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A *Hox* Gene, *Antennapedia*, Regulates Expression of Multiple Major Silk Protein Genes in the Silkworm *Bombyx mori*[*]

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***Hox* genes play a pivotal role in the determination of anteroposterior axis specificity during bilaterian animal development. They do so by acting as a master control and regulating the expression of genes important for development. Recently, however, we showed that *Hox* genes can also function in terminally differentiated tissue of the lepidopteran *Bombyx mori*. In this species, *Antennapedia* (*Antp*) regulates expression of *sericin-1*, a major silk protein gene, in the silk gland. Here, we investigated whether *Antp* can regulate expression of multiple genes in this tissue. By means of proteomic, RT-PCR, and *in situ* hybridization analyses, we demonstrate that misexpression of *Antp* in the posterior silk gland induced ectopic expression of major silk protein genes such as *sericin-3*, *fhxh4*, and *fhxh5*. These genes are normally expressed specifically in the middle silk gland as is *Antp*. Therefore, the evidence strongly suggests that *Antp* activates these silk protein genes in the middle silk gland. The putative *sericin-1* activator complex (middle silk gland-intermolt-specific complex) can bind to the upstream regions of these genes, suggesting that *Antp* directly activates their expression. We also found that the pattern of gene expression was well conserved between *B. mori* and the wild species *Bombyx mandarina*, indicating that the gene regulation mechanism identified here is an evolutionarily conserved mechanism and not an artifact of the domestication of *B. mori*. We suggest that *Hox* genes have a role as a master control in terminally differentiated tissues, possibly acting as a primary regulator for a range of physiological processes.**

Hox genes have a critical role in specifying identity along the anteroposterior axis during bilaterian animal development. In the fruit fly *Drosophila melanogaster*, *Hox* gene mutations can cause transformation of one segment into another and occasionally produce very striking phenotypes such as antenna-to-leg or haltere-to-wing transformation (1, 2). Each *Hox* gene encodes a protein with a homeodomain and acts as a transcription factor. *Hox* genes regulate a number of downstream genes as a master control gene during segment determination in arthropods or tissue development in vertebrates (3). This process is well illustrated by haltere and wing development in *Drosophila*. The fruit fly hind wing (haltere) is smaller and has a different shape from the forewing. These morphological differences are controlled by the *Hox* gene *Ultrabithorax* (*Ubx*),[3] a member of the *Bithorax Complex* group of genes that determine the identity of the metathoracic segment (2, 4). During haltere development, *Ubx* represses expression of wing-patterning genes at multiple points in development and does not simply act as an upstream activator of the haltere developmental cascade (5). Genes under the control of *Ubx* include transcriptional factors such as *vestigial*, *spalt-related*, and *achaete-scute*, and signaling molecules such as *wingless*, *decapentaplegic*, and *egfr/ras*, which all play key roles in wing development (5–10). Many *Ubx* downstream genes have recently been identified using technologies such as microarrays and chromatin immunoprecipitation; these studies revealed that hundreds of genes are potentially targets of *Ubx* (11–13). A number of *Hox* target genes have also been identified in *Drosophila* and vertebrates (14–16).

Although there has been extensive study of *Hox* functions during development, comparatively little is known about its functions in other biological processes. In mice, the homeobox transcription factor genes *Engrailed-1*/*2* and *Otx2* are known to be involved in the patterning and compartmentalization of the developing nervous system as well as in physiological regulation in adults (17). Recently, we identified a unique *Hox* gene function in the silkworm *Bombyx mori*, a lepidopteran species. Lepidopteran larvae produce silk to form cocoons and/or support larval molt. Silk genes are expressed at a very high level in the larval silk gland, and a number of studies have explored the transcriptional regulation mechanisms of these genes, mainly using biochemical approaches. The studies using *B. mori* revealed that the expression level and the spatial expression pattern of silk genes are determined by transcription factors such as *forkhead*, *Pit-1/Oct/Unc-86 (POU)-M1*, and *Arrowhead*

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[3] The abbreviations used are: *Ubx*, *Ultrabithorax*; POU, Pit-1/Oct/Unc-86; *Antp*, *Antennapedia*; *ser*, *sericin*; MIC, MSG-intermolt-specific complex; PSG, posterior silk gland; MSG, middle silk gland; *h-fib*, *h-fibroin*; *l-fib*, *l-fibroin*; *fhx*, *fibrohexamerin*; *fhxh*, *fibrohexamerin-like*.

TABLE 1
Oligonucleotide sequences of probes and competitors used for the EMSA
Lower case letters show extra sequences for labeling.

Name	Position	Sequence
ser3 −90	−114/−69	aattCAAGTGTATTAAACAAATAATTAATTATTTATTTTATTGGTAACTG
Sr-70	−81/−52	GAAGCGAAAATTTATTACTCTCTACGTAAG
Sr-70M2	−81/−52	GAAGCGAAAATTTGGTACTCTCTACGTAAG
fhxh4 −1660	−1682/−1637	aattGAAAAACGTAAATAAATTGATTTATGACAGAGTATATTTTTAACCG
fhxh5 −300	−324/−279	aattTTAATCTCTTGTCATCAATATTATAAATCTCATTTTTTGGCCCTTA

(18–22). We recently found that a *Hox* gene, *Antennapedia* (*Antp*), plays a key role in the expression of *sericin-1* (*ser1*), one of the major silk protein genes (23). Our analysis demonstrated that Antp protein is a component of a putative *ser1* activator called middle silk gland (MSG)-intermolt-specific complex (MIC); moreover, induced misexpression of *Antp* results in the induction of *ser1* expression in the posterior silk gland (PSG) where there is no expression in normal individuals (23). The silk gland is a terminally differentiated tissue, and we therefore speculated that *Antp* can regulate physiological as well as developmental processes.

The main question raised by our previous observations was whether *Hox* genes could play a fundamental role in physiological regulation. To answer this question here, we sought to identify novel *Antp*-regulated genes in the *Bombyx* silk gland. Using proteomic, RT-PCR, and *in situ* hybridization analyses, we found that *Antp* could induce expression of multiple major silk protein genes such as *ser3*, *fhxh4*, and *fhxh5* in the PSG. These genes are normally expressed only in the MSG. Moreover, MIC binds to the upstream regions of these genes, suggesting that *Antp* directly regulates their expressions. We also found that this pattern of gene expression is well conserved between *Bombyx mori* and the wild species *Bombyx mandarina*, indicating that this transcriptional regulation mechanism is an evolutionarily conserved process and not an artifact of silkworm domestication. Our results here support the speculation that *Hox* genes have a role as a key regulator in physiological regulation as well as in developmental processes. This finding provides further understanding of the functional evolution of *Hox* genes.

Experimental Procedures

Silkworm Strains—The silkworms were reared on an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) at 25 °C under a photoperiod of 12-h light:12-h dark for *daizo* and transgenic strains and 16-h light:8-h dark for the Kinshu × Showa strain. *daizo* was used as the wild type strain for gene expression analysis, and Kinshu × Showa was utilized for protein extraction for the electrophoretic mobility shift assay (EMSA). The *Ayfib-431a* (*Ayfib*-GAL4) transgenic strain was used for the PSG-specific GAL4 driver. This strain harbors the *Antheraea yamamai fibroin* promoter-GAL4 cassette and can induce PSG-specific gene expression in first instar larva or earlier stages (24, 25). The hs-GAL4 strain (modified from *Bmhsp70*-GAL4 (26)) was used for heat shock-induced gene expression in this or previous studies (21–23). The heat shock was a treatment of 42 °C for 2 h on the 1st day of fifth instar larvae (L5D1) following the method described previously (23). The UAS-*Antp* strain is described elsewhere (23). For misexpression analysis, UAS strains were crossed with GAL4 strains, and the genotype of the progeny was determined by screening for the transgenic marker (DsRed for GAL4 and AmCyan for UAS-*Antp*). The wild silkmoth *B. mandarina* was sampled from the field in the Shimonita and Maebashi areas of Japan, and their hybrid has been maintained in our laboratory for 19 generations.

EMSA—EMSA was carried out as described previously (23, 27). The protein extract was prepared from the posterior portion of the MSG of Kinshu × Showa larvae at L5D2. The sequences of the oligonucleotides and competitors are shown in Table 1. The protein-probe complexes were separated on 7% polyacrylamide gels. A 100-fold molar excess of unlabeled oligonucleotides was added to the reaction for the competition experiments. The antibodies used for the supershift assay are as described previously (23).

Sample Preparation and Two-dimensional Electrophoresis—Larvae of the *Ayfib*-GAL4/+ and *Ayfib*-GAL4/UAS-*Antp* strains were dissected at L5D3 to isolate PSGs. Sample preparation and electrophoresis were conducted as described previously (28). Briefly, the PSG was dissociated in lysis buffer (10 mg of tissue/300 μl of buffer) containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% Nonidet P-40, 5% 2-mercaptoethanol, 0.2% Ampholine (pH 3.5–10), and EDTA-free protease inhibitor. The dissociated tissue was subjected to repeated freeze/thaw cycles in liquid nitrogen and then stored at −80 °C until used for electrophoresis. The two-dimensional PAGE gels were stained with Coomassie Brilliant Blue.

Mass Spectrometry and Data Analysis—Mass spectrometry and data analysis were performed largely as described previously (28). Spots were excised from five or six gels, and amaZon SL (Bruker Daltonics, Bremen, Germany) was used for the MS analysis. The MS data were analyzed with Mascot software (29) using the amino acid sequence data in the National Center for Biotechnology Information data bank (www.ncbi.nlm.nih.gov/) and silkworm genome database (30).

RNA Extraction, cDNA Synthesis, and RT-PCR—RNA was extracted from each part of the silk gland of *B. mori daizo* strain (L5D3) and *B. mandarina* (just before spinning stage); additionally, RNA was obtained from the PSGs of *B. mori Ayfib*-GAL4/+ (L5D3), *Ayfib*-GAL4/UAS-*Antp* (L5D3), *hsp70*-GAL4/+ (L5D2), and *hsp70*-GAL4/UAS-*Antp* (L5D2) strains. The Illustra RNAspin MINI RNA Isolation kit (GE Healthcare) was used for RNA extraction from *hsp70*-GAL4/+ and *hsp70*-GAL4/UAS-*Antp* individuals and was used for cDNA synthesis using a PrimeScript RT-PCR kit (Takara, Otsu, Japan) (23). For other samples, ISOGEN (Nippongene, Tokyo, Japan) and the SV Total RNA Isolation System (Promega, Madison, WI) were

TABLE 2
Primers used for gene expression analysis

Gene name	Forward	Reverse
rp49	5′-CAGGCGGTTCAAGGGTCAATAC-3′	5′-TGCTGGGCTCTTTCCACGA-3′
ser1	5′-CAAAGACCGCCAACATGCGT-3′	5′-CAGCGTTCCAATTGGCCTGA-3′
ser2	5′-CACTTTTCGGGGGCTTAGTT-3′	5′-TTCGTATTCGGAACCTTTGC-3′
ser3	5′-AGTTGCTCTATTCCTGATAG-3′	5′-TGTCGTCGGAATTCTCACCA-3′
Antp	5′-ATGGATGGCTGCGATCAGCAG-3′	5′-TAAGGTTTACTGTGGCGAGGT-3′
fhxh4	5′-ACTGTGGCGGATGAGGTAGA-3′	5′-TCTGGGATTAGAGTCTTCGA-3′
fhxh5	5′-ACGAGGCGTGTCTTGCCCTT-3′	5′-GGTCCGTAATCACACGTGGC-3′
BGIBMGA010891	5′-GCATTTCCATTATTGCGTTG-3′	5′-ATGCAAAAACCTTGATGTCT-3′
BGIBMGA011308	5′-TCACAACACCTTAGTCAACT-3′	5′-CCTTCGTTCAAGAAACCAGC-3′
BGIBMGA007061	5′-CACTCTCCGATGAATTCATC-3′	5′-TCCGTTAGAATAGGTACATA-3′
h-fib	5′-TGCGCTCTGCAGTATGTCGC-3′	5′-CACTGTTTGATACGTATGGC-3′
l-fib	5′-TCGCCATCCTCAACGTTCAA-3′	5′-GACGATGCAGTACTCTTCAT-3′
fhx	5′-GATACCGGTGTTCCAGTTTG-3′	5′-GTGCAGCAGGGTCAGATCTT-3′

used for RNA extraction, and Superscript III (Life Technologies) was used for cDNA synthesis (25). KOD-FX polymerase (Toyobo, Osaka, Japan) was used for RT-PCR. Primer sequences are listed in Table 2.

Probe Synthesis for in Situ Hybridization—For *ser1* probe synthesis, a plasmid in which a partial fragment (106–775 bp; J01040) was inserted into the pSPT18 vector (Roche Diagnostics) was PCR-amplified using T7 and SP6 primers. The PCR product was utilized as the template for probe labeling. For *fhxh4* and *fhxh5*, L5D3 MSG-P cDNA from the *daizo* strain was amplified using primers 5′-ACTGTGGCGGATGAGGTAGA-3′ and 5′-GTAATACGACTCACTATAGGGCTCTGGGATTAGAGTCTTCGA-3′ (*fhxh4*) or 5′-ACGAGGCGTGTCTTGCCCTT-3′ and 5′-GTAATACGACTCACTATAGGGCGGTCCGTAATCACACGTGGC-3′ (*fhxh5*) and used for probe labeling. The labeling reaction was carried out with a digoxigenin RNA labeling kit (SP6/T7) following the manufacturer's protocol (Roche Diagnostics).

In Situ Hybridization Analysis—Silk glands were dissected from individuals of *Ayfib*-GAL4/+ and *Ayfib*-GAL4/UAS-*Antp* L5D3 and sequentially fixed with 100% methanol, 100% ethanol, and 4% paraformaldehyde. They were washed with PBS, treated with 0.2 N HCl for 20 min, and washed again with PBS. They were treated with 10 μg/ml proteinase K (Roche Diagnostics) at room temperature for 20 min and then washed with PBS. Prehybridization was carried out in hybridization buffer (5× SSC, 50% formamide, 50 μg/ml heparin, and 50 μg/ml salmon sperm DNA) at 50 °C for 1 h. The probe was then added at a final concentration of 100 ng/ml and incubated at 50 °C overnight. The silk glands were washed with 2× SSC and 0.1% Tween 20 at 50 °C for 20 min and then washed twice with 0.2× SSC and 0.1% Tween 20 at room temperature. For the antibody reaction, a blocking step was performed using 1% goat normal serum, the anti-digoxigenin antibody (Roche Diagnostics) was applied at a 1:4000 dilution, and the tissue was incubated at 37 °C for 1 h. The silk gland was washed with PBS, stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics), and counterstained with a graded series of ethanol. The image was captured using Nikon Ds-Vi1 (Nikon Corp., Tokyo, Japan) and processed using Photoshop CS5 (Adobe Systems Inc., San Jose, CA).

Results

Expression of sericin Genes following Antp Misexpression—*Antp* is a *Hox* transcription factor gene that shows MSG-specific expression in the silk gland (23, 31). The MSG is separated into three territories termed MSG-A, MSG-M, and MSG-P (Fig. 1, *A* and *A′*), all of which express *Antp* (Fig. 1*B*) (23). Ectopic expression of *Antp* in the PSG using a heat shock promoter and the PSG-specific *Antheraea yamamai fibroin* promoter resulted in the induction of *ser1* expression in the PSG (Fig. 1, *C* and *D*) (23). In the latter case, ectopic *ser1* was observed in some GAL4/+ PSGs (Fig. 1*D*), which might result from the transcriptional perturbation; in the silkworm, GAL4 frequently exhibits a toxic effect (32–34). We conclude that *ser1* is regulated by *Antp* because it was expressed in all *Ayfib*-GAL4/UAS-*Antp* PSGs and was also induced by hs-GAL4 (Fig. 1, *C* and *D*). *ser1* is normally expressed in the MSG-M and MSG-P but not in MSG-A (Fig. 1*B*). The absence of *ser1* expression in MSG-A may be associated with a lack of Antp protein expression or failure of MIC formation in this territory (23). Induction of *ser1* expression by ectopic *Antp* suggested that it was acting as a *ser1* activator in the MSG-M/MSG-P of normal individuals. In addition to *ser1*, *B. mori* has two *sericin* genes (*ser2* and *ser3*) that are expressed specifically in MSG-A and/or MSG-M (Fig. 1*B*) (35, 36). As a first step to identify novel *Antp* target genes, we investigated whether *ser2* and/or *ser3* could be induced by *Antp*. RT-PCR analyses showed that *ser3* but not *ser2* was induced in the PSG of individuals misexpressing *Antp* (Fig. 1*D*). *ser3* is expressed normally in the MSG-A and MSG-M (Fig. 1*B*); we suggest that Antp functions for the activation of *ser3* expression in MSG-M.

Antp Binds to the Promoter of ser3—Antp protein can bind to the promoter of *ser1* and directly activate its expression (23). We asked whether this was also the case for *ser3*. A search of the *ser3* upstream region identified a putative MIC binding site ((G/A)ATT(T/A)ATNA(T/C)) (27) at the −90 position (Fig. 2*A*). We carried out an EMSA using an oligonucleotide that included this element (ser3 −90) and found that the MSG extract showed significant binding to this probe (Fig. 2*B*, *lane 2*). This binding is likely mediated by MIC because the *ser1* −70 oligonucleotide (Sr-70) interfered with the binding, whereas the mutated probe (Sr-70M2) did not (Fig. 2*B*, *lanes 3* and *4*). The MIC is composed of Antp and its cofactors Extradenticle and Homothorax (23). The addition of antiserum against these

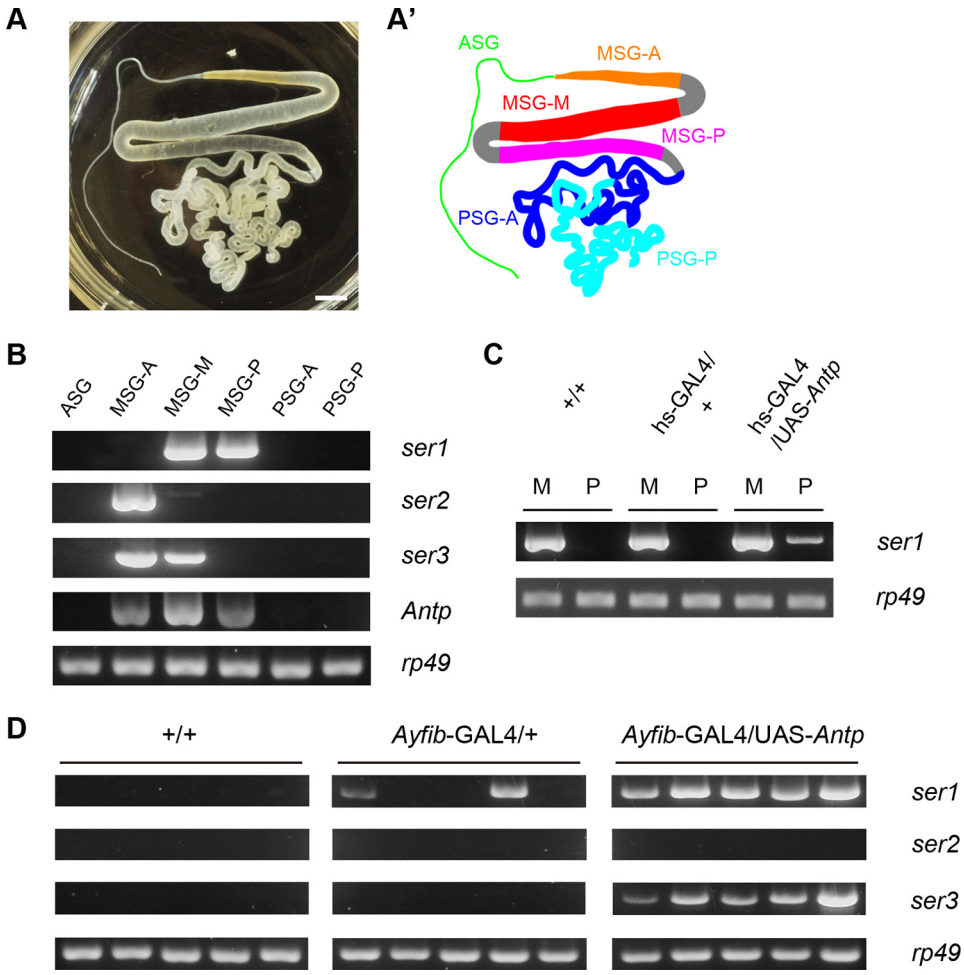

FIGURE 1. **Induction of *sericin* genes by *Antp*.** A photograph (*A*) and schematic drawing (*A'*) of the silk gland are shown. The MSG can be subdivided into three regions along the anteroposterior axis (MSG-A, MSG-M, and MSG-P). PSG can be also subdivided into two regions, although there is no apparent morphological difference (PSG-A and PSG-P). *Scale bar*, 0.3 cm. *B–D*, silk gland gene expression investigated by RT-PCR. *B*, expression of three *sericin* genes in *daizo* (wild type) strain. These genes are expressed specifically in MSG with different patterns of spatial expression. *C*, induction of *ser1* by heat shock-induced *Antp* misexpression. *M* and *P* indicate MSG and PSG, respectively. *D*, expression of *sericin* genes in the PSGs of +/+, *Ayfib*-GAL4/+, and *Ayfib*-GAL4/UAS-*Antp* individuals. Five individuals were investigated for each genotype. *ser1* and *ser3* are induced strongly by *Antp* misexpression. *ser1* expression is also apparent in two individuals of *Ayfib*-GAL4/+, possibly due to the transcriptional perturbation by GAL4. *ASG*, anterior silk gland.

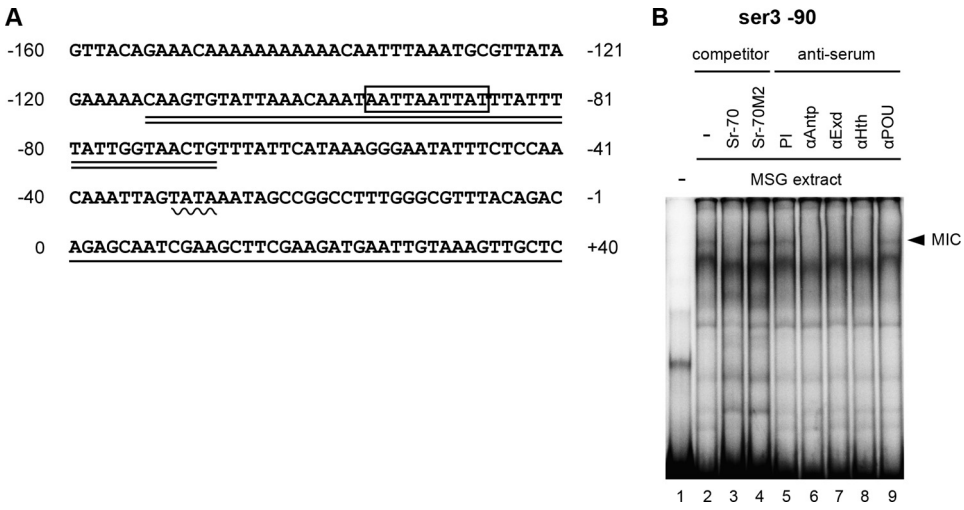

FIGURE 2. *A*, sequence of the putative promoter region for *ser3*. The *boxed* region indicates the hypothetical MIC binding site, the *wavy line* indicates the putative TATA box, the *underlined* sequence indicates the transcribed region, and the *double underlined* sequence indicates the oligonucleotide utilized for the EMSA analysis (ser3 −90). *B*, binding of MIC to the *ser3* promoter. The band representing the presumptive MIC binding is indicated with the *arrowhead*. The addition of the non-labeled Sr-70 oligonucleotide (*lane 3*) abolished the binding, whereas Sr-70M2 did not (*lane 4*). Antiserum against Antp (*lane 6*), Extradenticle (*Exd*) (*lane 7*), and Homothorax (*Hth*) (*lane 8*) also interfered with binding, but the preimmune serum (*lane 5*) or antiserum against POU-M1 (*lane 9*) did not show an effect.

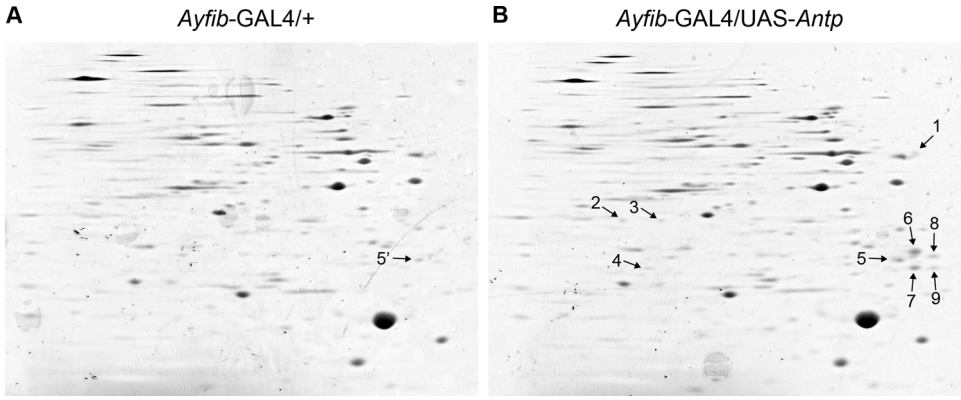

FIGURE 3. **Gel images of PSG proteins after two-dimensional electrophoresis.** The gels were stained with Coomassie Brilliant Blue. *A*, *Ayfib*-GAL4/+. *B*, *Ayfib*-GAL4/UAS-*Antp*. Spots detected specifically in *Ayfib*-GAL4/UAS-*Antp* are marked with *arrows* and *numbers*. Spot 5′ in the *Ayfib-GAL4*/+ gel indicates a spot that appears to be located at the identical position to spot 5 of *Ayfib*-GAL4/UAS-*Antp*.

proteins clearly inhibited binding, but the preimmune serum or antiserum against POU-M1 did not (Fig. 2*B*, *lanes 5–9*). This further suggests that the binding complex is certainly the MIC. We therefore conclude that *Antp* directly activates *ser3* expression by binding to its promoter sequence.

Identification of Novel Antp Target Genes—The fact that *ser3* was induced via ectopic *Antp* expression raises the possibility that genes other than *sericin* might also be regulated by *Antp*. To identify such genes, we carried out a proteomic analysis. PSG proteins were extracted from normal and *Antp*-misexpressing larvae at the 3rd day of the fifth instar and used for two-dimensional electrophoresis. Comparison of the gel images showed that the overall spot patterns were similar between the two experimental groups (Fig. 3). Nevertheless, we did identify nine spots that were apparently present only in the *Antp*-misexpressing PSG or showed stronger staining compared with the normal PSG (Fig. 3*B*, *arrows*). These spots were isolated and analyzed by mass spectrometry. The analysis showed that spot 1 was a protease inhibitor (BGIBMGA010891), spot 3 was acyl-coenzyme A dehydrogenase (BGIBMGA011308) and cathepsin B (BGIBMGA007061), and spots 5–9 were fibrohexamerin-like proteins Fhxh4 and Fhxh5 (Table 3). Spots 5–9 showed relatively strong staining in the gel image (Fig. 3). Thus, Fhxh4 and Fhxh5 were apparently strongly induced by *Antp* misexpression. Spot 5 also appeared to be present in normal individuals (Fig. 3, designated as spot 5′), but we suspect that this spot was some other protein(s) as Fhxh4/Fhxh5 transcripts were not detected in normal PSGs (see the description later). Fhxh4/Fhxh5 proteins are abundant in the cocoon of normal individuals (37) and are predicted to be major silk proteins similar to sericins.

Induction of the Identified Proteins Is Mediated by Transcriptional Activation—*Antp* encodes a transcription factor and is presumed to induce the identified proteins by transcriptional activation. An RT-PCR analysis confirmed this was the case for all the identified genes. mRNAs of *fhxh4* and *fhxh5* were induced strongly when *Antp* was misexpressed using the PSG promoter or the heat shock promoter (Fig. 4, *A* and *B*). Induction of the mRNAs was also observed for other genes when *Antp* was induced using the PSG promoter (Fig. 4*A*). We also examined *fhxh4* and *fhxh5* expression by *in situ* hybridization analysis and found that induction occurred in a widespread region of the PSG (Fig. 5).

In the silkworm, it is known that transgene expression is affected by position of insertion of the transposon (33, 38). The insertion can also cause disruption of endogenous genes (25) and this might possibly give rise to an artifactual effect that is irrespective of the transgene expression. It is therefore important to verify whether two or more independent transgenic strains can give identical results. We used another independent UAS-*Antp* strain (23) for this purpose and examined *fhxh4* and *fhxh5* expression in the PSG after *Antp* misexpression. A clear induction effect was also observed in this strain (Fig. 4*B*), indicating that induction was undoubtedly provided by Antp activity.

Gene Expression in the Normal Silk Gland—We next examined the expression of the genes described above in the normal silk gland. *Antp* is expressed specifically in the MSG (Fig. 1*B*) (23); it seems reasonable to presume that genes positively regulated by *Antp* also show this expression pattern. Our RT-PCR analysis showed that *fhxh4* and *fhxh5* are expressed strongly in the MSG-P and weakly in the MSG-M; they could not be detected in other regions of the silk gland (Fig. 6). This expression pattern correlates well with that of *ser1* (Fig. 1*B*). For other *Antp*-induced genes, we found that they were expressed not only in the MSG but also in other silk gland regions (Fig. 6).

Antp Binds to the Upstream Region of fhxh4 and fhxh5—The expression of *fhxh4* and *fhxh5* shows good agreement with the production of Antp protein. This suggests that Antp might directly activate their expressions. A search of upstream genome sequences revealed that a putative MIC binding site was present at around −1660 for *fhxh4* and −300 for *fhxh5* (Fig. 7, *A* and *B*). We carried out an EMSA using an oligonucleotide probe including these elements and found that the MSG extract could bind to both of these sequences (Fig. 7, *C* and *D*). A competition analysis and a supershift assay further supported the interpretation that this binding was mediated by the MIC (Fig. 7, *C* and *D*). Therefore, we conclude that Antp directly regulates *fhxh4* and *fhxh5* expression by binding to upstream sequences of these genes.

Effect of Antp Misexpression on PSG-specific Genes—In addition to their role as a transcriptional activator, *Hox* genes can

TABLE 3
Profile of PSG proteins expressed specifically in Antp-misexpressing individuals

Spot number	Gene number[a]	Gene name	Accession number	Annotation	Mascot score
1	BGIBMGA010891		AK383125	Protease inhibitor I8	62
2	ND				
3[b]	BGIBMGA011308		AK383321	Acyl-coenzyme A dehydrogenase	119
	BGIBMGA007061		AK383399	Cathepsin B	99
4	ND				
5[b]		*fhxh4*	XP_004922636	Fibrohexamerin-like	173
		fhxh5	XP_004922696	Fibrohexamerin-like	38
6		*fhxh4*	XP_004922636	Fibrohexamerin-like	83
7		*fhxh4*	XP_004922636	Fibrohexamerin-like	73
8		*fhxh4*	XP_004922636	Fibrohexamerin-like	83
9		*fhxh4*	XP_004922636	Fibrohexamerin-like	80

[a] Gene number in the silkworm genome database is shown. ND, not determined.
[b] These spots showed partial homology against two distinct proteins.

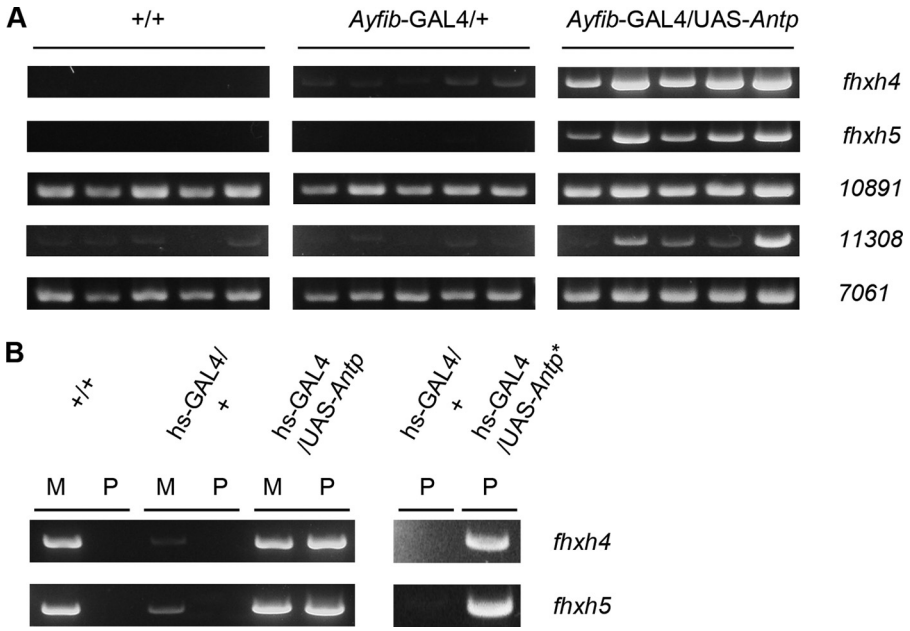

FIGURE 4. **Expression analysis of genes identified by proteomic analysis.** Expression was analyzed by RT-PCR. *A*, expression in the PSGs of +/+, *Ayfib*-GAL4/+, and *Ayfib*-GAL4/UAS-*Antp* individuals. *10891* indicates gene *BGIBMGA010891*, *11308* indicates *BGIBMGA011308*, and *7061* indicates *BGIBMGA007061*. *B*, induction of *fhxh4* and *fhxh5* by heat shock-induced *Antp* misexpression. *M* and *P* indicate MSG and PSG, respectively. Induction was also observed when *Antp* was misexpressed using an independent UAS-*Antp* strain (UAS-*Antp**). Gene expression was analyzed in fifth instar larvae.

also act as a repressor of expression (39). This suggests that *Antp* may function in the repression of PSG-specific genes. Fibroin component genes such as *h-fibroin* (*h-fib*), *l-fibroin* (*l-fib*), and *fibrohexamerin* (*fhx*) are known to be expressed strongly and specifically in the PSG (22, 40, 41). We examined the expression of these genes following misexpression of *Antp*. Previously, we showed that the heat shock promoter-induced misexpression of *Antp* did not affect *h-fib* expression (23). Here, we overexpressed *Antp* using the *fibroin* promoter and obtained a similar result (Fig. 8). In addition, this overexpression did not affect the expression of *l-fib* and *fhx* genes (Fig. 8). In a proteomic analysis, we found that none of the proteins that are expressed in the PSG showed a reduced expression level in individuals misexpressing *Antp* (Fig. 3). We conclude from these results that *Antp* has little or no effect on the expression of PSG-specific genes.

Gene Expression in the Silk Gland of a Wild Bombyx Species—B. mori is a domesticated species that has been selected for cocoon yield for more than 5000 years. It is possible that this long term artificial selection might have influenced the patterns of gene expression in this species such that the results here might not necessarily reflect events in a wild population. To determine whether the gene regulation mechanism identified here is also present in the natural environment, we examined the pattern of gene expression in *B. mandarina*, a wild relative of *B. mori*. We conducted an RT-PCR analysis of the *B. mandarina* silk gland and found that the gene expression patterns were similar in the two species (Fig. 9). Importantly, *Antp* showed MSG-specific expression in both species (Fig. 9; compare with Fig. 1*B*). Thus, we conclude that the gene regulation pattern in *B. mori* is not an artifact of domestication but reflects the natural situation.

Discussion

In this study, we confirmed that *Hox* genes regulate expression of multiple genes in the silk gland. Our analyses demonstrated that *Antp* induced the expression of a number of major silk protein genes such as *ser3*, *fhxh4*, and *fhxh5* in addition to *ser1* (Figs. 1*D*, 4, and 5). This finding is a significant contribution to our understanding of *Hox* functions because the roles of

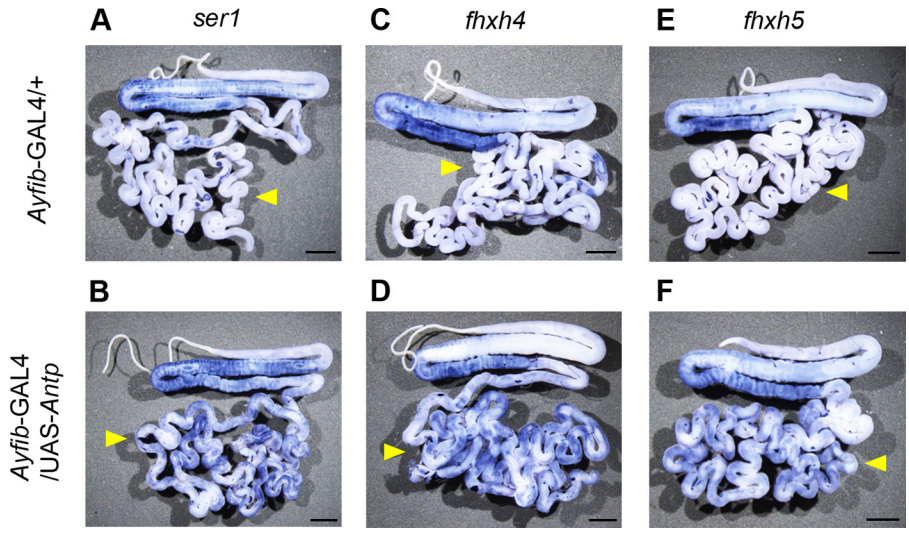

FIGURE 5. ***In situ* hybridization of *ser1* (*A* and *B*), *fhxh4* (*C* and *D*), and *fhxh5* (*E* and *F*).** *A*, *C*, and *E* are the silk glands of *Ayfib*-GAL4/+, and *B*, *D*, and *F* are those of *Ayfib*-GAL4/UAS-*Antp* individuals. *Yellow arrowheads* indicate the PSG. *Scale bar*, 0.3 cm.

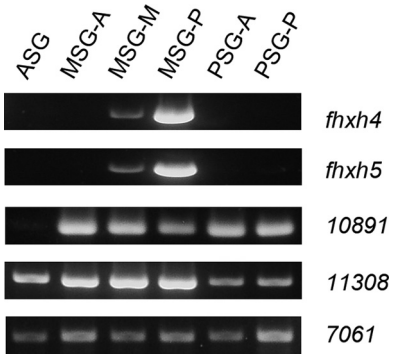

FIGURE 6. **RT-PCR analysis of gene expression in the silk gland of the *daizo* strain.** *ASG*, anterior silk gland.

these regulators in terminally differentiated tissue had not been elucidated previously. The silk gland is a good model for studying additional *Hox* functions because *Antp*-mediated regulation of *ser1* expression has already been studied in detail (23). Lepidopteran insects produce cocoons to protect the larvae and/or pupae from environmental dangers, and we speculate that the function of *Antp* in the silk gland indicates its role as a regulator of physiological processes. We found that individuals misexpressing *Antp* in the silk gland exhibited little or no morphological changes despite the expression of the gene from an early larval stage (see Fig. 5, *B*, *D*, and *F*) (25). This further suggests that *Antp* acts in regulatory pathways that are distinct from those required for development. Our finding that *Antp* controls expression of multiple genes in the silk gland indicates its role as a master control gene in this tissue. This result also implies that acquisition of *Antp* expression in the MSG could have been one of the critical events that enabled the silkworm to be able to produce a cocoon. We investigated *Antp* expression in another lepidopteran species and, interestingly, found that MSG-specific expression was also present in *B. mandarina* (Fig. 9). This finding suggests that *Antp* function might be conserved in the genus *Bombyx*. In contrast, *Antp* is expressed strongly in the anterior silk gland/MSG and the PSG of the eri silkworm (*Samia ricini*), a Saturniidae species (data not shown), indicating that it might have distinct biological functions in this family. One possibility is that *Antp* regulates *sericin* expression in the MSG and *fibroin* in the PSG in a similar manner as *B. mori forkhead* (19, 43).

Our misexpression analysis identified seven *Antp*-induced genes (*ser1*, *ser3*, *fhxh4*, *fhxh5*, *BGIBMGA010891*, *BGIBMGA011308*, and *BGIBMGA007061*; Figs. 1*D* and 4*A*). Among these genes, expression of *ser1*, *fhxh4*, and *fhxh5* was specific to MSG-M and MSG-P in the normal silk gland (Figs. 1*B* and 6). We found that MIC could bind to the −1660 sequence of *fhxh4* and −300 sequence of *fhxh5* (Fig. 7, *C* and *D*), and we presume that *Antp* directly activates their expression by binding to these elements. The MIC binding consensus sequence was also present at −190 of *fhxh4*, but our analysis failed to detect binding to this region (data not shown). We therefore hypothesize that not all consensus sequences are recognized by the MIC. *fhxh4*/*fhxh5* expression was confined to the middle and posterior portions of the MSG, and this expression shows a good correlation with the territories in which the MIC is formed (23). In addition to transcriptional activation by MIC, a POU-type transcription factor, POU-M1, might act to repress *fhxh4*/*fhxh5* in the MSG-A and/or in the MSG-M; its role in *ser1* repression has already been demonstrated (20). *ser3* is normally expressed in the MSG-A/MSG-M (Fig. 1*B*), and our finding that MIC can bind to position −90 (Fig. 2*A*) indicates that *Antp* directly activated its transcription in the MSG-M. The MIC is not formed in the MSG-A (23), and we speculate that there is probably another activation mechanism in this region of the gland. *ser3* transcription is absent in the MSG-P; this may be due to expression of a factor such as *invected*, a putative transcriptional repressor (22, 23). Other *Antp*-induced genes showed more widespread expression (Fig. 6), and it is unclear whether *Antp* is indeed involved in the transcriptional regulation of these genes. There are two possible scenarios. First, *Antp* activates their expression in MSG-M/MSG-P, whereas other factor(s) act in other locations. Second, *Antp* regulates these genes only in particular biological contexts such as embryogenesis. It should be feasible to discriminate

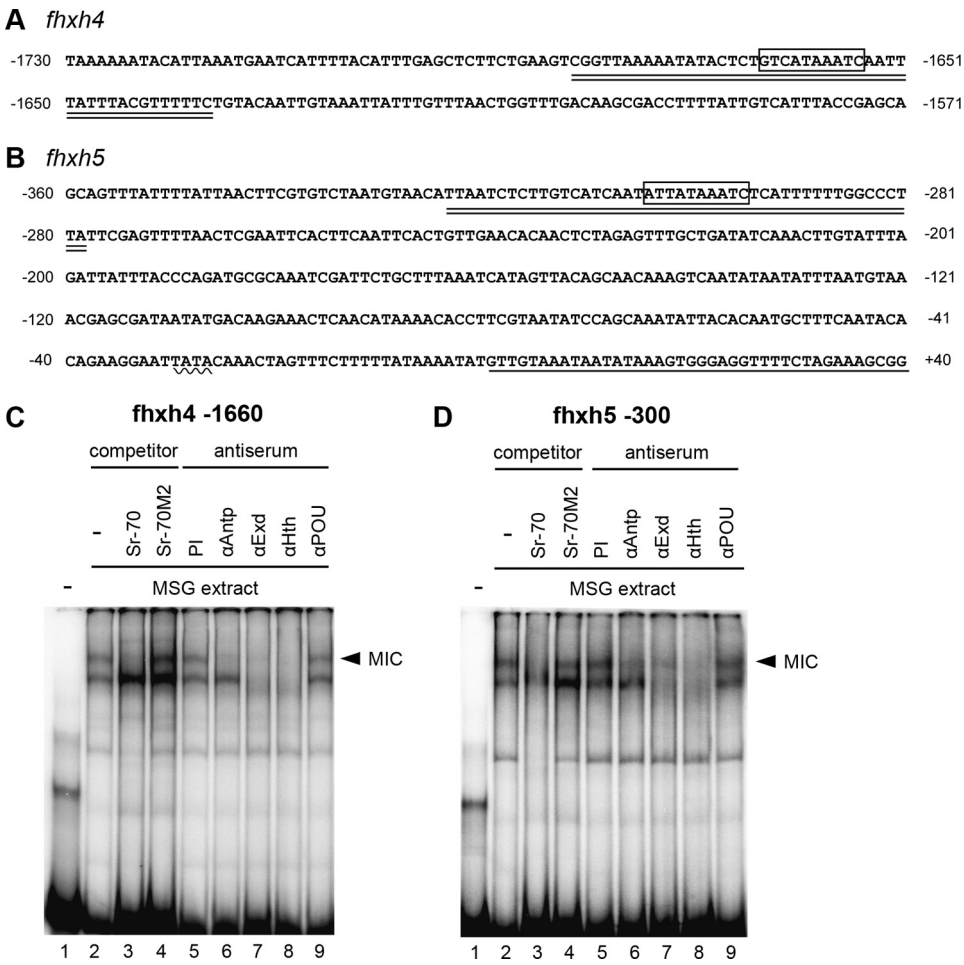

FIGURE 7. *A* and *B*, sequence of the upstream region of *fhxh4* (*A*) and *fhxh5* (*B*). The *boxed* region indicates the hypothetical MIC binding site, the *wavy line* indicates the putative TATA box, the *underlined* sequence indicates the transcribed region, and the *double underlined* sequence indicates the oligonucleotide utilized for the EMSA analysis (fhxh4 −1660 and fhxh5 −300). *C* and *D*, binding of MIC to the upstream region of *fhxh4* (*C*) and *fhxh5* (*D*). The band that represents the presumptive MIC binding is indicated with the *arrowhead*. The binding was abolished by the non-labeled Sr-70 probe but not by the Sr-70M2 probe (*lanes 3* and *4*). Interference with the binding was also apparent after addition of antisera against Antp, Extradenticle (*Exd*), and Homothorax (*Hth*) but not after addition of anti-POU-M1 (*POU*) serum or preimmune (*PI*) serum (*lanes 5–9*).

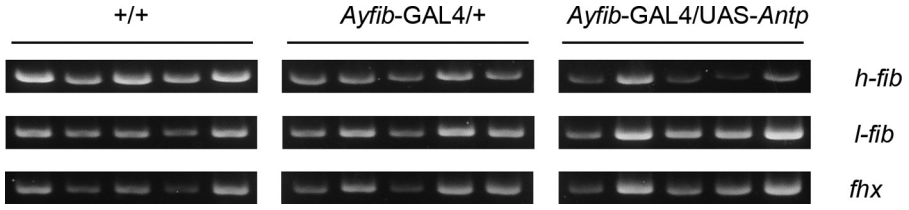

FIGURE 8. **RT-PCR analysis of expression of *h-fib*, *l-fib*, and *fhx* in the PSG of *Antp*-misexpressing individuals.**

between these possibilities using *Antp* knockdown and/or knock-out in the MSG.

The *fhxh* genes were recently identified and found to show weak homology to *fhx* (30). In total, eight *fhxh* genes have been identified in the silkworm, and all are aligned tandemly in the genome. Of these genes, only *fhxh4* and *fhxh5* are expressed in the silk gland and produce major silk proteins. At present, the biological roles of Fhxh4/5 are unknown. Fhx is involved in the appropriate organization and secretion of the fibroin complex (44), and the presence of a putative glycosylation site that is essential for Fhx function in Fhxh4/5 (data not shown) (45) indicates that they might act in a similar manner. In a two-dimensional gel analysis, the Fhxh4 protein was detected as a multiple cluster of spots (Fig. 3 and Table 3), supporting the interpretation that it undergoes post-translational modifications in the silk gland. Recently *fhxh* genes were also found in *S. ricini*, and interestingly some are expressed strongly in the anterior silk gland/MSG (42). These proteins retain the glycosylation sites (42), and it is possible that they have conserved biological functions.

Here we succeeded in identifying a number of novel *Hox* target genes in the silk gland using a proteomics approach. However, this method provides limited information due to limitation in sensitivity, and it is possible that *Antp* might regulate a larger numbers of genes. Currently, we are in the process of trying to identify further candidate genes using RNA

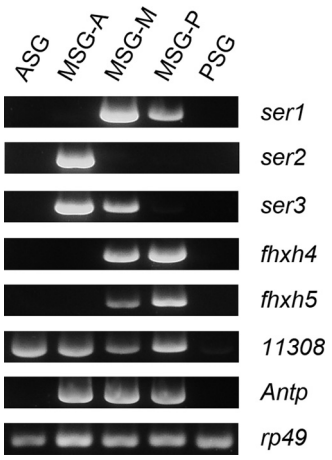

FIGURE 9. **RT-PCR analysis of gene expression in the silk gland of *B. mandarina*.** *11308* indicates the *BGIBMGA011308* gene. *ASG*, anterior silk gland.

sequencing analysis; this approach is expected to produce more information on the number of genes regulated by *Hox* genes in biological pathways unrelated to the developmental process.

Author Contributions—T. T. designed and carried out all of the experiments with the exceptions described below. K. U. generated transgenic silkworm strains. M. K. conducted experiments concerned with heat shock-induced Antp expression, RNA sampling, and cDNA synthesis. Shi. T. carried out the EMSA experiment. H. K. performed proteomic and mass spectrometry analysis. Shu. T. and T. Y. provided instructions. T. T. wrote the paper with support from all authors. H. S. supervised the work.

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