Inhibition of the binding of MSG-intermolt-specific complex, MIC, to the *sericin-1* gene promoter and *sericin-1* gene expression by POU-M1/SGF-3

Mai Kimoto • Tsuyuki Kitagawa • Isao Kobayashi • Tomohiro Nakata • Asato Kuroiwa • Shigeharu Takiya

M. Kimoto • T. Kitagawa • T. Nakata • A. Kuroiwa • S. Takiya
Graduate School of Life Science, Hokkaido University, North 10, West 8, Kita-ku, Sapporo 060-0810, Japan

A. Kuroiwa • S. Takiya (mail mark)
Division of Biological Sciences and Center for Genome Dynamics, Faculty of Science, Hokkaido University, North 10, West 8, Kita-ku, Sapporo 060-0810, Japan
e-mail: takiya@sci.hokudai.ac.jp (S. Takiya)

I. Kobayashi
National Institute of Agrobiological Sciences, 1-2 Owashi Tsukuba, Ibaraki 305-8634, Japan

Abstract

The *sericin-1* gene encoding a glue protein is expressed in the middle silk gland (MSG) of the silkworm, *Bombyx mori*. A member of the class III POU domain transcription factors, POU-M1, was cloned as the factor bound to the SC site of the *sericin-1* promoter and has been proposed to be a positive transcription factor. In this study, we analyzed the expression pattern of the *POU-M1* gene in fourth and fifth instars in comparison with the pattern of the *sericin-1* gene. The *POU-M1* gene was expressed strongly in the region anterior to the *sericin-1*-expressing portion of the silk gland at both feeding stages. As the *sericin-1*-expressing region expands from the posterior to middle portions of the MSG in the fifth instar, the *POU-M1*-expressing region retreated from the middle to anterior portion. Introduction of the expression vector of POU-M1 into the silk glands by gene gun technology repressed promoter activity of the *sericin-1* gene, suggesting that POU-M1 regulates the *sericin-1* gene negatively. An *in vitro* binding assay showed that POU-M1 bound not only to the SC site but also to other promoter elements newly detected *in vivo.* Another spatiotemporal specific factor MIC binds to these elements, and POU-M1 competed with MIC to bind at the -70 site essential for promoter activity. These results suggest that POU-M1 is involved in restricting the anterior boundary of the *sericin-1*-expressing region in the silk gland by inhibiting the binding of the transcriptional activator to the promoter elements.

Keywords *Bombyx mori*, Sericin-1, Silk gene, Silk gland, Homeodomain, Gene gun

Introduction

The silkworm *Bombyx mori* produces massive silk proteins in the silk gland at the last instar to make cocoons, but a certain amount of silk proteins are constantly synthesized throughout all larval instars except for each molting stage (Maekawa and Suzuki, 1980; Couble *et al.*, 1983; Ishikawa and Suzuki, 1985). The silk gland can be divided into distinct subparts on the basis of its morphology and function. The silk genes encoding fibrous proteins (fibroin heavy chain, fibH; light chain, fibL) and a glycoprotein (fibrohexamerin, fhx) are specifically expressed in the posterior silk gland (PSG), while those encoding glue proteins (sericin-1, sericin-2, sericin-3) are found in the middle silk gland (MSG) (Couble *et al.*, 1987; Takasu *et al.*, 2010). Studies of such stage- and tissue-specific expression of the silk genes contribute to understanding the fundamental mechanisms of eukaryotic transcriptional regulation. Several protein factors have been proposed to be involved in transcriptional regulation of the silk genes (Fukuta *et al.*, 1993; Mach *et* al., 1995; Hui *et al.*, 1990; Takiya *et al.*, 1997, 2011), and the gene expression profiles of those factors in the silk gland have been characterized individually (Kimoto *et al.*, 2010) or by genome-wide analysis (Li *et al.*, 2011), but their roles *in vivo* remain unclear.

Expression of the *sericin-1* gene is localized in the posterior part of the MSG through the first to fourth instars and expands to the middle part in the final instar (Matsunami *et al.*, 1998; Takasu *et al.*, 2010). Several promoter elements of the *sericin-1* gene have been detected *in vitro* (Matsuno *et al.*, 1989, 1990), and the *Bombyx* fork head (Fkh) binds to the SA site (-103/-85) and POU-M1 binds to the SB (-149/-135) and SC (-204/-183) sites, respectively (Fukuta *et al.*, 1993; Mach *et al.*, 1995). POU-M1 has been proposed to be the activator of

the *sericin-1* gene, because removal of the SC site decreased promoter activity *in vitro*. In embryo and early larval instars, however, POU-M1 is expressed in the anterior silk gland (ASG) and anterior part of MSG where *sericin-1* is not expressed (Kokubo *et al.*, 1997; Matsunami *et al.*, 1998). Instead, the recent work identified a novel activator candidate, termed MIC (MSG-intermolt-specific complex), which binds to the TAAT-containing elements of *sericin-1* promoter (Takiya *et al.*, 2011). Here we sought to determine the role of POU-M1 in the transcriptional regulation of the *sericin-1* gene.

POU-M1 is a *Bombyx* homologue of *Drosophila* Drifter belonging to the class III POU domain proteins (Fukuta *et al.*, 1993). Class III POU domain genes have been also identified in zebrafish, *Xenopus* and mammals, and they are expressed predominantly in the nervous system (Ryan and Rosenfeld, 1997). For example, *Drosophila* Drifter is required for expression of the Dopa decarboxylase gene in selected dopaminergic neurons (Johnson and Hirsh 1990). Later, Drifter was also found to be necessary for tracheal morphogenesis and development of the wing imaginal disc as a direct activator of other transcription factors (Anderson *et al.*, 1996; Certel *et al.*, 2000). In addition, Drifter regulates innate immune defense by activating antimicrobial peptide genes (Junell *et al.*, 2010). Thus, Drifter has been shown to be involved in multiple developmental events in the fly. Similarly, in *Bombyx*, POU-M1 is expressed in not only the silk glands but in various organs such as the tracheal system, central nervous system, prothoracic glands and salivary glands in embryogenesis (Kokubo *et al.*, 1997; Matsunami *et al.*, 1998).

In this study, we analyzed the expression pattern of *POU-M1* in the silk gland in late larval instars. The expression region of *POU-M1* was anterior to that of *sericin-1* in the MSG. We demonstrated that the ectopic expression of *POU-M1* resulted

in reduction of the activity of the *sericin-1* promoter in the silk gland. Electrophoretic mobility shift assay (EMSA) showed that POU-M1 could compete with MIC to bind to the elements of the *sericin-1* promoter. From these results, we discuss the role of POU-M1 in the regulation of *sericin-1* gene expression.

Materials and Methods

Animals

Eggs of the silkworm *B. mori* (Kinshu x Showa) were purchased from Ueda Sanshu (Ueda, Japan). Larvae were reared at 25 °C under LD 16:8 on an artificial diet obtained from Nippon Nosan-Kogyo (Yokohama, Japan). The larvae were staged as described previously (Kiguchi and Agui, 1981; Suzuki *et al.*, 1990).

RNA extraction and RT-PCR

Total RNA was extracted from the silk gland using the Illustra RNAspin Mini RNA Isolation kit (GE Healthcare) according to the manufacturer's instructions. Silk glands were dissected from day 2 of the fourth instar, fourth molting instar and day 2 of the fifth instar. They were divided into three parts of the MSG (anterior, middle and posterior), and two parts of the PSG (anterior and posterior). MSGs were separated at natural bending points and shortened by cutting out the turns to reduce cross-contamination of mRNA among regions (Fig. 1A, Couble *et al.*, 1987). cDNA was synthesized using 1 µg total RNA with a PrimeScript RT-PCR kit (Takara) according to the manufacturer's instructions. Reverse transcription reactions were performed in a final volume of 20 µl, and 1 µl of the cDNA was used as a template for PCR. PCR amplification was performed in a volume of 50 µl with ExTaqHS polymerase (Takara). *Sericin-1* was amplified for 20 cycles with forward primer 5'-ATAGTCGTCTTATCATCG-3' and reverse primer 5'-AACGCAATCAAAGTGCAG-3'. *Ribosomal protein 49* (*Rp49*) was amplified for 20 cycles with forward primer 5'-CATACAAGATGGCTATAAGACC-3' and reverse primer 5'-GTGAGCTGCTGGG CTCTTTC-3'. Endogenous *POU-M1* was amplified

for 35 cycles with forward primer 5'-CGGAATTCGGAGCGGGATCAGCCCGAGGAG-3' and reverse primer 5'-CTGTCGACGTGGCGTCATCCTCTTCTCTTT-3', and transfected *FLAG-POU-M1* was amplified with forward primer 5'-AGGAATTCATGGACTACAAGGACGACGATG-3' and reverse primer 5'-AAGGTCGACTTAGTGCGCTGCCAGTGTGTG-3'. PCR products were detected on a 12% polyacrylamide gel for *sericin-1* and 1% agarose gels for the other genes.

Real-time RT-PCR

Real-time RT-PCR was performed using the ABI 7300 PCR system and Power SYBR Green PCR Master mix (Applied Biosystems). The PCR reaction mixture (10 µl) contained 1 x SYBR Green PCR Master mix, 0.4 µM of each primer, and 0.5 µl of cDNA. The amplification conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec. The *sericin-1* was amplified with forward primer 5'-ATAGTCGTCTTATCATCG-3' and reverse primer 5'-AACGCAATCAAAGTGCAG-3'. *POU-M1* was amplified with forward primer 5'-GAGCCTGCCGACATGAAGTA-3' and reverse primer 5'-ACGTCCAAGGGTTCCCTATC-3'. *GAPDH* was amplified with forward primer 5'-TGTGCTCCGTGCTTCTATT-3' and reverse primer 5'-GCCATGGGTGGAATCATACT-3'. Dissociation curves were obtained to confirm the specificity of the amplified DNA. The data were normalized separately with GAPDH. PCR reactions were performed twice on the same cDNA sample.

In situ hybridization

Probes were obtained by PCR with cDNA of MSG-A for *POU-M1* and pBmS9014 (Okamoto *et al.*, 1982) for *sericin-1*. The position of *POU-M1* and *sericin-1* probes corresponded to the nucleotide sequence 1-432 (AY334012) and 106-775 (J01040), respectively.

Sense and antisense digoxigenin (DIG)-labeled riboprobes were synthesized by *in vitro* transcription using DIG RNA Labeling mix (Roche Diagnostics). The silk glands isolated from day 2 fifth instars were frozen in Tissue-tek (Sakura Finetek) and kept at -80°C until use. The silk glands were sectioned at 4-μm thickness using a cryostat and thaw-mounted onto Matsunami adhesive silane-coated slides (Matsunami Glass). Tissues were then fixed by incubating in a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.76 mM KH_2PO_4, pH7.4) for 10min and washed with PBS for 5 min three times. The sections were treated with 10 ng/ml proteinase K (Wako) in PBS for 10 min. The slides were washed again with PBS and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine/HCl for 10 min. After prehybridization for 2 h at room temperature in 55% formamide, 6 x SSPE (0.15 M NaCl, 8.65 mM NaH_2PO_4 and 1.25 mM EDTA, pH7.4), 0.5 x Denhardt's solution and 0.5 μg/ml tRNA, the sections were incubated at 65°C for 18 h in the same buffer containing 100-250 ng/ml DIG-labeled riboprobes. The hybridized sections were washed three times in 0.2 x SSC at 65°C for 20 min. The slides were incubated in 1% blocking reagent (Roche Molecular Biochemicals) in DIG buffer 1 (100 mM maleic acid, 150 mM NaCl, 0.01% TritonX-100, pH7.5) for 1 h and then in 1:5000-diluted anti-DIG antibody in DIG buffer 1 containing 1 % blocking reagent for 30 min. After washing twice with DIG buffer 1 for 15 min and equilibrating with freshly prepared DIG buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH9.5) for 5 min, the sections were incubated at room temperature with a Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) tablet (Roche Molecular Biochemicals) in H_2O for a few minutes (for *sericin-1*) or 24 h (for *POU-M1*). Slides were rinsed by repeated soaking in PBS, and decolored in a graded series of ethanol.

Electrophoretic Mobility Shift Assay (EMSA)

Tissue extracts from each portion of the silk gland were prepared as described (Tsuda and Suzuki, 1981; Takiya *et al.*, 1990). EMSA was carried out as reported previously (Takiya *et al.*, 1997, 2005). The probe listed in Fig. 5B was incubated with protein solutions, and probe-protein complexes were analyzed on 7% polyacrylamide gels. In the competition assays, 100 pmol of the oligonucleotides listed in Fig. 5B was added as a competitor against the probe DNA. In the hypershift assay of POU-M1, serum against the C1 peptide of POU-M1 as described by Fukuta *et al.* (1993) was added to the EMSA reaction mixtures. To prepare recombinant POU-M1 protein, the ORF was amplified with primers 5'-CGGAATTCATGGCGGCGACCACGTACATGC-3' and 5'-GTGTCGACTTAGTGCGCTGCCAGTGTGTTGG-3' by PCR, and cloned between EcoRI and SalI sites of pGEX6P1 vector. We sequenced the pGEX6P1-POU-M1 plasmid and original SGF-3/POU-M1 cDNA plasmid obtained by Fukuta *et al.* (1993), and found that both nucleotide sequences were the same as the sequence of the POU-M2 gene registered in the DNA database. GST-POU-M1 fusion protein was prepared as described previously (Takiya *et al.*, 2005), and purified using glutathione-Sepharose beads. The glutathione-Sepharose beads bound by the fusion protein were washed with PBS several times, once with 50 mM Tris-HCl pH7.0, and then once with proteolysis buffer (50 mM Tris-HCl pH7.0, 150mM NaCl, 1mM EDTA and 1 mM DTT). The beads were resuspended in a small amount of proteolysis buffer, and PreScission protease (60 units/ml beads) was added to the suspension. The mixture was rotated at 4 °C overnight, centrifuged and the supernatant was recovered. The recombinant protein was analyzed with SDS-PAGE, and amount of the protein was measured by the method of Bradford with Protein Assay reagent (BioRad). The

recombinant POU-M1 was used after dilution with a large amount of NP40 buffer (50 mM Tris-HCl pH7.9, 12.5 mM $MgCl_2$ 100mM NaCl, 0.1 mM EDTA, 20% glycerol, 10% NP40 and 1 mM DTT) or dialyzed against NP40 buffer.

Construction of the reporter and expression plasmids

The XbaI-Gal4VP16 fragment was amplified from the plasmid pBac[A3-GAL4VP16, 3xP3-EGFP] (Kobayashi et al., 2011) using the primers 5′-ACTAGTTCTAGAATGAAGCTACTGTCTTCT-3′, 5′-ACTAGTGGAACAACACTCAACCCTATCTCG-3′ and inserted into the pTA vector (TOYOBO, Japan). To construct the plasmid pBac[XbaI-Gal4VP16]R, the XbaI-Gal4VP16 *Spe*I fragment and the 3xP3-DsRed2-UTR *Bgl*II fragment of the plasmid pBacA3GAL4/3xP3DsRed (Uchino et al., 2006) were inserted into the *Nhe*I and *BamH*I-*Bgl*II site of the plasmid pBacMCS (Uchino et al., 2006), respectively.To construct Gal4VP16 expression vector induced from the *Bombyx actin A3* gene promoter, the *A3* promoter (-129/+540) was amplified by PCR with pA3-LacZ (Mange *et al.*, 1997) using primers 5'-GCTCTAGATGCGCGTTACCATATATG -3' and 5'-GTTCTAGACTTGAATTAGTCTGCAAG-3' was inserted into the XbaI site of the pBac[XbaI-Gal4VP16]R. To construct the POU-M1 expression vector induced by the yeast transcriptional activator Gal4, the PCR fragment of the *POU-M1* ORF sequence was inserted into the BlnI site of the pBacMCS[UAS-3xP3-EGFP] (Sakudoh *et al.*, 2007). The ORF sequence of *POU-M1* was amplified by PCR using primers 5'-AGCCTAGGATGGACTACAGAGGACGACGATGACAAAATGGCGGCGACCACGTACATG-3' and 5'-AGTCTAGATTAGTGCGCTGCCAGTGTGTG-3'. To construct PGV-ser1, a PCR fragment of the *sericin-1* gene promoter (-1602/+47) was inserted between XhoI and SmaI sites of the *Photinus* luciferase vector using PGV Basic (Toyo Ink) (Takiya *et al.*, 2011). The internal control vector pRL-A3 was

constructed by insertion of the PCR fragment of the *A3* gene promoter (-128/+28) between the EcoRI and XhoI sites of the *Renilla* luciferase vector pRL-null (Promega) (Nishita and Takiya, 2009).

Gene transfection and luciferase activity assay

The expression plasmids and reporter plasmids were introduced together into silk glands from day 1 fifth instar larvae with a gene gun (BioRad) according to Takiya *et al.* (2011) with several modifications. Tangsten particles (25 mg) were mixed with pGAL4-A3 (80 µg), pUAS-POU-M1 (80 µg), PGV-*sericin-1* (100 ug) and pRL-A3 internal control DNA (10 µg) and treated according to the manufacturer's instructions. A 70-cm Tefzel tube (BioRad) was coated with tungsten particles coated with DNA and cut into 12-mm pieces. The particles were introduced into silk glands of day 1 fifth instars using N_2 gas at 100 psi. The silk glands were then transplanted into larvae at the same stage. Luciferase activity of the middle silk gland was measured 48 h after transfection with a Dual-Luciferase Reporter Assay system (Promega) using Luminescencer PSN AB-2200 (ATTO). The pRL-A3 vector was used to normalize the transfection efficiency of each assay.

Results

Complementary expression patterns of the *sericin-1* and *POU-M1* genes

When invagination of the silk glands starts at embryogenesis, POU-M1 is detected in the entire silk glands, but the signal is diminished in the posterior region of the elongating silk gland. After hatching, POU-M1 is not detectable in the regions where *sericin-1* and *fibroin* genes are expressed in the early instars (Kokubo *et al.*, 1996; Matsunami *et al.*, 1998). To compare localizations of the *sericin-1* and *POU-M1* gene expressions in the silk gland from fourth to fifth instars, we first performed RT-PCR. MSG was separated into three portions at natural turns and each portion was referred to as the anterior, middle or posterior part of the MSG (MSG-A, MSG-M and MSG-P, respectively), but the joint bend regions themselves were removed carefully (Fig. 1A). The *sericin-1* transcripts were detected specifically in MSG-P in day 2 fourth instars, were almost undetectable at the fourth molting stage, and were then expressed in MSG-M and -P in day 2 fifth instars (Fig. 1B). In contrast, the expression of *POU-M1* was high in ASG, MSG-A and MSG-M, weakened in MSG-P, and slightly in PSG in fourth instars. *POU-M1* was expressed with a similar pattern to fourth instars but was strong in each region at the fourth molting stage. In the fifth instar, the expression level was decreased significantly in MSG-M and became undetectable in MSG-P and the entire PSG. We next analyzed the expression levels of the *sericin-1* and *POU-M1* genes quantitatively with real-time RT-PCR (Fig. 2). The results were almost consistent with those presented above. Notably, in MSG-M, where *sericin-1* mRNA was undetectable before the fifth instar, the expression of *POU-M1* in the fourth instar was more than 5-fold stronger than in the

fifth instar.

POU-M1 are expressed in the trachea (Kokubo *et al.*, 1997; Matsunami *et al.*, 1998) and numerous fine tracheal branches are attached the silk gland cells. Although we eliminated tracheae from the silk gland tissues carefully, it is possible that the RNA fractions prepared from silk glands still contained some RNA from tracheae. We determined the localization of *POU-M1* and *sericin-1* transcripts in the MSG cells using *in situ* hybridization (Fig. 3). Because of some technical difficulties with whole mount immunohistochemistry of the silk gland of the last instar, sections of the silk glands were used for hybridization. *Sericin-1* mRNA was detected in MSG-M and -P cells in the fifth instar, while *POU-M1* mRNA was detected in MSG-A and was undetectable in MSG-M and -P. Thus, the *sericin-1* and *POU-M1* genes were expressed complementary to each other in the MSG in both fourth and fifth instars.

POU-M1 repressed the promoter activity of the *sericin-1* gene

POU-M1 protein was first identified as a factor binding to the SC site, which has been presumed as a positive *cis*-element of the *sericin-1* gene (Matsuno *et al.*, 1990; Fukuta *et al.*, 1993); however, these genes exhibited opposite expression patterns spatially in MSG, as described above. To investigate functions of POU-M1 for the *sericin-1* gene expression, we conducted gene transfer experiments (Fig. 4). The expression vectors carrying the Gal4-VP16 gene with the *Bombyx actin* gene *A3* promoter (A3/GAL4VP16), *POU-M1* gene with the UAS promoter (UAS/POU-M1) induced by Gal4 and *Photinus*-luciferase gene with the *sericin-1* promoter (PGV-ser(-1642/+47)) were introduced together into silk glands in the fifth instar using a gene gun. The silk glands were then transplanted into the hemolymphatic cavity of the host larvae and luciferase activity of MSGs was analyzed two days

later (Fig. 4B). These experiments were repeated using different preparations of these expression vectors, and the same results were obtained.

Expression of the transgene was confirmed by RT-PCR with primers specific to transcripts from the UAS/POU-M1 vector. *POU-M1* mRNA transcribed from the transfected DNA was detected at various levels according to samples, reflecting the transfection efficiency (Fig. 4C). *Sericin-1* promoter activity was decreased clearly when the POU-M1 expression vector was co-transfected (Fig. 4B). These results suggested that POU-M1 is a negative regulator of the *sericin-1* gene.

POU-M1 and MIC bound to the same promoter elements of the *sericin-1* gene

The -70 promoter element (the transcription start site is denoted as +1) is indispensable for expression of the *sericin-1* gene *in vivo* (Takiya *et al.*, 2011). MIC was detected as a factor that binds to the -70 and other promoter elements with spatiotemporal specificity identical to that of the *sericin-1* gene expression (see Fig. 6A). As shown in Fig. 5A, the MIC-binding sites of -320, -180 and -70 elements exist alternatively with high-affinity binding sites for POU-M1, SC and SB in the *sericin-1* promoter. Nucleotide sequences of the MIC-binding elements were different from typical sequences recognized by POU domain proteins, but contain AT-rich sequences that might be recognized by homeodomain (Fig. 5B). We prepared recombinant POU-M1 protein (rPOU-M1) and examined its binding activity to these elements by EMSA. rPOU-M1 bound to the -70, -180 and -320 elements in a dose-dependent manner (Fig. 5C).

The specificity of the binding activity of POU-M1 was confirmed by competition experiments with mutant

oligonucleotides (Fig. 5D). Sr-70M1, M2 and M4 competitors with mutations in the AT-rich sequences could not compete with POU-M1 binding to the Sr-70 probe, whereas the Sr-70M3 competitor with mutations outside of the AT-rich sequence competed as efficiently as the wild type Sr-70 oligonucleotide. Similarly, the oligonucleotides that mutated at the putative homeodomain-binding sites lost the ability to compete with the wild type Sr-180 and Sr-320 probes. The SC oligonucleotide, including a typical binding element of POU-M1 upstream of the *sericin-1* gene, could compete with all MIC binding probes at low doses.

POU-M1 inhibited the binding of MIC at -70 element *in vitro*

A gel shift assay using MSG-P extract also confirmed that MIC and POU-M1 could bind to the same DNA elements, Sr-70 (Fig. 6). Although the expression level of the *POU-M1* gene in MSG-P was quite low (Fig. 1-3), DNA-binding activity of POU-M1 was detectable in the MSG-P extract. Since the binding sequence of POU-M1 overlapped with the MIC sites, it was predicted that excess POU-M1 inhibits the binding of MIC. When rPOU-M1 was added, the MIC signal decreased as the amount of rPOU-M1 increased (Fig. 6). In addition to bands of the rPOU-M1 monomer, those corresponding to the homodimer appeared and increased in a dose-dependent manner.

Discussion

POU-M1 was detected as a factor binding to the SC site in the promoter of the *sericin-1* gene, and has been proposed as its transcriptional activator; however, we have demonstrated that the *sericin-1* and *POU-M1* genes were expressed complementarily in the subparts of the MSG in the last and penultimate instars. Similar complementary expression of these genes in MSG from the embryonic stage to the second instar was also reported (Matsunami *et al*., 1998). In this study, we examined the effects of POU-M1 on *sericin-1* gene expression *in vivo* using gene gun technology and organ transplantation. The promoter activity of the *sericin-1* gene was repressed when the expression vector of *POU-M1* was co-transfected into silk glands, suggesting that POU-M1 represses the *sericin-1* gene.

Removal of the high affinity POU-M1-binding site SC from -239 to -174 decreased promoter activity *in vitro* (Matsuno *et al*., 1989; Fukuta *et al*., 1993); however, the region contained not only the POU-M1 binding element but also part of the MIC binding element in the -180 region. MIC is presumed to be the activator of the *sericin-1* gene from its same spatiotemporal specificity as that of *sericin-1* gene expression (Takiya *et al*., 2011); therefore, the effects of deletion of the SC site might be led by the deletion of the MIC-binding element. In the present study, we showed that POU-M1 could bind to the MIC-binding elements. POU-M1 inhibited the binding of MIC at -70 element, which is indispensable for the promoter activity of *sericin-1 in vivo*. From these results, we speculate that POU-M1 represses promoter activity of the *sericin-1* gene by inhibiting the binding of the transcriptional activator to the promoter elements.

POU domain proteins bind diverse sets of DNA sequences flexibly due to their bipartite DNA-binding domain consisting

of a POU-specific domain and POU homeodomain (Herr and Cleary, 1995). They recognize not only optimal octamer sequences, but also typical homeodomain-binding elements including TAAT motif (Stepchenko *et al.*, 1997). Consistent with this observation, POU-M1 has been found to bind different kinds of sequences. First, POU-M1 binds to the SC site of the *sericin-1* promoter that contains an octamer-like sequence ATGAATAA (Matsuno *et al.*, 1990; Fukuta *et al.*, 1990). It has been also shown that POU-M1 bound to some octamer-like elements of the *fibroin* promoter and the *Diapause hormone and pheromone biosynthesis-activating neuropeptide* (*DH-PBAN*) promoter (Takiya *et al.*, 1997; Zhang *et al.*, 2004). Meanwhile, it was demonstrated that POU-M1 binds to the AT-rich elements of its own gene promoter and autoregulates itself negatively *in vitro* (Xu *et al.*, 1994).

In this study, we showed that POU-M1 bound to AT-rich MIC binding sites in the *sericin-1* promoter, although with affinity lower than that for the SC site. It has been proposed that the affinity of a binding site provides a mechanism to respond to different concentrations of transcription factors (Parker *et al.*, 2011; Rowan *et al.*, 2010). POU-M1 could repress the *sericin-1* gene at a high concentration, observed in regions anterior to the *sericin-1*-expressing portion in the silk gland, via all low-affinity elements by competing with MIC. On the other hand, binding of POU-M1 to the high-affinity SC site (-204/-183) may disturb the binding of MIC to the -180 site (-182/-171), because the SC site is adjacent to the MIC binding site. Accordingly, POU-M1 could repress *sericin-1* partly via the SC site at a low concentration. Further investigations are necessary to understand the regulation mechanism of the *sericin-1* gene expression by POU-M1.

Besides Fkh and FMBP-1, POU-M1 can bind to the intronic and upstream modulator elements of the *fibroin-H* gene (Takiya *et al.*, 1997). *Fibroin-H* is expressed specifically in the PSG,

and the expression level of the *POU-M1* is low in the PSG; however, in the anterior regions of the silk gland, POU-M1 could also suppress *fibroin-H*. During embryogenesis, the expression of POU-M1 is detected in many invaginating regions, including whole silk glands, salivary glands and tracheal system placodes. Later it diminishes only in the posterior portion of the elongating silk glands prior to expression of the silk genes (Kokubo *et al.*, 1997, Matsunami *et al.*, 1998). Fkh is a transcriptional activator of the silk genes and is expressed in the entire MSG, PSG, and also in the trachea (Julien *et al.*, 2002; Kokubo *et al.*, 1996; unpublished result). It is possible that POU-M1 plays a role in suppression of the silk genes in the anterior parts of the silk gland and other tubular tissues, including the trachea, from organogenesis to the last instar.

Acknowledgments

We are grateful to Dr. Nishita and Ms. Kosaka for helpful discussions and technical assistances.

References

Anderson, M.G., Certel, S.J., Certel, K., Lee, T., Montell, D.J. & Johnson W.A. 1996. Function of the *Drosophila* POU domain transcription factor drifter as an upstream regulator of breathless receptor tyrosine kinase expression in developing trachea. *Development* **122**, 4169-4178.

Certel, K., Hudson, A., Carroll, S.B. & Johnson, W.A. 2000. Restricted patterning of vestigial expression in *Drosophila* wing imaginal discs requires synergistic activation by both Mad and the drifter POU domain transcription factor. *Development* **127**, 3173-3183.

Couble, P., Moine, A., Garel, A. & Prudhomme J.-C. 1983. Developmental variations of a nonfibroin mRNA of *Bombyx mori* silkgland, encoding for a low-molecular-weight silk protein. *Dev. Biol.* **97**, 398-407.

Couble, P., Michaille, J.-J., Garel, A., Couble, M.-L. and Prudhomme, J.-C. 1987. Developmental switches of sericin mRNA splicing in individual cells of *Bombyx mori* silkgland. *Dev. Biol.* **124**, 431-440.

Fukuta, M., Matsuno, K., Hui, C.C., Nagata, T., Takiya, S., Xu, P.X., Ueno, K. & Suzuki, Y. 1993. Molecular cloning of a POU domain-containing factor involved in the regulation of the *Bombyx sericin-1* gene. *J. Biol. Chem.* **268**, 19471-19475.

Herr, W. & Cleary, M.A. 1995. The POU domain: versatility in transcriptional regulation by a flexible two-in-one DNA-binding domain. *Genes Dev.* **9**, 1679-1693.

Hui, C.-C., Matsuno, K. & Suzuki, Y. 1990. Fibroin gene promoter contains a cluster of homeodomain binding sites that interact with three silk gland factors. *J. Mol. Biol.* **213**, 651-670.

Ishikawa, E. & Suzuki, Y. 1985. Tissue- and stage-specific expression of sericin genes in the middle silk gland of *Bombyx mori*. *Devlop. Growth Differ.* **27**, 73-82.

Johnson W.A. & Hirsh, J. 1990. Binding of a *Drosophila* POU-domain protein to a sequence element regulating gene expression in specific dopaminergic neurons. *Nature* **343**, 467-470.

Julien, E., Bordeaux, M.-C., Garel, A. & Couble, P. 2002. Fork head alternative binding drives stage-specific gene expression in the silk gland of *Bombyx mori*. *Ins. Biochem. Mol. Biol.* **32**, 377-387.

Junell, A., Uvell, H., Davis, M.M., Edlundh-Rose, E., Anttonsson, A., Pick, L. and Engstrom, Y. 2010. The POU transcription factor Drifter/Ventral veinless regulates expression of *Drosophila* immune defence genes. *Mol. Cell. Biol.* **30**, 3672-3684.

Kiguchi, K. & Agui, N. 1981. Ecdysteroid levels and developmental events during larval moulting in the silkworm, *Bombyx mori*. *J. Insect Physiol.* **27**, 805-812

Kimoto, M., Yamaguchi, M., Fujimoto, Y. and Takiya, S. 2010. Expression profiles of the genes for nine transcription factors and their isoforms in the posterior silk gland of the silkworm *Bombyx mori* during the last and penultimate instars. *J. Insect Biotech. Sericol.* **79**, 31-43.

Kobayashi, I., Kojima, K., Uchino, K., Sezutsu, H., Iizuka, T., Tatematsu, K., Yonemura, N., Tanaka, H., Yamakawa, M., Ogura, E., Kamachi, Y. and Tamura, T., 2011. An efficient binary system for gene expression in the silkworm, Bombyx mori, using GAL4 variants. *Arch. Insect Biochem. Physiol.* **76**, 195e210.

Kokubo, H., Takiya, S., Mach, V. & Suzuki, Y. 1996. Spatial and temporal expression pattern of *Bombyx fork head/SGF-1* gene in embryogenesis. *Dev. Genes Evol.* **206**, 80-85.

Kokubo, H., Xu, P.-X., Xu, X., Matsunami, K. & Suzuki, Y. 1997. Spatial and temporal expression pattern of POU-M1/SGF-3 in *Bombyx mori* embryogenesis. *Dev. Genes Evol.* **206**, 494-502.

Li, J.-Y., Yang, H.-J., Lan, T.-Y., Wei, H., Zhang. H.-R., Chen, M., Fan, W., Ma, Y.-Y. & Zhong, B.-X. 2011. Expression profiling and regulation of genes related to silkworm posterior silk gland development and fibroin synthesis. *J. Proteome Res.* **10**, 3551-3564

Mach, V., Takiya, S., Ohno, K., Handa, H., Imai, T. & Suzuki, Y. 1995. Silk gland factor-1 involved in the regulation of *Bombyx* sericin-1 gene contains fork head motif. *J. Biol. Chem.* **270**, 9340-9346.

Maekawa, H. & Suzuki, Y. 1980. Repeated turn-off and turn-on of fibroin gene transcription during silk gland development of *Bombyx mori. Dev. Biol.* **78**, 394-406.

Mange, A., Julien, E., Prudhomme, J.-C. & Couble, P. 1997. A strong inhibitory element down-regulates SRE-stimulated transcription of the A3 cytoplasmic actin gene of *Bombyx mori*.

J. Mol. Biol. **265**, 266-274.

Matsunami, K., Kokubo, H., Ohno, K. & Suzuki, Y. 1998. Expression pattern analysis of SGF-3/POU-M1 in relation to sericin-1 gene expression in the silk gland. *Devlop. Growth Differ.* **40**, 591-597

Matsuno, K., Hui, C.-C., Takiya, S., Suzuki, T., Ueno, K. & Suzuki, Y. 1989. Transcription signals and protein binding sites for sericin gene transcription *in vitro*. *J. Biol. Chem.* **264**, 18707-18713

Matsuno, K., Takiya, S., Hui, C.-C., Suzuki, T., Fukuta, T., Ueno, K. & Suzuki, Y. 1990. Transcriptional stimulation *via* SC site of *Bombyx* sericin-1 gene through an interaction with a DNA binding protein SGF-3. *Nucleic Acids Res.* **18**, 1853-1858.

Okamoto, H., Ishikawa, E. & Suzuki, Y. 1982. Structural analysis of sericin genes. Homologies with fibroin gene in the 5′ flanking nucleotide sequences. *J. Biol. Chem.* **257**, 15192-15199

Parker, D.S., White, M.A., Ramos, A.I., Cohen, B.A. & Barolo, S. 2011. The cis-regulatory logic of hedgehog gradiant responses: Key roles for Gli binding affinity, competition, and cooperativity. *Sci. Signal.* **4**, ra38.

Rowan, S., Siggers, T., Lachke, S.A., Yue, Y., Bulyk, M.L. & Maas, R.L. 2010. Precise temporal control of the eye regulatory gene *Pax6* via enhancer-binding site affinity. *Genes Dev.* **24**, 980-985.

Ryan, A.K. & Rosenfeld, M.G. 1997. POU domain family values: flexibility, partnerships, and developmental codes. *Genes Dev.*

11, 1207-1225.

Sakudoh, T., Sezutsu, H., Nakashima, T., Kobayashi, I., Fujimoto, H., Uchino, K., Banno, Y., Iwano, H., Maekawa, H., Tamura, T., Kataoka, H. & Tsuchida, K. 2007. Carotenoid silk coloration is controlled by a carotenoid-binding protein, a product of the *Yellow blood* gene. *Proc. Natl. Acad. Sci. USA* **21**, 8941-8946.

Stepchenko, A.G., Luchina, N.N. & Pankratova E.V. 1997. Cysteine 50 of the POU_H domain determines the range of targets recognized by POU proteins. *Nucleic Acids Res.* **25**, 2847-2853.

Suzuki, Y., Obara, T., Takiya, S., Hui, C.-C., Matsuno, K., Suzuki, T., Suzuki, E., Ohkubo, M. & Tamura, T. 1990. Differential transcription of the fibroin and sericin-1 genes in cell-free extracts. *Develop. Growth Differ.* **32**, 179-187.

Takasu, Y., Hata, T., Uchino, K. & Zhang, Q. 2010. Identification of Ser2 proteins as major sericin components in the non-cocoon silk of *Bombyx mori*. *Insect Biochem. Mol. Biol.* **40**, 339-344.

Takiya, S., Hui, C.-C. & Suzuki, Y., 1990. A contribution of the core-promoter and its surrounding regions to the preferential transcription of the fibroin gene in posterior silk gland extracts. *EMBO J.* **9**, 489-496.

Takiya, S., Kokubo, H. & Suzuki, Y. 1997. Transcriptional regulatory elements in the upstream and intron of the fibroin gene bind three specific factors POU-M1, Bm Fkh and FMBP-1. *Biochem. J.* **321**, 645-653.

Takiya, S., Ishikawa, T., Ohtsuka, K., Nishita, Y. & Suzuki, Y. 2005. Fibroin-modulator-binding protein-1 (FMBP-1) contains a novel DNA-binding domain, repeats of the score and three amino acid peptide (STP), conserved from *Caenorhabditis elegans* to humans. *Nuleic Acids Res.* **33**, 786-795.

Takiya, S., Inoue, H. & Kimoto, M. 2011. Novel enhancer and promoter elements indispensable for the tissue-specific expression of the *sericin-1* gene of the silkworm *Bombyx mori*. *Insect Biochem. Mol. Biol.* **41**, 592-601.

Tsuda, M. & Suzuki, Y. 1981. Faithful transcription initiation of fibroin gene in a homologous cell-free system reveals an enhancing effect of 5' flanking sequence far upstream. *Cell* **27**, 175-182.

Uchino, K., Imamura, M., Sezutsu, H., Kobayashi, I., Kojima, K., Kanda, T. & Tamura, T. 2006. Evaluating promoter sequences for trapping an enhancer activity in the silkworm *Bombyx mori*. *J. Insect Biotech. Sericol.* **75**, 89-97.

Xu, P.X., Fukuta, M., Takiya, S., Matsuno, K., Xu, X. & Suzuki, Y. 1994. Promoter of the POU-M1/SGF-3 gene involved in the expression of *Bombyx* silk genes. *J. Biol. Chem.* **269**, 2733-2742.

Zhang, T.-Y., Kang, L., Zhang, Z.-F. & Xu, W.-H. 2004. Identification of a POU factor involved in regulating the nuron-specific expression of the gene encoding diapause hormone and pheromone biosynthesis-activating neuropeptide in *Bombyx mori*. *Biochem. J.* **380**, 255-263.

Figure legends

Fig. 1 RT-PCR analysis of *sericin-1* and *POU-M1* in the silk glands. Total RNA was prepared from each portion of the silk glands indicated in (A) in the fourth (IV) and fifth (V) instar and fourth molting (IVm) stages (B). *Rp49* (*ribosomal protein 49*) was used as an internal control.

Fig. 2 Real-time RT-PCR analysis of *sericin-1* and *POU-M1* in the silk glands. The same total RNA preparations as in Fig. 1 were used. *Sericin-1* and *POU-M1* mRNA levels were normalized separately to *GAPDH*. Real-time PCR was performed twice on each sample and the average is shown as a graph. Expression levels of *ser-1* and *POU-M1* are shown as the fold difference calculated relative to the levels in the PSG-P of day 2 fifth instars.

Fig. 3 Localization of *sericin-1* and *POU-M1* mRNA in the middle silk gland. Silk glands were collected from day 2 fifth instars. Continuous sections (4 μm) were hybridized with digoxigenin-labeled sense or antisense probes for *sericin-1* or *POU-M1*. Signals were detected by an alkaline phosphatase reaction with the NBT/BCIP substrate. Bar represents 10 μm.

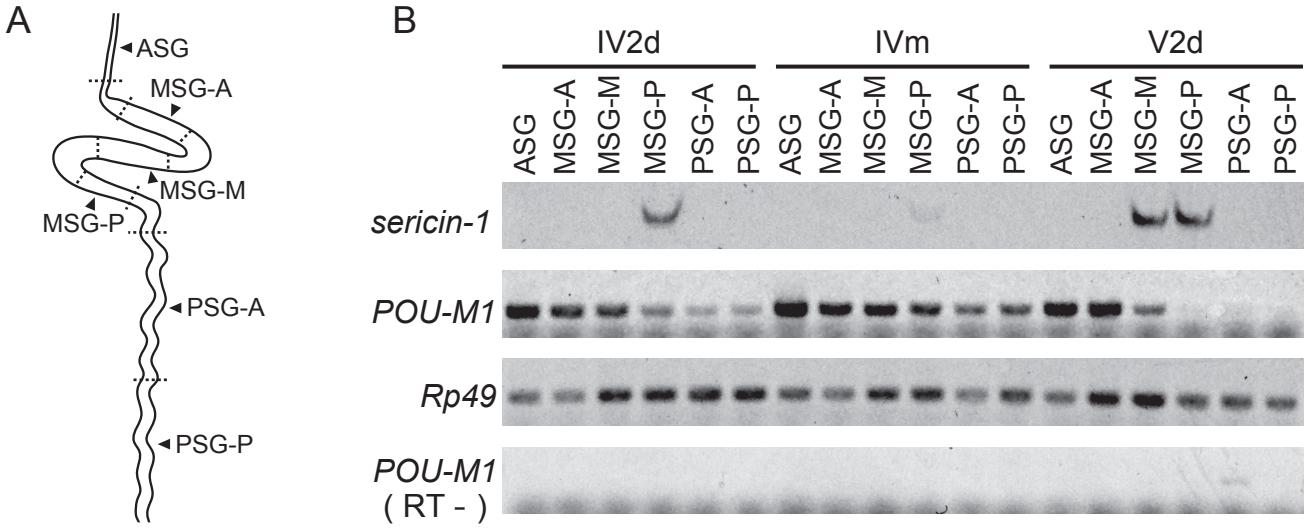
Fig. 4 Repression of promoter activity of the *sericin-1* gene by cotransfection of the *POU-M1* expression vector. (A) Expression vectors and reporter constructs transfected into the silk glands by a gene gun are shown. (B) Relative transcriptional activities of the *sericin-1* promoter in the MSG was measured after co-transfection with *POU-M1* expression vector (pBac[UAS/POU-M1]) or control empty vector (pBacMCS[UAS-3xP3-EGFP]). PGV-ser1 firefly luciferase activities were normalized by pRL-A3 Renilla luciferase activities. Error bars represent S.D., and the p value indicated

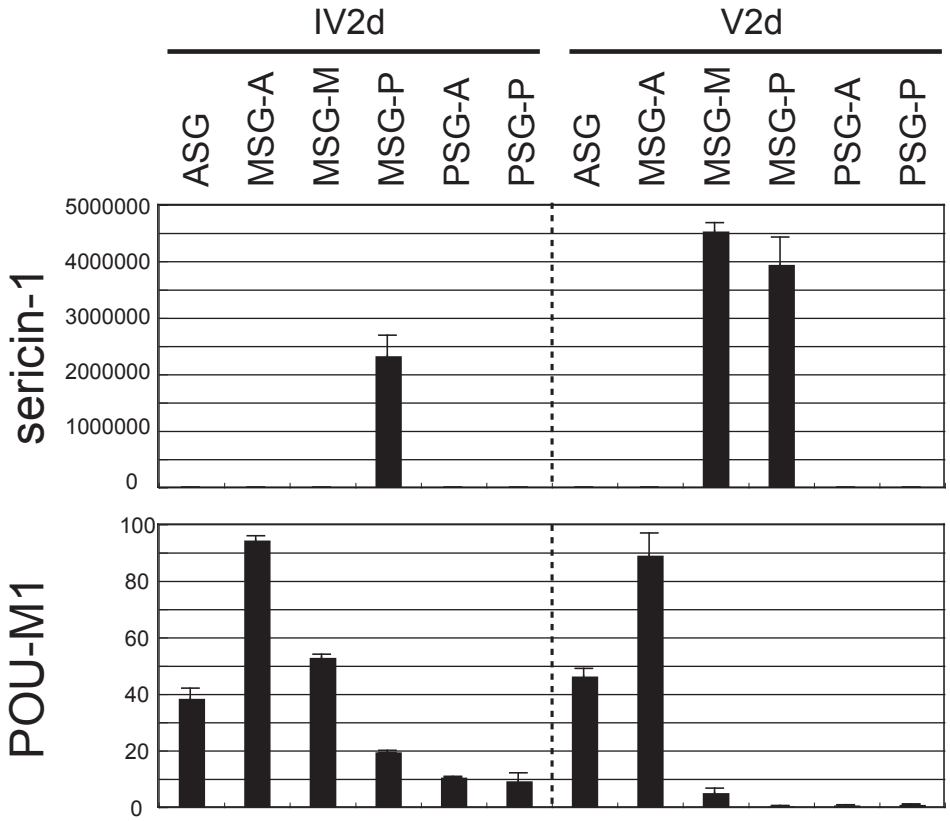
with * is <0.001. (C) Expression of ectopic *POU-M1* was confirmed by RT-PCR with primers specific to transcripts from pBac[UAS/POU-M1] (shown in A with arrows). Transfection efficiency was different for each bombardment. Total RNA without reverse transcription (RT-minus) and cDNA of MSG-A from untransfected larva were used as negative controls for RT-PCR.

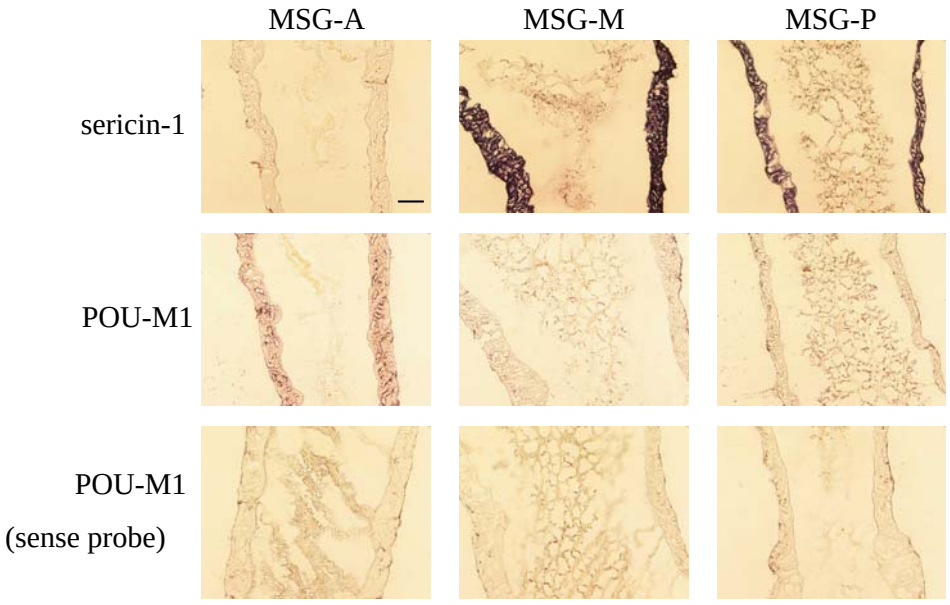
Fig. 5 Binding of POU-M1 to the MIC-binding sites of the *sericin-1* promoter. (A) Relative positions of the MIC-binding sites (-70, -180, -320) and POU-M1 binding sites (SB, SC) of the *sericin-1* promoter are shown. (B) Nucleotide sequence of the probes and competitors used for EMSAs. Putative POU-M1-binding elements are indicated by a box. (C) In the binding assay, 0.04 pmol (lane 1), 0.2 pmol (lane 2), 1 pmol (lane 3, 5, 9 and 13), 2 pmol (lane 6, 10 and 14), 4 pmol (lane 7, 11 and 15) of recombinant POU-M1 protein (rPOU-M1) were incubated with each probe shown at the top of the panel. Reaction mixture without POU-M1 protein was loaded onto lanes 4, 8 and 12. (D) The competitive effects of the mutant oligonucleotides on rPOU-M1 binding were examined. Then, 4 pmol rPOU-M1 was incubated with probes and competitors, as indicated.

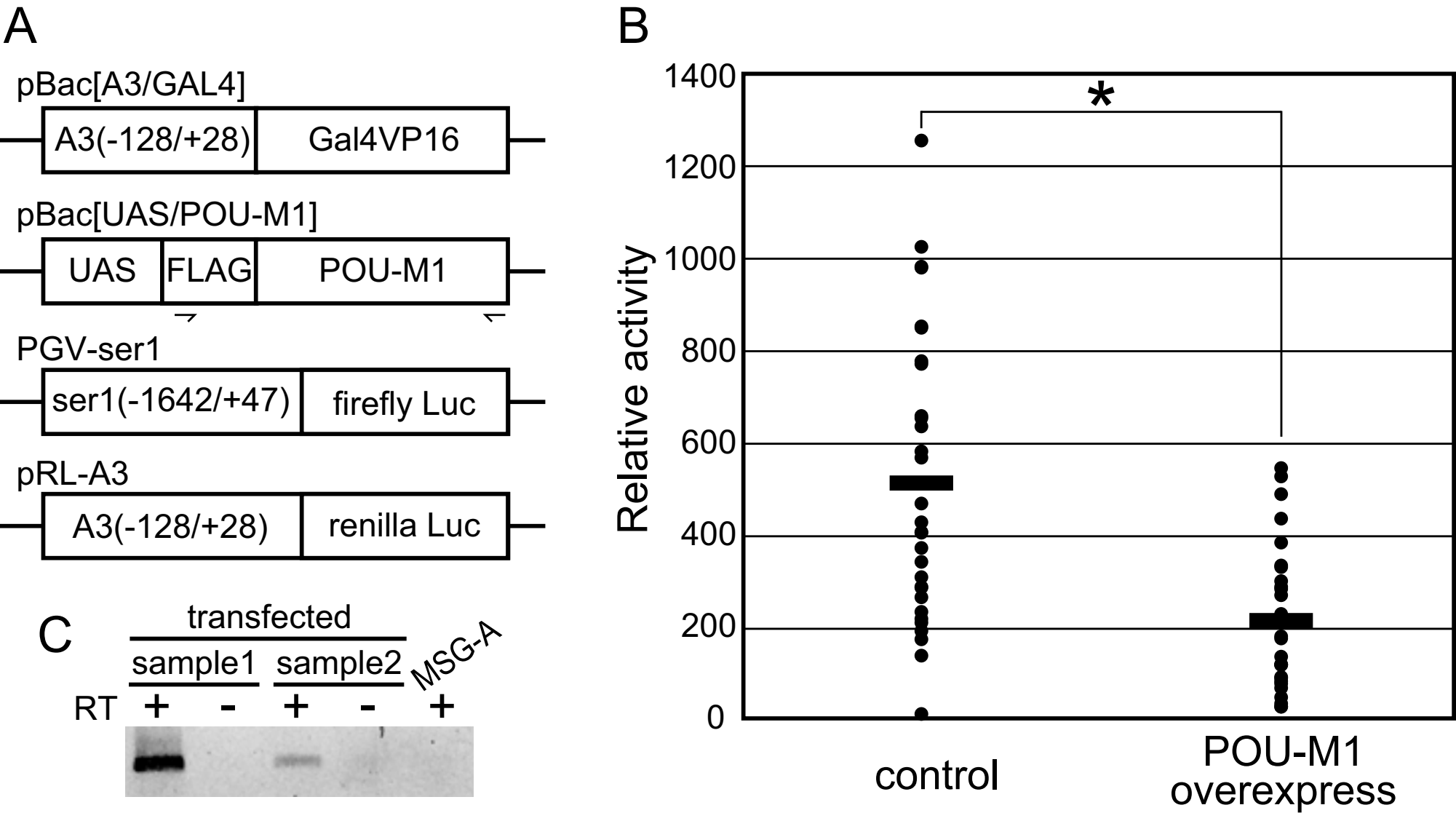
Fig. 6 Competition of MIC binding to the -70 site of the *sericin-1* promoter by POU-M1. (A) EMSA was carried out by the incubation of staged extracts prepared from the entire MSG (MSG) or PSG (PSG), and the posterior region of MSG (MSG-P) with the -70 probe. Nucleotide sequence of the top strand of the -70 probe is 5'-AAATGTTTGAAGAAGCGAAAATTTATTACTCTCTACGTAAGCTTGATCA-3'. -; no extract, IVm; extract from the fourth molting larvae, V2; extract from day 2 fifth instar larvae, IV3; extract from day 3 fourth instar larvae. (B) The -70 probe (50 fmol) was incubated with V2 MSG-P extracts (10 µg) in the presence or absence (-) of recombinant POU-M1 protein (rPOU-M1). To each

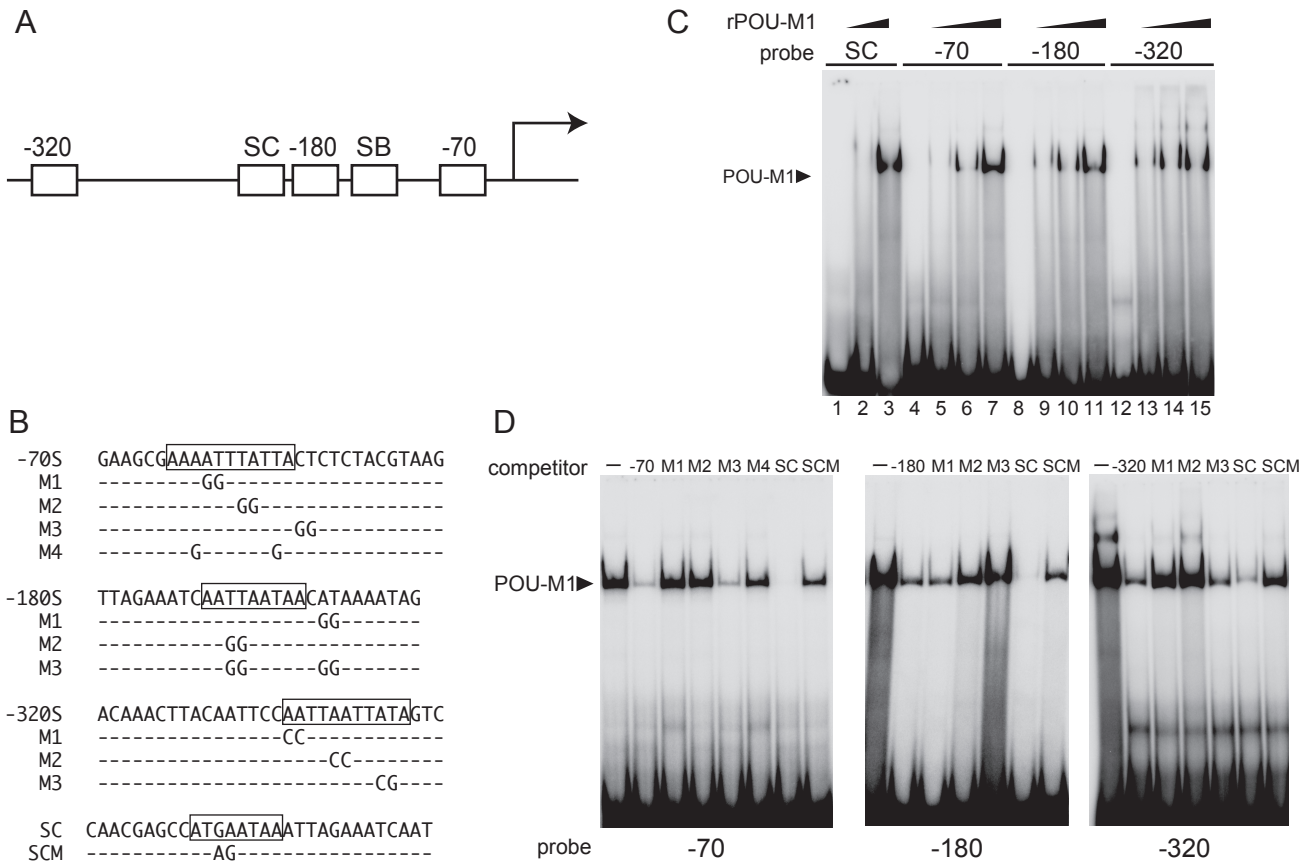
reaction, 5, 50 and 500 fmol or 5 pmol of rPOU-M1 was added. Hypershift products (*asterisk*) of rPOU-M1 were appeared by the addition of anti-POU-M1 antibody (anti-POU).

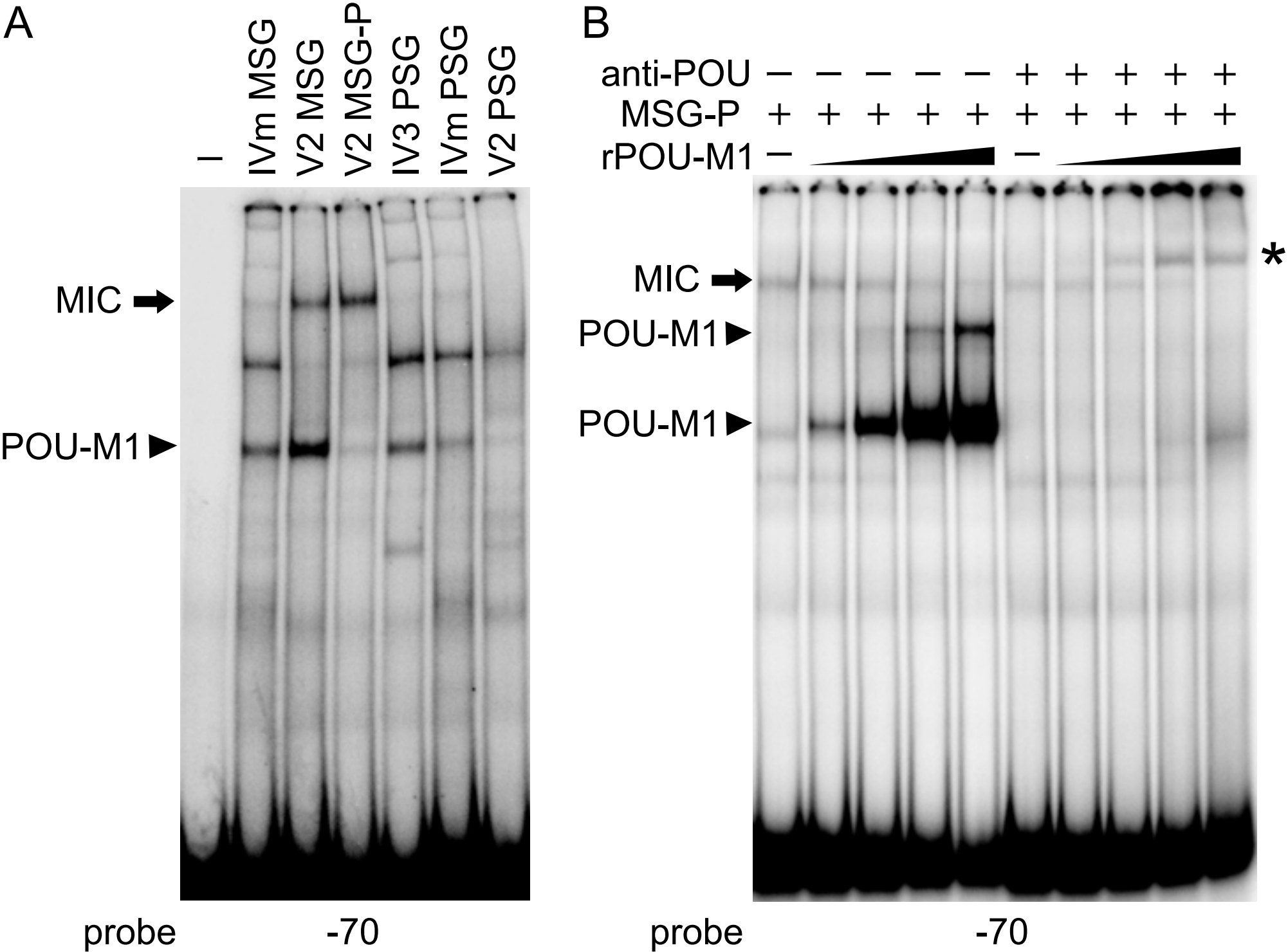

A
ASG
MSG-A
MSG-M
MSG-P
PSG-A
PSG-P
B
IV2d
IVm
V2d
ASG
MSG-A
MSG-M
MSG-P
PSG-A
PSG-P
sericin-1
POU-M1
Rp49
POU-M1
(RT -)


IV2d
V2d
ASG
MSG-A
MSG-M
MSG-P
PSG-A
PSG-P
ASG
MSG-A
MSG-M
MSG-P
PSG-A
PSG-P
sericin-1
5000000
4000000
3000000
2000000
1000000
0
POU-M1
100
80
60
40
20
0


MSG-A
MSG-M
MSG-P
sericin-1
POU-M1
POU-M1
(sense probe)


A
pBac[A3/GAL4]
A3(-128/+28)
Gal4VP16
pBac[UAS/POU-M1]
UAS
FLAG
POU-M1
PGV-ser1
ser1(-1642/+47)
firefly Luc
pRL-A3
A3(-128/+28)
renilla Luc
C
transfected
sample1
sample2
MSG-A
RT + - + - +
B
Relative activity
1400
1200
1000
800
600
400
200
0
*
control
POU-M1
overexpress


A
-320
SC
-180
SB
-70
B
-70S GAAGCGAAAATTTATTACTCTCTACGTAAG
M1 ---------GG--------------------
M2 ------------GG-----------------
M3 ----------------GG------------
M4 --------G------G---------------
-180S TTAGAAATCAATTAATAACATAAAATAG
M1 -------------------GG-------
M2 -----------GG---------------
M3 -----------GG------GG-------
-320S ACAAACTTACAATTCCAATTAATTATAGTC
M1 ----------------CC------------
M2 --------------------CC--------
M3 ------------------------CG----
SC CAACGAGCCATGAATAAATTAGAAATCAAT
SCM ----------AG-----------------
C
rPOU-M1
probe
SC
-70
-180
-320
POU-M1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
D
competitor
-70 M1 M2 M3 M4 SC SCM
-180 M1 M2 M3 SC SCM
-320 M1 M2 M3 SC SCM
POU-M1
probe
-70
-180
-320


A
–
IVm MSG
V2 MSG
V2 MSG-P
IV3 PSG
IVm PSG
V2 PSG
MIC
POU-M1
probe
-70
B
anti-POU – – – – – + + + + +
MSG-P + + + + + + + + + +
rPOU-M1 –
–
MIC
POU-M1
POU-M1
*
probe
-70