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Analysis of tissue-specific region in sericin 1 gene promoter of *Bombyx mori*

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Abstract

The gene encoding sericin 1 (*Ser1*) of silkworm (*Bombyx mori*) is specifically expressed in the middle silk gland cells. To identify element involved in this transcription-dependent spatial restriction, truncation of the 5′ terminal from the sericin 1 (*Ser1*) promoter is studied in vivo. A 209 bp DNA sequence upstream of the transcriptional start site (−586 to −378) is found to be responsible for promoting tissue-specific transcription. Analysis of this 209 bp region by overlapping deletion studies showed that a 25 bp region (−500 to −476) suppresses the ectopic expression of the *Ser1* promoter. An unknown factor abundant in fat body nuclear extracts is shown to bind to this 25 bp fragment. These results suggest that this 25 bp region and the unknown factor are necessary for determining the tissue-specificity of the *Ser1* promoter.

Keywords: *Bombyx mori*; Sericin 1 promoter; Tissue-specificity; Ectopic expression; *Autographa californica* multiple nuclear polyhedrosis virus

The silkworm, *Bombyx mori*, is a domestic insect that has been used in silk production for several thousand years. Silk protein synthesis occurs in a pair of silk glands that can be divided into three distinct compartments: posterior silk gland (PSG), middle silk gland (MSG), and anterior silk gland (ASG). Fibroin and its linked subunits are expressed exclusively in PSG, whereas the sericins are produced specifically in MSG [1,2]. The silk gland constitutes an attractive model for understanding the molecular mechanism governing spatially and temporally programmed transcription [3].

Six kinds of sericin protein molecules are coded by two genes, *Ser1* and *Ser2*, through alternative splicing [4,5]. The *Ser1* gene encodes the four major constituents of sericins. Their mRNAs are 10.5, 9.0, 4.0, and 2.8 kilonucleotides in length [4,6]. Within the upstream sequence of the *Ser1* gene, three specific protein binding sites: SA (−103 to −85), SB (−149 to −135), and SC (−204 to −183) have been characterized by in vitro transcription and DNase I footprinting [7]. Several factors were shown to interact with these elements. Silk gland factor I (SGF-1) that binds to the SA site exists specifically in the silk gland extract. It was thought to be required initially for the development of silk glands and subsequently utilized in the transcription control of genes encoding silk protein [8]. SC binding factor was particularly abundant in the MSG extract. Removal of this SC element curbed in vitro transcription in crude MSG nuclear extracts. By mobility shift and nuclease protection assays, SGF-3/POU-M1 was found to interact with the SC site [9,10]. Analysis of the expression pattern of SGF-3 and its relationship to sericin 1 gene expression in vivo suggested that there might be another unidentified factor or mediator required for in vivo transcription [11].

In recent years, the PSG specificity of fibroin promoters has been studied utilizing newly established transgenic

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techniques [12] or transient in vivo gene expression systems like the ballistic method [13,14]. However, these techniques require special apparatus and skilled workers. Current knowledge concerning the *Ser1* promoter and factors associated with transcription regulation has been either partially or totally derived from in vitro assays. The mechanism responsible for the tissue-specific expression of the *Ser1* gene still remains poorly understood. In our recent work, we found that some strains of silkworm were permissive to *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), which could serve as a novel in vivo gene delivery tool for the silkworm [15,16]. The enhanced green fluorescent protein (EGFP) gene, located downstream of 1.6 kb *Ser1* promoter, was mainly expressed in MSG [17]. A slight leaky expression was found in the posterior and anterior silk gland, which could be attributed to the lack of some mechanisms related to chromatin topology in the regulation of gene expression [18].

In the present study, we are interested in studying the possible cis-acting region(s) that takes part in determining the tissue-specific expression of the sericin 1 gene in vivo. The observation is focused on the expression of EGFP in MSG and the fat body. Using the AcMNPV vector and the EGFP reporter gene, successive deletions from the 5′ upstream region of the *Ser1* promoter were carried out. Following the observation of ectopic expression in the fat body, overlapping dissections of the related region were assayed and a relative ectopic expression inhibitory region was identified. Moreover, electrophoretic mobility shift assays (EMSAs) were performed for a preliminarily analysis of the protein binding to this inhibitory region.

Materials and methods

Cells and silkworm larvae. Sf9 insect cells were cultured in Grace's medium (Invitrogen) supplemented with 10% (V/V) FCS (Invitrogen), and 100 μg/ml streptomycin and 100 U/ml penicillin at 27 °C in a humidified incubator. Larvae of *B. mori* strain 54A (kindly provided by Sericultural Research Institute, Chinese Academy of Agricultural Sciences) were used in this experiment. The larvae were fed with mulberry leaves and reared at 25 °C.

5′ truncation of the sericin 1 gene promoter. pFFa2-SerEGFP, which contains the sericin 1 promoter, the complete EGFP gene and *Ser1* 3′ flanking sequence including the polyA signal site sequence was constructed previously in our laboratory [17]. The sense primers appended with an *Nco*I site (in bold) were designed as follows: P1.2, 5′ CATG**CCATGG**T TGAGAGGAAATCG 3′(nucleotide positions −1188 to −1174), P0.92, 5′ CATG**CCATGG**TAACATTTCCCGG 3′ (nucleotide positions −863 to −850), P0.64, 5′ CATG**CCATGG**TGCACAATGCTAAT 3′ (nucleotide positions −586 to −572), P0.43, 5′ CATG**CCATGG**CGAAATTTGTG AT 3′ (nucleotide positions −377 to −362), P0.27, 5′ CATG**CCATGG**CG GGTCTCAACGA 3′ (nucleotide positions −217 to −204), P0.18, 5′ CA TG**CCATGG**AACAAAAACAATAACTT 3′ (nucleotide positions −124 to −107). Each of the sense primers above were paired with an antisense primer Pser 5′ GG*GGTACC*GGTCTTTGGATCG 3′ (nucleotide positions +36 to +49, with a *Kpn*I site shown in italics) to acquire the 5′ truncated sericin 1 promoter fragment. PCR was performed under the following conditions: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 90 s, and an extra 72 °C extension for 10 min. The PCR products were digested with *Nco*I and *Kpn*I and cloned into the same sites of pFFa2-SerEGFP, which resulted in pFFa2-1.2serEGFP, pFFa2-0.92serEGFP, pFFa2-0.64serEGFP, pFFa2-0.43serEGFP, pFFa2-0.27serEGFP, and pFFa2-0.18serEGFP. These recombinant plasmids were confirmed by restriction endonuclease digestion and DNA sequencing.

Partial deletion of the tissue-specificity related region. Primers used in this section are presented in Table 1. Generation of these recombinant plasmids is schematically shown in Fig. 1. SerΔ1AB promoter sequence was obtained using primer pairs (P1F-528 and Pser). This PCR product was digested with *Nco*I and *Kpn*I, cloned into the same sites of pFFa2-0.64serEGFP, and resulted in pFFa2-serΔ1ABEGFP. To produce the serΔ2AB promoter fragment, the Δ2A fragment was amplified by PCR using PFsacII and P2R-547, while Δ2B was acquired using P2F-499 and Pser. These two PCR products were digested with *Xba*I and ligated by T4 DNA ligase. The SerΔ2AB promoter sequence was amplified from the ligation mixture using primer pairs (PFsacII and Pser). It was digested with *Sac*II and *Kpn*I and then inserted into the same sites of pFFa2-0.64serEGFP to generate pFFa2-serΔ2ABEGFP. pFFa2-serΔ3ABEGFP, pFFa2-serΔ4ABEGFP, pFFa2-serΔ5ABEGFP, and pFFa2-serΔ6ABEGFP were obtained similarly.

Transfection and construction of recombinant AcMNPV. The donor plasmids were transfected into *E. coli DH10BacΔEGT* [15] cells to make recombinant bacmids. Recombinant baculovirus bacmids were verified by PCR analysis using the *EGFP*-specific primer $P_{EGFP\text{-}1}$ (5′ AAGCTTGTC GACAGATCTGCATGCATGGTGAGC 3′) and the M13 reverse

Table 1
Primers used in recurrent deletion of MSG specificity related region

Primers	Sequence	Site in Ser1 promoter
PFSacII	*5′ GAAGTGCAAATTGCCCGTCGT 3′*	
P1F-528	5′ CATG**CCATGG**AAAACAGCACACACACT 3′	−528 to −511
P2R-547	5′ GACCTCTAGACTTTTATGCATTATTTTA 3′	−564 to −547
P2F-499	5′ CTAGTCTAGATTTGACGCACACACGC 3′	−499 to −483
P3R-521	5′ GTGCTCTAGACTGTTTTCTTTATTGATT 3′	−538 to −521
P3F-476	5′ GACCTCTAGACTATTTATTGTCAAACTTT 3′	−476 to −457
P4R-477	5′ CCAGTCTAGATACATGCGTGTGTGCGT 3′	−494 to −477
P4F-426	5′ GTCGTCTAGATTAAATATTGTT 3′	−426 to −411
P5R-451	5′ GACCTCTAGACAAAAGTTTGACAATAA 3′	−471 to −454
P5F-389	5′ GACGTCTAGATAGTGTAGTCTTGGCGAA 3′	−389 to −371
P6R-426	5′ CAGGTCTAGATTCTCAGTTTGAACACAG 3′	−444 to −426
P6F-378	5′ CTGGTCTAGATTGGCGAAATTTGTGAT 3′	−378 to −362

Primer *PFsacII* was the sequence upstream of *Sac*II site lies in ampicillin resistant gene. Primer P1F-528 was appended with *Nco*I site (in bold) and paired with Pser (seen in Materials and methods) to abandon the 1AB fragment (−585 to −528 bp) in sericin 1 promoter. The rest primers in this table were appended with *Xba*I (underlined) restriction endonuclease sites, respectively.

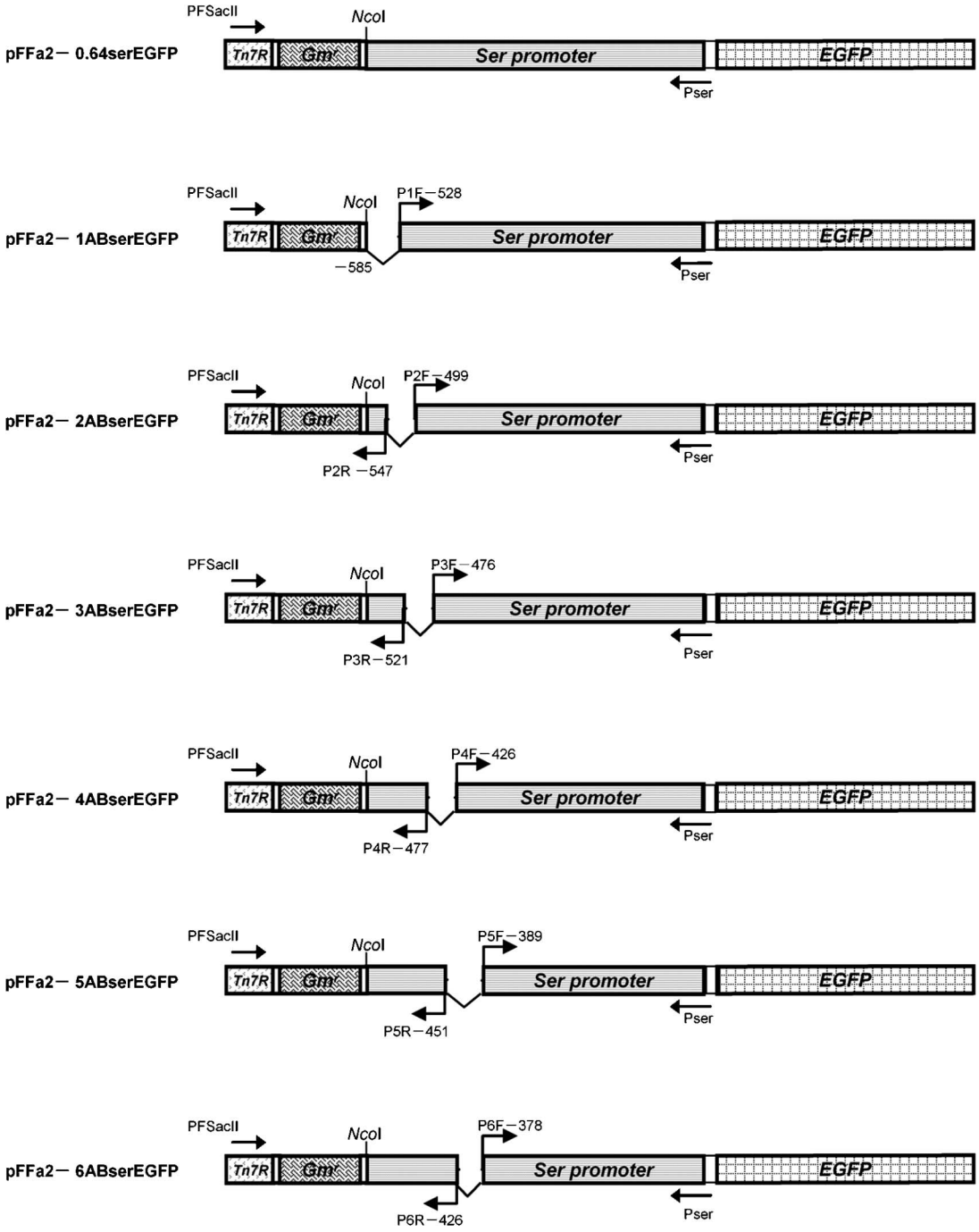

Fig. 1. Diagram of plasmid structures generated for overlapping deletion study. The plasmids containing different overlapping deletions in the −586 to −378 region of the sericin 1 gene promoter are shown schematically. The bent arrow indicates the transcription initiation site. The Sericin 1 promoter sequence was abbreviated to *Ser* promoter. The blank region linked by bent lines symbolizes the deletion region in the sericin 1 promoter.

primer (5′ CAGGAAACAGCTATGAC 3′). M13 forward (5′ GTTT TCCCAGTCACGAC 3′) and M13 reverse primers were used to determine the purity of the recombinant bacmid. This process is schematically shown in Fig. 2.

Transfections with various bacmid DNA constructs into Sf9 cells were carried out using Cellfectin reagent in 6-well plates (10^6 cells per well in 2 ml medium). Generation and large-scale harvest of budded virus (BV) of the recombinant baculovirus were performed according to the instruction manual "Bac-to-Bac baculovirus expression systems" (Invitrogen) using the Sf9 cell line.

Characterization of BV production and injection of silkworm larvae. Viruses were collected by centrifugation at 35,000*g* for 60 min. The viruses were resuspended in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4–7.6) supplemented with 1% (v/v) fetal bovine serum and stored at −70 °C. Virus titer was determined by a Tissue Culture Infectious Dose 50 ($TCID_{50}$) method. Silkworm larvae in early fifth instar were used for infection by recombinant viruses. Ten microliters of the viral solution (1×10^6PFU/larva) was injected into the hemocoel of the larvae. Control larvae were injected with EGT-null AcMNPV (1×10^6PFU/larva) [15].

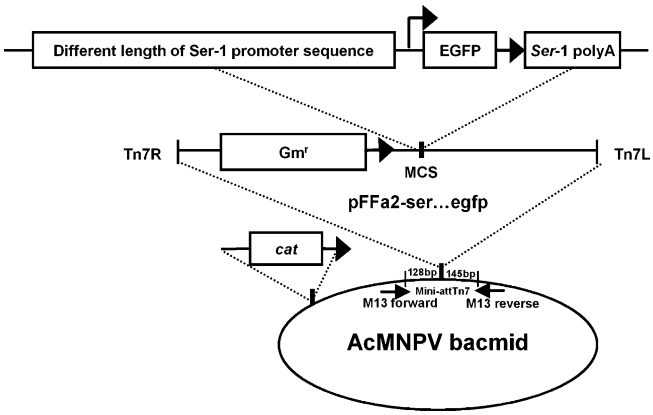

Fig. 2. Schematic representation of the recombinant bacmid procedure. The EGFP cassette was cloned into the multiple cloning site (MCS) of pFFa2 to form the recombinant donor plasmid. When the recombinant plasmid was transfected into *E.coli DH10BacΔEGT*, the EGFP cassette was transposed into the polyhedron site of AcMNPV bacmid by Tn7-based transposition.

Inspection of fluorescence in tissues. About five days post-injection, tissues (silk glands and fat bodies) were dissected out and washed twice in ice-cold PBS. The samples were observed and photographed with a fluorescence microscope (Leica MZ FL III, Switzerland, with GFP Plus fluorescence filter set).

Preparation of nuclear extracts. Nuclear extracts from the MSGs and fat bodies of 2-day-old fifth instar larvae of *B. mori* were performed essentially as described [19,20] with slight modifications. Briefly, the 2-day-old fifth instar larvae were dissected. MSGs and fat bodies were squeezed out. Those tissues were washed twice in pre-chilled PBS before being frozen immediately under liquid nitrogen and stored at −80 °C until use.

All of the following procedures were carried out at 0–4 °C. Ten grams of MSGs and fat bodies were thawed, minced, and suspended in 40 ml buffer A (10 mM Hepes-KOH, pH7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF). After 10 min incubation on ice, the homogenates were centrifuged at 1000*g* for 5 min. The supernatants were removed and the cell pellets were resuspended in three packed-tissue volumes of buffer A containing 0.1% Nonidet P40 (NP-40). Cells were incubated on ice for 10 min and then Dounce homogenized 30 times. The homogenates were filtered through six layers of gauze and nuclei were pelleted by centrifugation of the filtrates at 3300*g* for 15 min at 4 °C. The nuclear pellets were resuspended in 1/2 packed-pellet volumes of hypotonic buffer B (20 mM Hepes-KOH, pH 7.9, 20% v/v glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) and then 1/2 packed-pellet volumes of high salt extraction buffer C (20 mM Hepes-KOH, pH 7.9, 20% v/v glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) was added to suspension with gentle mixing. These churning mixtures were incubated on ice for 30 min and centrifuged again for 30 min at 25,000*g*. The supernatant was stored at −80 °C until use. The protein concentration of each extract was determined according to the method of Bradford [21].

Electrophoretic mobility shift assay. After annealing, a double-stranded probe was obtained by filling the single-stranded ends with dGTP, dCTP, dTTP, and [α-P^{32}]dATP by Klenow enzyme (Fig. 6). The binding reaction was carried out in a 15 μl mixture containing 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 1 μg poly(dI–dC).poly(dI–dC), 0.1 ng of P^{32} labeled probe, and 10 μg of extract protein and 1 μl BSA (2 mg/ml), in the presence or absence of 10 ng of unlabeled competitors. The reactions were incubated for 30 min at 4 °C. Protein–DNA complexes were visualized on 5% polyacrylamide gels in 0.5× TBE (50 mM Tris–borate, pH 8.3, and 1 mM EDTA) at 4 °C. The gels were exposed for autoradiography.

Results

The −378 to −586 region of the sericin 1 gene promoter affects the tissue-specificity

5′ truncated promoters were amplified and cloned into the donor plasmids. Recombinant plasmids were verified by PCR and restriction endonuclease digestion (Fig. 3). Recombinant AcMNPVs carrying different truncations of the 5′terminal *Ser1* promoter were then obtained as described in the Materials and methods section and named Ac1.2serEGFP, Ac0.92serEGFP, Ac0.64serEGFP, Ac0.43serEGFP, Ac0.27serEGFP, and Ac0.18serEGFP. The recombinant virus (Ac1.6serEGFP) was derived from pFFa2-SerEGFP [17].

Five to six days post injection silkworm larvae showed typical symptoms of infection. After dissection, silk glands and their surrounding tissues were subjected to fluorescent observation. Fluorescence derived from EGFP indicated that the promoter directed protein expression in tissue cells successfully. The fluorescence was limited to silk glands until deletion of the *Ser1* promoter to position −586 (visible in the Ac0.64serEGFP group). There was no visible expression in other tissues such as the integument, fat body, and tracheal bush (Fig. 4A, group Ac0.64serEGFP). Ectopic expression was observed when sequences upstream from −377 were deleted. Ubiquitous fluorescence obviously appeared in larvae injected with Ac0.43serEGFP. Further deletion of sequences upstream from −217 resulted in no fluorescence (group Ac0.27serEGFP, Fig. 4A), as did the deletion of sequences upstream from −124 (group Ac0.18serEGFP, Fig. 4A). Silk glands and fat bodies from the Ac0.64serEGFP and Ac0.43serEGFP groups were removed from the larvae and examined in dishes containing PBS. Fluorescence was observed in the silk glands from both groups as well as in the fat bodies from the Ac0.43serEGFP group but not from the Ac0.64serEGFP group

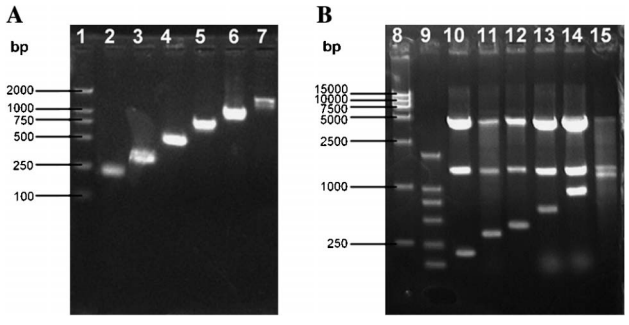

Fig. 3. Verification of the recombinant plasmids with 5′ truncation of sericin 1 promoter sequence by PCR and digestion by restriction endonuclease. Lanes 2–7 in (A) showed the PCR products of successive deletions of the sericin 1 promoter (180, 270, 430, 640, 920, and 1200 bp, respectively). Results of verification pFFa2-0.18serEGFP, pFFa2-0.27serEGFP, pFFa2-0.43serEGFP, pFFa2-0.64serEGFP, pFFa2-0.92serEGFP, and pFFa2-1.2serEGFP by*Nco*I/*Sph*I are shown in lanes 10–15 in (B), respectively. Lanes 1 and 9 showed the DL2000 DNA marker, while DL15000 DNA marker is shown in lane 8. The band lengths are indicated by arrows.

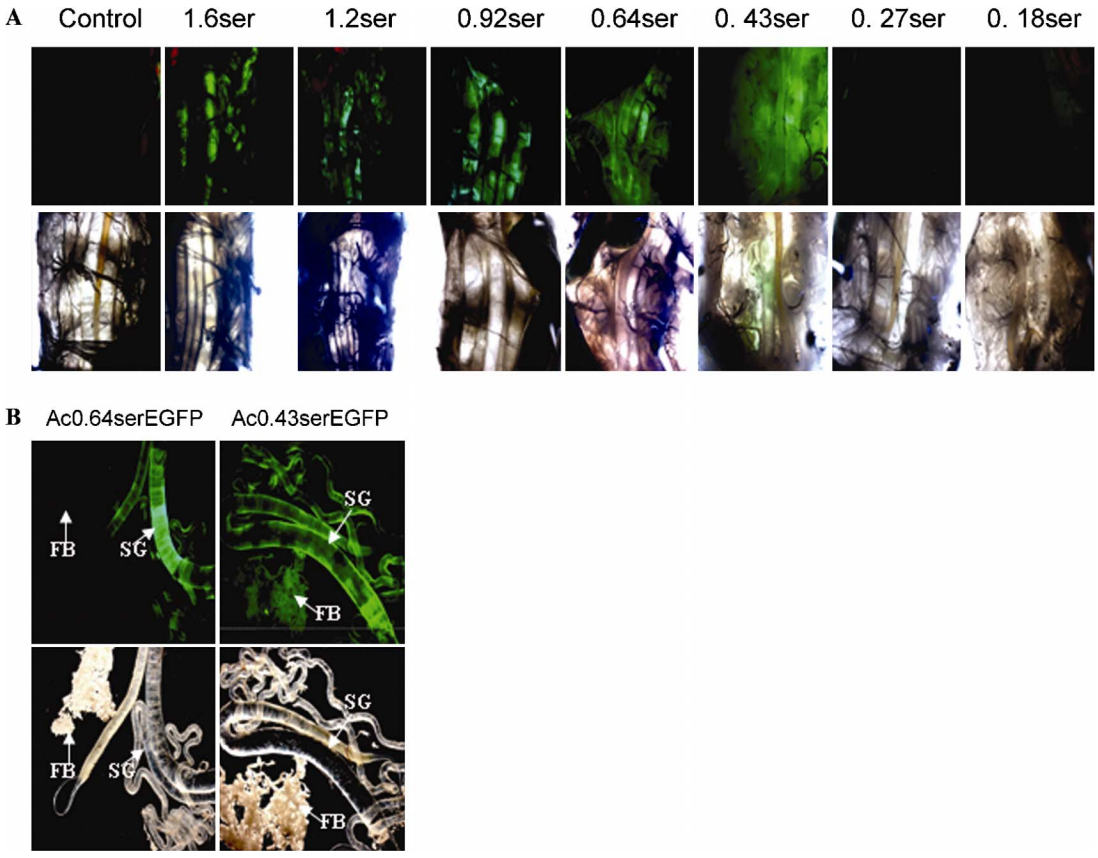

Fig. 4. In vivo expression of EGFP driven by the 5′-deletion mutants of the sericin 1 gene promoter. Photographs in the upper panels show fluorescent images while the lower panels show the light image. (A) Fluorescent observation of larvae injected with different recombinant viruses. 1.6ser to 0.18ser was short for group Ac1.6serEGFP, Ac1.2serEGFP, Ac0.92serEGFP, Ac0.64serEGFP, Ac0.43serEGFP, Ac0.27serEGFP, and Ac0.18serEGFP, respectively. (B) Silk glands and fat-bodies from larvae injected with Ac0.43serEGFP and Ac0.64serEGFP were observed by fluorescent microscopy (Leica MZ FL III, Switzerland). Scan bars, 1 mm. Arrows indicate: FB, fat bodies; SG, silk glands.

(Fig. 4B). These results suggest that the region from −586 to −378 upstream from the transcriptional start site was necessary to suppress the ectopic expression of the *Ser1* promoter. We named this the tissue-specificity related region.

Overlapping deletion analysis of tissue-specificity related region in vivo

By overlapping deletion of the tissue-specificity related region, six recombinant AcMNPVs were constructed, namely AcserΔ1ABEGFP, AcserΔ2ABEGFP, AcserΔ3ABEGFP, AcserΔ4ABEGFP, AcserΔ5ABEGFP, and AcserΔ6ABEGFP. Five days post injection, larvae that had been infected with AcserΔ3ABEGFP showed fluorescence derived from EGFP in both the silk glands and the fat body. Fluorescence in the remaining groups was limited to the silk glands (Fig. 5). These results indicated that the 3AB fragment (−521 to −476 upstream from the transcriptional start site) was responsible for the suppressive expression of the sericin 1 gene in the fat body. A 21 bp region upstream from the transcriptional start site, −521 to −500, overlapped between fragments 2AB and 3AB (Fig. 1). Thus, it appeared that the repression element of the *Ser1* promoter in the fat body was located in the region −500 to −476 upstream from the transcriptional start site.

Preliminary analysis of the possible DNA binding protein

The specificity of the DNA–protein interactions was ascertained by the presence of the expected shift band. When a probe labeled with [α-P^{32}]dATP was used in the EMSA reaction, one clear complex was obtained in the fat body extracts (Fig. 6, lane 3). The probe formed a faint DNA–protein complex with nuclear extracts from MSG migrating to the same position (Fig. 6, lane 2). When excess cold probe was added to the reaction mixture, the migrating protein complex disappeared (Fig. 6, lanes 4 and 5). This unidentified protein might take part in determining the tissue-specificity of the *Ser*1 gene along with the above repressing region.

Discussion

Previous analysis of transcription regulatory elements of the sericin 1 gene promoter focused on the region of basic transcriptional activity of the promoter. Matsuno et al. [7] reported that the −331 *Ser*1 promoter retained complete

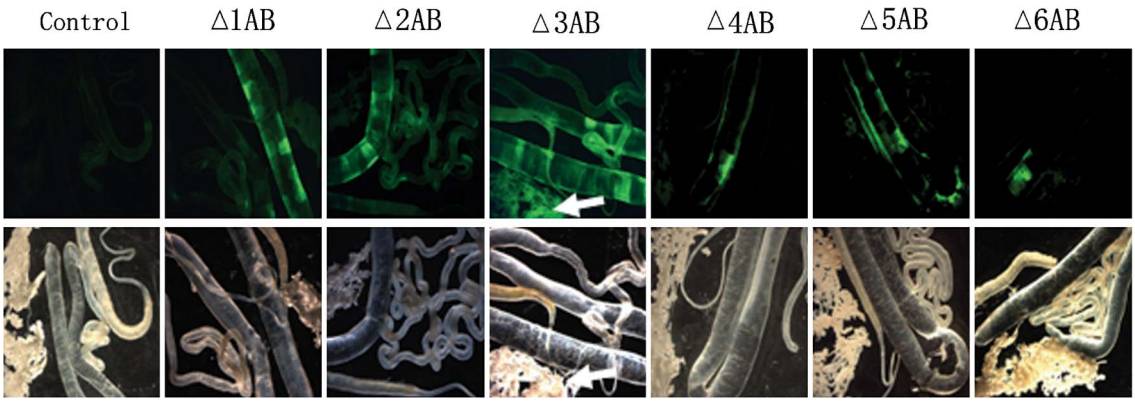

Fig. 5. Expression of EGFP driven by overlapping deletion of tissue-specific region in *sericin 1* promoter. Photographs in the upper panels show fluorescent images while lower panels show light images. Fifth instar larvae injected with AcserΔ1ABEGFP, AcserΔ2ABEGFP, AcserΔ3ABEGFP, AcserΔ4ABEGFP, AcserΔ5ABEGFP, and AcserΔ6ABEGFP (10^6 pfu/larva), respectively. Five days post injection, larvae in each group were dissected and observed. Results of these groups are shown schematically as Δ1AB–Δ6AB, respectively (Leica MZ FL III, Switzerland). Scan bars, 1 mm.

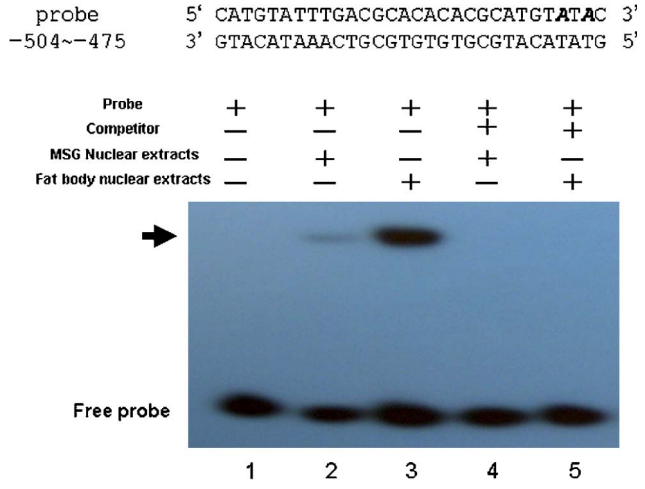

Fig. 6. EMSA with nuclear extracts from MSGs and fat bodies. Nuclear extracts from MSGs and fat bodies were used. For the competition experiments, a 100-fold excess of cold probe was added to the reaction mixture before the addition of the labeled probe. Arrowhead indicates the position of the specific DNA–protein complexes. The nucleotide sequence of the probe is shown in the upper panel. Bold letters denote labeled nucleotides. The position of the probe on the 5′-upstream region is indicated in the lower panel.

activity by in vitro transcription. The regions to be analyzed by foot printing were within −331 to −50 of the 5′ upstream sequence of the *Ser1* gene. The three known protein binding sites (SA, SB, and SC) were within this region. In the present work, with the advantage of rAcMNPV system, 1.6 kb *Ser1* promoter was analyzed in vivo. The regulatory and transcriptional mechanisms of the *Ser1* gene were focused on the tissue-specificity of the *Ser1* promoter.

In this study, a 209 bp region (−586 to −378 relative to the transcriptional start site) was observed necessary to suppress the ectopic expression of *Ser1* promoter in tissues other than silk gland. Furthermore, a 25 bp region (−500 to −476 relative to the transcriptional start site) was ascertained to play an important role in suppressing ectopic expression of the gene driven by the *Ser1* promoter. Considering that loss of this 25 bp region resulted in ectopic expression of the reporter gene in the fat body, we proposed that there might be some unknown factor or mediator in the fat body which recognized motif(s) within this 25 bp region. Gel shift assays verified that an unidentified protein existed more abundantly in the fat body than that in the MSG, supporting the above hypothesis. The activity of *Ser1* promoter was suppressed in the fat body because of negative regulation by the unidentified factor. This unidentified factor might be involved in determining the tissue specificity in a negative manner by recognizing and interacting with motif(s) within this 25 bp region. Deletion of the binding site for this unknown factor from the *Ser1* promoter was necessary to activate expression of the downstream reporter gene in fat body. This would explain why we could see fluorescence in the fat body with the serΔ3AB promoter (Fig. 5). These findings might also answer to what Matsunami et al. [11] thought that there might be another unidentified factor or mediator required for in vivo transcription.

Simultaneous, some other possible binding sites were found existed in this 209 bp region of the *Ser1* promoter, particularly, similar sequences to the octamer-like element which was recognized by the POU-domain protein [9,25]. The POU-domain is a highly conserved region found in a number of transcription factors and products of developmentally controlled genes [23,24]. More than one octamer-like element was identified in the 5′ upstream sequence and even in the intron of the silkworm fibroin gene. Several binding proteins have been identified as stimulators of the transcription of fibrion genes [25]. Furthermore, the SC region of *Ser1* promoter contains octamer-like sequences. It was shown that the insertion of five repeats of the SC oligonucleotides to a promoter resulted efficiently in increasing the transcription activity of 8.8-fold [7,26]. These indicated that there have been some positive elements and factors involved in regulating the expression of silk gene. Based on the present reports, we proposed that the tissue-specific expression of *Ser*1 is under the control of

activators as well as repressors, which was similar to that of the *fibl* promoter [22].The specific expression of the sericin 1 gene could be the result of the coordination of multiple factors and cis-acting elements.

Moreover, high efficiency of gene transfer as is demonstrated by expression of EGFP reporter in vivo indicates that the recombinant AcMNPV could serve as a vector for transcription regulation research in silkworms. This method is valuable also for its convenience and easy to be examined, and it gives us a good tool in studying the functional genomic of silk gene. In general, these studies may contribute to understanding the mechanisms of tissue-specific gene expression and benefit to improving the future production of silk-manufacture industry.

Acknowledgments

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