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*

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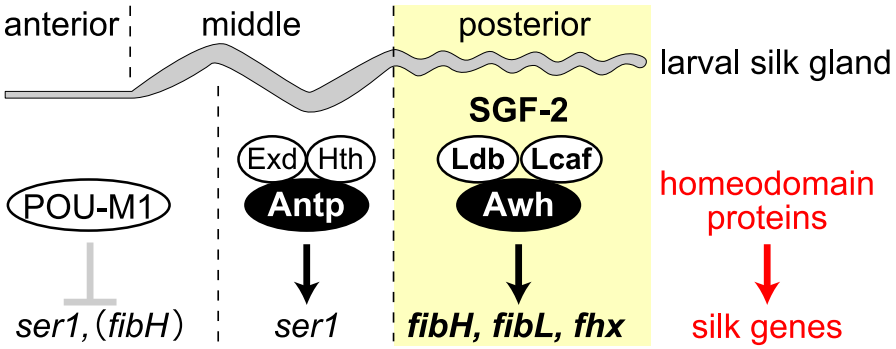
Abstract

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



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).

89 The promoter of *fibH* has been analyzed in detail. Cell-free transcription
90 assays using silk gland extracts revealed that a 200 base-pair upstream
91 region of the promoter is essential for its transcriptional activity (Tsuda and
92 Suzuki, 1983). Sequence -30 to +6 acts as the core promoter and the proximal
93 region from -94 to -32 is occupied by SGF-1/BmFkh, which is proposed to
94 play important roles in silk gland development and silk gland-specific
95 expression of the silk genes (Tsuda and Suzuki, 1981; Takiya *et al.,* 1990;
96 Hui *et al.,* 1990; Julien *et al.,* 2002; Mach *et al.,* 1995; Takiya *et al.,* 1997).
97 Recently, it was reported that Bmsage, a basic helix-loop-helix transcription
98 factor expressed specifically in the silk gland, stimulates expression of *fibH*
99 through interaction with Fkh, and is probably responsible for the massive
100 production of the silk proteins at the last instar (Zhao et al. 2014). The distal
101 part from -238 to -73 of *fibH* promoter contains a cluster of
102 homeodomain-binding sites and is required for PSG-specific transcriptional
103 enhancement (Suzuki *et al.,* 1986; Hui *et al.,* 1990). These homeodomain
104 binding sites are bound by SGF-2, SGF-3/POU-M1 and FMBP-1, though
105 FMBP-1 is not a homeodomain protein (Takiya et al., 1997, 2005; Hui *et al.,*
106 1990; Fukuta *et al.,* 1993).
107 The SGF-2 complex is detected specifically in PSG (Hui *et al.* 1990), and
108 mainly consists of a LIM-homeodomain transcription factor Arrowhead
109 (Awh), LIM domain-binding protein (Ldb) and Lcaf belonging to a
110 sequence-specific single-stranded DNA-binding protein (SSDP) family (Ohno

111 *et al.,* 2013). Ldb proteins are known as cofactor for LIM-homeodomain
112 proteins (Matthews and Visvader, 2003), and SSDP proteins are presumed to
113 play a role in the stabilization of Ldb (Chen *et al.*, 2002; Xu *et al.,* 2007;
114 Wang *et al.,* 2010). Furthermore, Lcaf would mediate long-range
115 enhancer-promoter communication on chromatin by its self-oligomerization
116 activity (Ohno *et al.*, 2013).

117 Enforced expression of *Awh* in transgenic silkworms induces ectopic
118 expression of *fibH* in MSG, but, it has not been tested whether misexpression
119 of *Awh* activates two other fibroin genes *fibL* and *fhx*, and whether is
120 sufficient for activation of *fibH* in other tissues. In this study, we show that
121 *Awh* is expressed in PSG specifically in wild type silkworms, while other
122 components of SGF-2 are expressed ubiquitously in most tissues.
123 Overexpression of *Awh* using transgenic silkworms resulted in ectopic
124 expression of the *fibH*, *fibL* and *fhx* genes in the silk gland with subregional
125 specificities. However, tissues other than the silk gland remained unaffected.
126

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 *Bln*I 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.*,

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

192 tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) tablet
193 (Roche). Tissues were then cleared with a graded series of ethanol.
194

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

217 silkworms that allow heat-inducible expression of *Awh.* In this study, the
218 transgenic silkworms were treated at 42°C for two hours on day 1 of the fifth
219 instar, and gene expression was analyzed 1 day after the heat treatment.
220 Expression of *fibH* was observed in MSG at the fifth instar as same as the
221 fourth instar (Fig. 2A). We further investigated time course of the induction
222 of *Awh* and *fibH* after heat treatment. *Gal4* was expressed both immediately
223 and transiently after heat treatment, followed by *Awh* induction in MSG
224 within 6 hours (Fig. 2B). The ectopic expression of *fibH* was then observed
225 after another 6 hours and lasted at least 2 days. Induction of two other
226 PSG-specific fibroin genes, *fibL* and *fhx,* was observed in MSG with almost
227 the same time course in the transgenic silkworms (Fig. 2B). The data
228 suggests that Awh acts as a common activator of these fibroin genes.
229
230 3.3 SGF-2 binds to the promoter of *fibL* and *fhx*
231
232 To investigate whether SGF-2 can bind to the promoter region of *fibL* and
233 *fhx*, we first compared nucleotide sequence of *fibH*, *fibL* and *fhx* promoters.
234 Although paired binding elements existing in the E and C sites of *fibH*
235 promoter was not observed around the promoter of *fibL* and *fhx*, half
236 elements like the -1620 region in the *fibH* upstream enhancer were found at
237 the -230 and -50 regions of *fibL* promoter as well as the +100 region of *fhx*
238 intron (Fig. 3).

239 Binding of SGF-2 to these elements was examined in EMSAs (Fig. 4A, B,
240 C). A specific band was detected with the fibH-200 probe corresponding to
241 the E site using PSG extracts on day 2 of the fifth instar, but not on PSG
242 extracts of the fourth molting stage or MSG extracts on day 2 of the fifth
243 instar (Fig 4A). The band was supershifted by the addition of anti-Awh
244 antiserum into EMSA reactions, and was competed with fibL-50 and fhx+100
245 oligonucleotides as well as fibH-200 and fibH-120 oligonucleotides,
246 suggesting that the fibL-50 and fhx+100 regions contain SGF-2-binding
247 elements. Using these oligonucleotides as probes, EMSAs were performed,
248 and SGF-2-like complexes were detected, though the background signals
249 were often high under the conditions used here, and mobility of the band
250 with the fibL-50 probe was delayed (Fig. 4B).
251
252 3.4 The *fhx* core promoter is a binding site for SGF-2
253
254 Horard *et al.* (1997) showed that PSGF site (-45 to -39) is a key element
255 for PSG-specific expression of *fhx*, and combination of SGFB site (-74 to -56)
256 with the PSGF site is sufficient for the PSG-specific expression. The SGFB
257 site contains binding elements for Fkh and FMBP-1 (Fig. 3), but the
258 sequences of the SGFB and PSGF sites do not coincide with the typical
259 sequence (CAATTA) for SGF-2 binding. However, we found that the
260 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 oligonucleotide 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

283 and MSG-P were extremely low in spite of the expression of *Awh* in all parts
284 of MSG (Fig. 5A). To confirm the expression pattern of *fibH* in the silk gland
285 of *Awh* transgenic silkworms, whole-mount *in situ* hybridization of *fibH* was
286 performed using silk glands of heat-treated fifth instar larvae. In addition to
287 the normal expression of *fibH* in PSG, ectopic accumulation of *fibH* mRNA
288 was detected in MSG-M, as in the RT-PCR findings (Fig. 5B). Similarly,
289 expression levels of the *fibL* and *fhx* genes were higher in MSG-M than in
290 MSG-A and MSG-P of the transgenic silkworms.

291 We further investigated whether ectopic expression of the fibroin genes is
292 induced in the anterior silk gland (ASG) or other tissues. Although *Awh*
293 overexpression occurred throughout the body, expression of the fibroin genes
294 was restricted to the silk gland (Fig. 5A). In ASG, where no silk genes are
295 expressed in the wild-type silkworms, *fibL* and *fhx* were induced by the *Awh*
296 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 (TATATAAATA) 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

341 silk gland-specific factor, sage, activates *fibH* expression through interaction
342 with Fkh (Zhao *et al.* 2014). Another factor capable of restricting *fibH* gene
343 expression is a POU-homeodomain protein POU-M1 expressed highly in
344 ASG and MSG-A (Kokubo *et al.* 1997; Matsunami *et al.* 1998; Kimoto *et al.*
345 2012). Our previous analysis showed that POU-M1 represses *ser1* expression
346 through competition with the binding sites of an activator complex MIC for
347 *ser1* (Kimoto *et al.,* 2012). Since a typical and high-affinity POU-M1-binding
348 sequence exists within the C site of *fibH* promoter (Fig. 6, Hui *et al.,* 1990), it
349 is possible that POU-M1 competes the binding of SGF-2 to the C site and
350 regulates *fibH* negatively. On the other hand, no typical POU-M1-binding
351 sequence is found near the SGF-2-binding sites around promoter of *fibL* and
352 *fhx*, which were expressed ectopically in ASG by the misexpression of *Awh*
353 (Figs. 3 and 5).

354 Expression levels of the fibroin genes were generally low in MSG-P under
355 the conditions of *Awh* overexpression. The homeobox gene *invected* (*in*), a
356 homolog of *engrailed* (*en*) (Simmonds *et al.,* 1995; Gustavson *et al.,* 1996),
357 was expressed highly in the MSG-P (Kimoto *et al.,* 2014). The
358 engrailed-family proteins are known to recruit the co-repressor groucho and
359 repress target genes through the use of chromatin modifications (Tolkunova
360 *et al.,* 1998; Peel *et al.,* 2006). These homeodomain proteins might be
361 involved in regulation of the fibroin genes. Inhibition of mRNA accumulation
362 of the fibroin genes by a territory-specific digestion in MSG-P cells or

363 region-specific translational regulation of Awh mRNA is also possible in the
364 region-specific induction of the fibroin genes in the transgenic silkworms,
365 though such a mechanism is not yet known.

366 We have demonstrated that LIM-homeodomain protein Awh activates all
367 fibroin genes in the silk gland. Expression of *ser1* is activated by the Hox
368 protein Antp, and repressed by the POU-homeodomain protein POU-M1,
369 (Kimoto *et al.,* 2012, 2014; Takiya *et al.*, 2011). Thus,
370 homeodomain-containing transcription factors appear to play roles in
371 region-specific expressions of the silk genes in the larval silk gland in
372 conjunction with other non-homeodomain transcription factors expressed in
373 the silk gland such as Fkh, sage and FMBP-1.
374

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574 **Figure legends**

575

576 **Fig. 1.** Gene expression of the components of SGF-2 and three fibroin
577 proteins in the silk gland and various tissues. RT-PCR was performed with
578 total RNA from each part of the silk gland and other tissues of wild-type day
579 2 fifth instars. Awh, Ldb and Lcaf are SGF-2 components. The ribosomal
580 protein 49 (Rp49) gene was used as a control. MSG-A, -M and -P are the
581 anterior, middle and posterior parts of the middle silk gland (MSG),
582 respectively. CNS: central nervous system.

583

584 **Fig. 2.** Misexpression of *Awh* induces ectopic expression of *fibH* in MSG. (A)
585 RT-PCR was performed with total RNA from the MSG or PSG of non-heated
586 wild-type strains (+/+) on day 2 of the fifth instar or +/hs-Gal4 strain
587 carrying hs-Gal4 transgene alone or two hs-Gal4/UAS-Awh strains (Awh
588 TG1, 2) 24 hr after heat treatment on day 1 of the fifth instar. (B) Time
589 course of the gene expression in MSG of transgenic silkworms after heat
590 treatment. RT-PCR was performed with total RNA from MSG of *Awh*
591 transgenic silkworms (hs-Gal4/UAS-Awh) after heat treatment on day 1 of
592 the fifth instar. The dissection time after heat treatment is shown at the top
593 of the panels. Expression of *fhx* was detected at 12 hours after heat
594 treatment by RT-PCR with 24 cycles in this figure, but further data for *fhx*
595 used in this paper was obtained through RT-PCR with 21 cycles.

596

597 **Fig. 3.** Binding sites of transcription factors on the promoter of *fibH, fibL* and
598 *fhx.* Nucleotide sequence around the promoter including the first intron of
599 *fibH*, *fibL* and *fhx* is shown (Tsujimoto and Suzuki, 1979; Kikuchi *et al.,*
600 1992; Couble *et al,.* 1985). Number +1 represents the transcriptional start
601 site. TATA box is marked with a box. Single underlines indicate protein
602 coding regions. Double underlines indicate LIM-homeodomain recognition
603 sequences (*fibH*) and putative LIM-homeodomain binding sequences (*fibL*
604 and *fhx*). Broken lines indicate FMBP-1-binding elements, and arrows
605 indicate Fkh-binding elements. Wavy underline indicates a typical and
606 high-affinity POU-M1 binding sequence. Brackets indicate the E and C sites
607 of *fibH* promoter.

608

609 **Fig. 4.** Binding of SGF-2 to the promoter of *fibL* and *fhx*. (A) EMSA was
610 carried out with fibH-200 probe and extracts from entire MSG on day 2 of the
611 fifth instar (V2 MSG), PSG on the fourth molting stage (IVm PSG) or PSG on
612 day 2 of the fifth instar (V2 PSG). For competition assays, 100-fold excess of
613 unlabeled oligonucleotides indicated at the top of panel was added into
614 EMSA reactions. For supershift assay, anti-Awh antiserum (+αAwh) or
615 anti-POU-M1 antiserum (+αPOU) was added. (B) Each double stranded
616 oligonucleotide probe was used for EMSA using IVm PSG extract or V2 PSG
617 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. 1

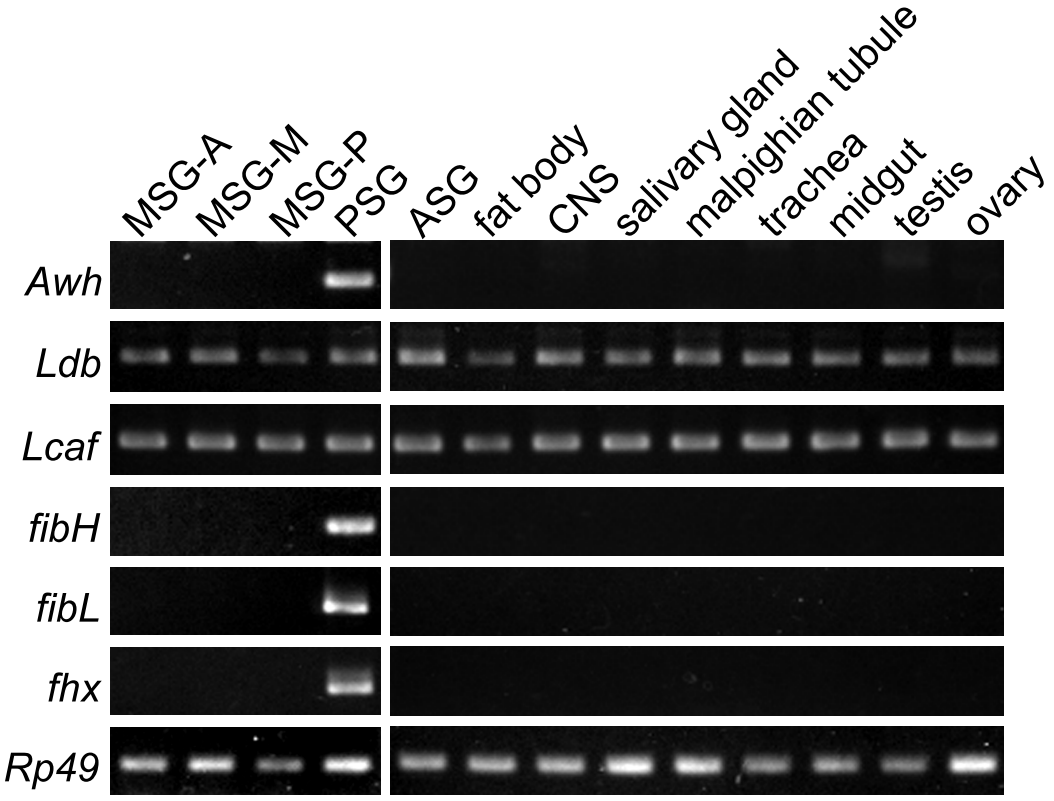
MSG-A
MSG-M
MSG-P
PSG
ASG
fat body
CNS
salivary gland
malpighian tubule
trachea
midgut
testis
ovary
Awh
Ldb
Lcaf
fibH
fibL
fhx
Rp49

Fig. 2

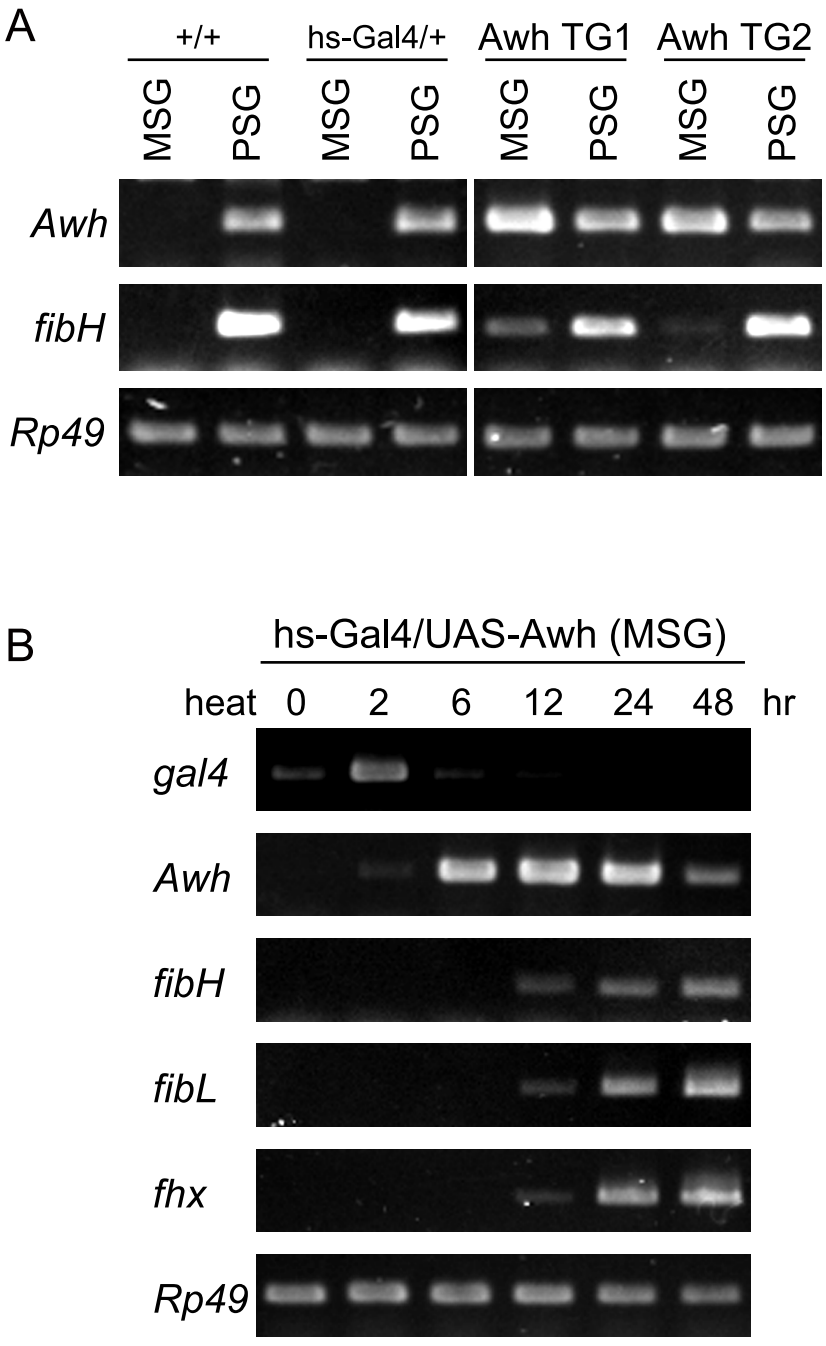
A
+/+
hs-Gal4/+
Awh TG1
Awh TG2
MSG
PSG
MSG
PSG
MSG
PSG
MSG
PSG
Awh
fibH
Rp49
B
hs-Gal4/UAS-Awh (MSG)
heat 0 2 6 12 24 48 hr
gal4
Awh
fibH
fibL
fhx
Rp49

Fig. 3

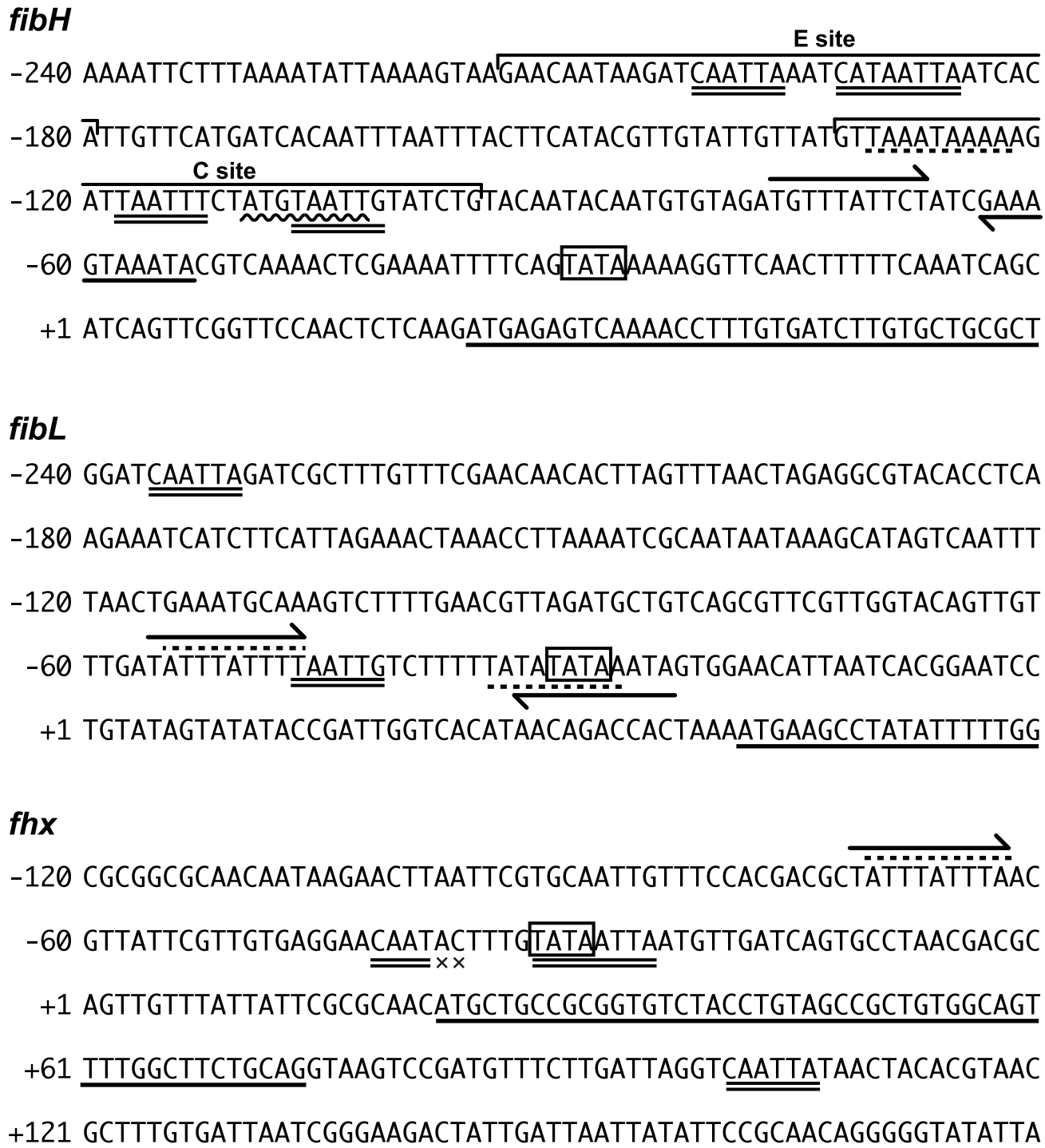
fibH
E site
-240 AAAATTCTTTAAAATATTAAAAGTAAGAACAATAAGATCAATTAAATCATAATTAATCAC
-180 ATTGTTCATGATCACAATTTAATTTACTTCATACGTTGTATTGTTATGTTAAATAAAAAG
C site
-120 ATTAATTTCTATGTAATTGTATCTGTACAATACAATGTGTAGATGTTTATTCTATCGAAA
-60 GTAAATACGTCAAAACTCGAAAATTTTCAGTATAAAAAGGTTCAACTTTTTCAAATCAGC
+1 ATCAGTTCGGTTCCAACTCTCAAGATGAGAGTCAAAACCTTTGTGATCTTGTGCTGCGCT
fibL
-240 GGATCAATTAGATCGCTTTGTTTCGAACAACACTTAGTTTAACTAGAGGCGTACACCTCA
-180 AGAAATCATCTTCATTAGAAACTAAACCTTAAAATCGCAATAATAAAGCATAGTCAATTT
-120 TAACTGAAATGCAAAGTCTTTTGAACGTTAGATGCTGTCAGCGTTCGTTGGTACAGTTGT
-60 TTGATATTTATTTTAATTGTCTTTTTATATATAAATAGTGGAACATTAATCACGGAATCC
+1 TGTATAGTATATACCGATTGGTCACATAACAGACCACTAAAATGAAGCCTATATTTTTGG
fhx
-120 CGCGGCGCAACAATAAGAACTTAATTCGTGCAATTGTTTCCACGACGCTATTTATTTAAC
-60 GTTATTCGTTGTGAGGAACAATACTTTGTATAATTAATGTTGATCAGTGCCTAACGACGC
+1 AGTTGTTTATTATTCGCGCAACATGCTGCCGCGGTGTCTACCTGTAGCCGCTGTGGCAGT
+61 TTTGGCTTCTGCAGGTAAGTCCGATGTTTCTTGATTAGGTCAATTATAACTACACGTAAC
+121 GCTTTGTGATTAATCGGGAAGACTATTGATTAATTATATTCCGCAACAGGGGGTATATTA

Fig. 4

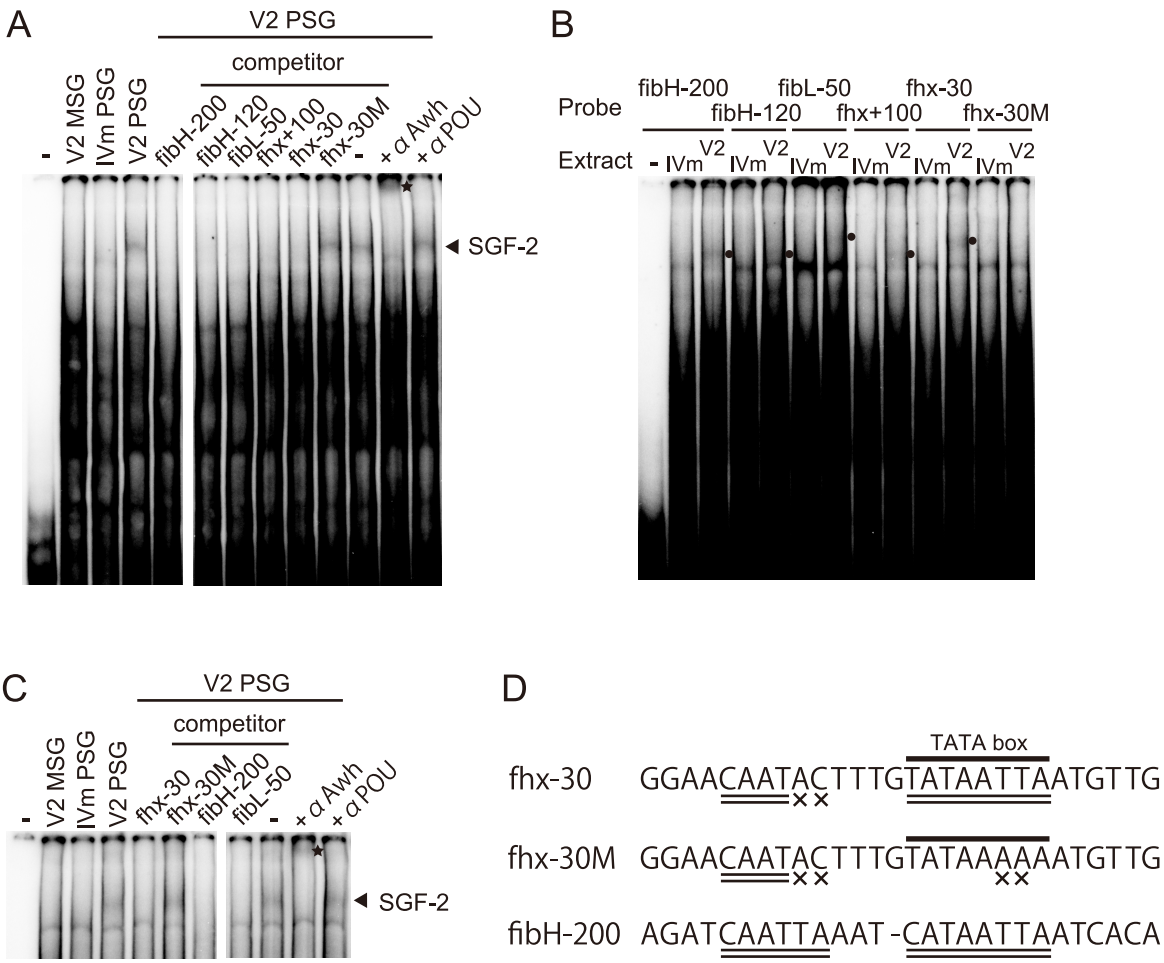
A
V2 PSG
competitor
- V2 MSG IVm PSG V2 PSG fibH-200 fibH-120 fibL-50 fhx+100 fhx-30 fhx-30M - +αAwh +αPOU
SGF-2
B
Probe
fibH-200 fibH-120 fibL-50 fhx+100 fhx-30 fhx-30M
Extract
- IVm V2 IVm V2 IVm V2 IVm V2 IVm V2 IVm V2
C
V2 PSG
competitor
- V2 MSG IVm PSG V2 PSG fhx-30 fhx-30M fibH-200 fibL-50 - +αAwh +αPOU
SGF-2
D
TATA box
fhx-30 GGAACAATACTTTGTATAATTAATGTTG
fhx-30M GGAACAATACTTTGTATAAAAAATGTTG
fibH-200 AGATCAATTAAAT-CATAATTAATCACA

Fig. 5

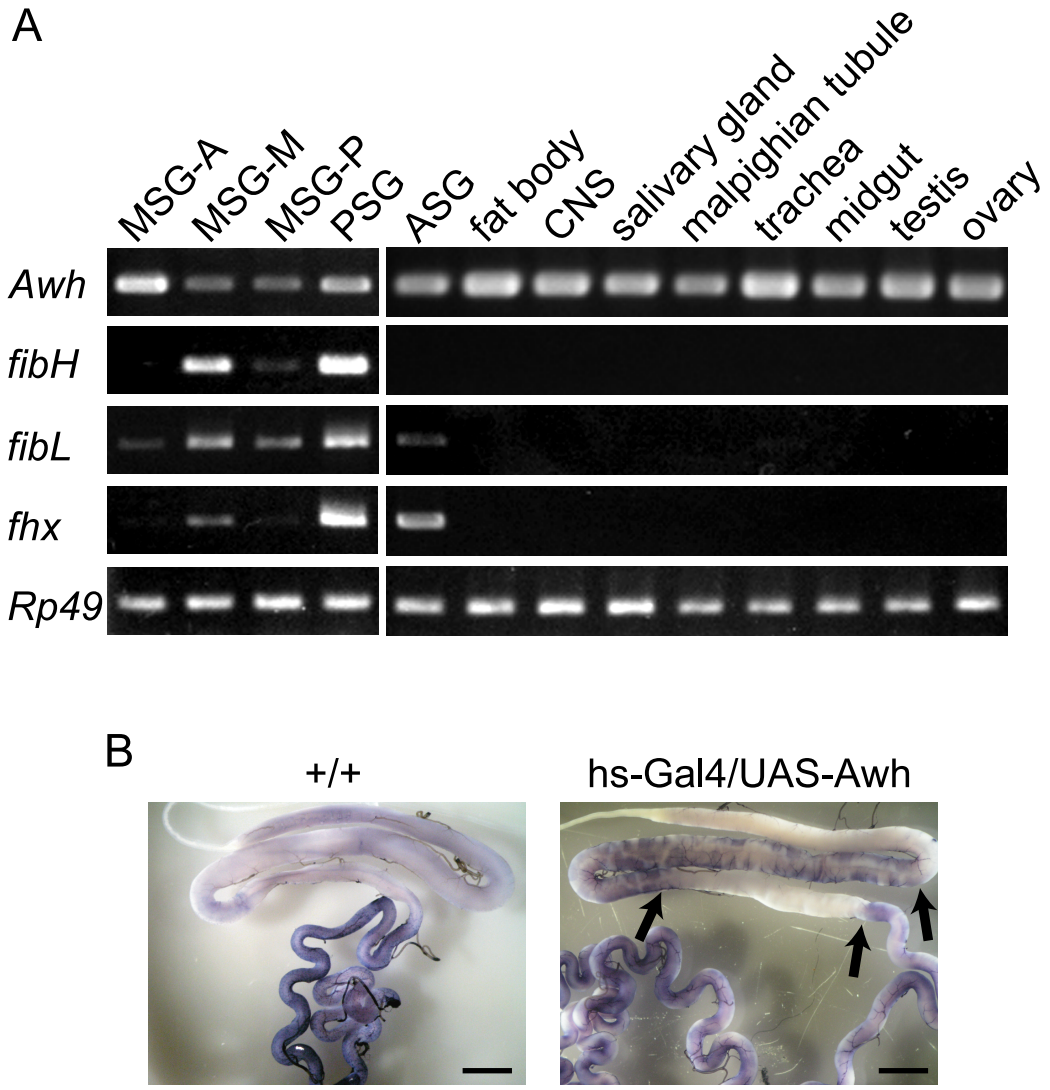
A
MSG-A
MSG-M
MSG-P
PSG
ASG
fat body
CNS
salivary gland
malpighian tubule
trachea
midgut
testis
ovary
Awh
fibH
fibL
fhx
Rp49
B
+/+
hs-Gal4/UAS-Awh

Table S1. Primers and PCR cycles for each gene.

Gene		Primers	Accession no.	Cycles
fibH	F	5'-TCCAACTCTCAAGATGAG-3'	AF226688	21
	R	5'-TATCCTTGATGAGTGCTG-3'		
fibL	F	5'-ACCATCGGTGACCATCAATC-3'	AF541967	21
	R	5'-TTAGACGTGAACCTGGCTG-3'		
fhx	F	5'-TGGCAGTTTTGGCTTCTGCAG-3'	X04226	21[*2]
	R	5'-TCAGTGCTGGCAACCGAAGTC-3'		
Awh	F	5'-ATGAAGACGGAGCACCGCA-3'	AB687553	35
	R	5'-TTGTGATAGCCCTCCGAGTCAC-3'		
Ldb	F	5'-AAGAGGTTACAGCAGAGGAC-3'	AB687554	33
	R	5'-TTTCTGGAAGAGCGTTGTC-3'		
Lcaf	F	5'-ATGTATGCCAAGGGCAAGG-3'	AB687556	35
	R	5'-TGGCGGTCCATTGAAATCATTC-3'		
Rp49	F	5'-CATACAAGATGGCTATAAGACC-3'	AY769302	21
	R	5'-GTGAGCTGCTGGGCTCTTTC-3'		

*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.

Probe		Nucleotide sequence*2
fibH-200	T *1	5'-aattCAATAAGATCAATTAAATCATAATTAATCACATTGTTCATGATCAC-3'
	B	5'-aattGTGATCATGAACAATGTGATTAATTATGATTTAATTGATCTTATTG-3'
fibH-120	T	5'-aattATGTTAAATAAAAAGATTAATTTCTATGTAATTGTATCTGTACAAT-3'
	B	5'-aattATTGTACAGATACAATTACATAGAAATTAATCTTTTTATTTAACAT-3'
fibL-50	T	5'-aattGGTACAGTTGTTTGATATTTATTTTAATTGTCTTTTTATATATAAA-3'
	B	5'-aattTTTATATATAAAAAGACAATTAAAATAAATATCAAACAACTGTACC-3'
fhx-30	T	5'-aattCGTTGTGAGGAACAATACTTTGTATAATTAATGTTGATCAGTGCCT-3'
	B	5'-aattAGGCACTGATCAACATTAATTATACAAAGTATTGTTCCTCACAACG-3'
fhx-30M	T	5'-aattCGTTGTGAGGAACAATACTTTGTATAAAAAATGTTGATCAGTGCCT-3'
	B	5'-aattAGGCACTGATCAACATTTTTTATACAAAGTATTGTTCCTCACAACG-3'
fhx+100	T	5'-aattGATGTTTCTTGATTAGGTCAATTATAACTACACGTAACGCTTTGTG-3'
	B	5'-aattCACAAAGCGTTACGTGTAGTTATAATTGACCTAATCAAGAAACATC-3'

*1 T: Top strand, B: Bottom strand

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