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<td>B 5’-aattATTGACAGACATCATGAAATTATCTAGATTTATGACAT-3’</td>
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<tr>
<td>fibL-50</td>
<td>5’-aattGGGATACAGTTGATATTATTTATTATGTCTTTATATATAAA-3’</td>
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<tr>
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<td>B 5’-aattTTTATATATAAAAAGACAATTAAAAATATACAAACATGTACC-3’</td>
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<tr>
<td>fhx-30</td>
<td>5’-aattCGGATGTGAGGGAACATGTTGATAATATGTTGATCGTCTAT-3’</td>
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<td>B 5’-aattAGGCACTGATCAACATTAATATGACCATTGTTCCATCAACAG-3’</td>
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<tr>
<td>fhx-30M</td>
<td>5’-aattCGGATGTGAGGGAACATGTTGATAATATGTTGATCGTCTAT-3’</td>
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<td>B 5’-aattAGGCACTGATCAACATTTTATACAAATGATTGTTCCATCAACAG-3’</td>
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<tr>
<td>fhx+100</td>
<td>5’-aattGATGTTTTTATTAGGTGTCATAACTACGTTACGTTTGAAC-3’</td>
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<tr>
<td></td>
<td>B 5’-aattCAGAAACCGCTTACGTTTATGTTGACCTAAATCAAGAAACATC-3’</td>
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</tbody>
</table>

*1 T: Top strand, B: Bottom strand

*2 The lower case letters show extra sequences for labeling with Klenow polymerase and [α-32P]dATP.