Molecular cloning of a POU domain transcription factor involved in regulation of *Bombyx* sericin-1 gene

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Contents

Abstract

The POU domain is a highly conserved region found in a number of transcription factors and products of developmental control genes. This report presents here the isolation and characterization of a POU domain-containing cDNA (POU-M1) from the middle silk gland of *Bombyx mori*. It encodes a protein with a POU domain identical to that of the *Drosophila Cf1-a* protein. By mobility shift and nuclease protection assay, the POU-M1 protein and the putative silk gland factor SGF-3 were found to interact in an inditinguishable manner with the SC region of the sericin-1 gene, which is a key *cis*-acting element involved in the stimulation of sericin-1 gene transcription through the interaction with SGF-3. Antibodies raised against the synthetic oligopeptides corresponding to the two regions of putative POU-M1 sequence reacted specifically to both the POU-M1 protein and the SGF-3. Northern blot hybridization and Western blotting revealed that the POU-M1 expression is regulated both temporally and spatially during the silk gland development. It is concluded that the POU-M1 protein is identical to SGF-3 and proposed that the differential expression of the POU-M1 gene is probably involved in the transcriptional regulation of the silk protein genes.

Introduction

The POU domain is a DNA-binding region consisting of 75-82 amino acids POU-specific domain, a short variable linker region, and a POU-specific homeodomain of 60 amino acids (for reviews, see Ruvkin and Finney, 1991; Herr *et al.*, 1988). It was originally found in three mammalian transcription factors, the pituitary-specific Pit-1/GHF-1, the ubiquitous Oct-1, and the predominantly B cell specific Oct-2, and the product of the cell lineage control gene *Unc-86* of *Caenorhabditis elegans* (Herr *et al.*, 1988 and references therein). By means of sequence similarity, several other mammalian POU domain genes have also been identified (He *et al.*, 1989; Monuki *et al.*, 1990; Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Scholer *et al.*, 1990; Suzuki, N. *et al.* 1990). All of them were shown to interact with an octamer-like sequence (see Fig. 2 of Ruvkin and Finney, 1991) and to activate transcription *via* an octamer motif near the TATA box. The *Drosophila Cf1-a* protein, which interacts with a DNA element required for expression of the dopa decarboxylase gene in selected dopaminergic neurons (Johson and Hirsh, 1990), was also found to possess a POU domain similar to those of the mouse Oct-6 (Suzuki, N. *et al.*, 1990) and the human Brn-1 and Brn-2 (He *et al.*, 1989) proteins. These POU domain genes are likely regulatory genes controlling transcription of distinct sets of genes during development. The findings that two dwarf mutations in

mice are null mutations in the Pit-1/GHF-1 gene (Li *et al.*, 1990) provide further support on the roles of POU transcription factors in development. Recently, the maternally expressed POU domain transcription factor, the Oct-3/4 protein (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Scholer *et al.*, 1990), has also been shown to be required for the first embryonic cell division in mice (Rosner *et al.*, 1990).

Suzuki and his colleagues have been studying the developmental regulation of the silk protein genes in *Bombyx mori* (see Suzuki, Y. *et al.*, 1990 for a review). Among them, the sericin-1 gene is expressed exclusively in the middle silk gland while the fibroin gene is specific to the posterior silk gland. Both genes are actively expressed during the intermolts but repressed during the molting stages. Several silk gland proteins have been identified as putative regulatory factors involved in the transcriptional control of the fibroin and sericin-1 genes (Hui *et al.*, 1990; Matsuno *et al.*, 1989, 1990). One of these proteins, SGF-3, was found to bind with a high affinity to the SC region of the sericin-1 gene (Matsuno *et al.*, 1990) and the distal upstream region of the fibroin gene (Hui *et al.*, 1990). These regions are known to be important for an efficient transcription of these genes in the silk gland extracts (Matsuno *et al.*, 1990; Tsuda and Suzuki, 1983; Suzuki *et al.*, 1986). A multimer of the SC region gave transcriptional enhancement in extracts prepared from the middle silk gland where the

sericin-1 gene is specifically expressed but that of a mutant SC region giving a reduced affinity for SGF-3 did not (Matsuno *et al.*, 1990). Mobility shift assays revealed that SGF-3 is far more abundant in the middle silk gland of the 2-day-old fifth-instar larvae than in the posterior silk gland (Hui *et al.*, 1990; Matsuno *et al.*, 1990; Suzuki, Y., unpublished). These observations suggest that the SGF-3 is a key regulatory factor in the transcriptional control of the sericin-1 gene.

The SGF-3 was proposed as an octamer binding protein (Hui *et al.*, 1990). Since high affinity SGF-3 binding sites, such as the SC and fibroin distal upstream regions, also contain octamer-like sequences, it has been speculated that SGF-3 might possess a POU domain similar to the mammalian octamer-binding proteins. This report describes the isolation and characterization of a POU domain cDNA (POU-M1) from the middle silk gland. It has been shown that the POU-M1 protein and SGF-3 bind to the SC region in an indistinguishable manner and they share immunologically closely related structures. The expression of POU-M1 gene is developmentally regulated, and the POU-M1 transcript was restricted to the middle silk gland during the fifth intermolt.

Materials and Methods

Cloning and Sequencing of POU-M1 cDNA.

A set of fully degenerated primers for polymerase chain reaction (PCR) were synthesized according to the two highly conserved regions (for the sequences, see Fig. 1) among POU domain proteins as described by He *et al.* (1989). The sequence for forward primer is TT(C/T)AA(A/G)(G/C)(A/T)N(A/C)GN(A/C)GNAT(A/T/C)AA-(A/G)(T/C)TNGG and the sequence for reverse primer is (T/C)TGNC(T/G)N(T/C)(T/G)(A/G)TT(A/G)CA(A/G)AACCANACNC, where N stands for a mixture of A, T, G and C. First strand cDNAs were generated from poly(A)$^+$ RNAs of the middle and the posterior silk glands of 2-day-old fifth-instar *B. mori* larvae (a Kanebo hybrid strain, Kin-shu x Sho-Wa) according to standard procedures (Ausubel *et al.*, 1987). PCR was performed 40 cycles at 94 °C for 1 min, 45 °C for 1 min, and 55 °C for 3 min with 50 ng of cDNA and 200 pmoles each of the primers in a 100 µl reaction mixture as recommended by Perkin-Elmer Cetus. The reaction products of an appropriate size were subcloned into pBluescript II (Stratagene) and the POU domain sequence was confirmed by sequencing with a Sequenase protocol (United States Biochemicals). The PCR clone was then used as a probe to screen a middle silk gland lambda gt11 cDNA library (Sambrook *et al.*, 1989). After subcloning the cDNA insert into pBluescript II, the complete nucleotide sequence of the longest cDNA clone was

determined.

***In vitro* Transcription/Translation.**

The pBluescript II subclone containing the entire open reading frame was used as template for *in vitro* transcription. *In vitro* transcription and RNA capping were performed as recommended by the manufacturer (Stratagene). An aliquot of the synthesized mRNA was translated in a rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-methionine and the translated products were analyzed by an SDS-PAGE.

Mobility Shift and DNaseI Footprinting Assays.

The oligonucleotide probes used for mobility shift assay were shown in Fig. 3B. Binding reaction (10 µl) was carried out in a binding buffer containing 25 mM HEPES-NaOH (pH7.9), 25 mM NaCl, 5 mM $MgCl_2$, 8.7% glycerol, 1 µg poly(dI-dC)·poly(dI-dC), 1 µg sonicated salmon testis DNA and 0.1 ng oligonucleotide probe labeled by filling-in reaction with Klenow enzyme. The reaction was started by adding proteins and kept on ice for 30 min. Protein-DNA complexes were visualized on a 5% polyacrylamide gel (Matsuno *et al.*, 1990). The probe DNA containing -331 to -50 sericin-1 upstream region used for footprinting assay was described previously (Matsuno *et al.*, 1989). Footprinting was performed as described (Matsuno *et al.*, 1989) except that the time for the DNaseI treatment was increased to 10 min for the *in vitro* translated products. Nuclear extracts from the middle or posterior silk gland of 2-day-old fifth-instar or fourth molting stage larvae

were prepared as described (Matsuno *et al.*, 1989).

RNA Extraction and Blot-Hybridization (Northern) Analysis.

Total RNA was isolated by using an acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) and poly(A)$^+$ RNA was enriched by oligo (dT)-cellulose chromatography. The RNA was electrophoresed on 1% agarose-1.1 M formaldehyde gels (Ausubel *et al.*, 1987) and transferred onto Nylon membranes (Biodyne, PALL). Blots were hybridized with the whole POU-M1 cDNA probe labeled by a random primer method. A control probe which hybridizes with an abundantly expressed transcript of unknown identity was also used to check the integrity of RNA.

Synthetic Peptides and Immunization.

Rabbits were immunized by injecting 2 mg of BSA conjugated oligopeptides synthesized according to the deduced POU-M1 amino acid sequence by a Peptide synthesizer (Applied Biosystems Inc.) mixed with complete Freund's adjuvant. Following the subsequent 3 boosts with 2 weeks interval of the oligopeptiides mixed with incomplete Freund's adjuvant, blood samples were taken and the serums were tested for anti-POU-M1 activity. IgG was purified from each serum by GammaBind G column (GENEX), and used for further experiments.

Hyper-shift Assay.

Affinity purified IgGs were added to the mobility shift reaction mixtures with or without competitor peptides,

incubated further for 20 min and tested for mobility shift as described (Matsuno *et al.*, 1990).

Western Blotting.

Proteins of nuclear extracts were separated by an SDS-PAGE, and transferred to nitrocellulose membrane (Schleicher & Schuell). Immunoblotting was performed with the rabbit IgGs described above and horse radish peroxidase-labelled goat anti-rabbit IgG (CAPPEL) (Amanai *et al.*, 1991).

Results

Isolation and Sequence Analysis of POU-M1 cDNA. Based on the assumption that SGF-3 is a POU domain protein, a PCR method was used to clone POU domain sequences from the cDNA of the middle silk glands. PCR primers corresponding to the two highly conserved regions of the POU domain (Fig. 1) were prepared by the method described by He *et al.* (1989) which were successfully used in the isolation of a number of mammalian POU domain sequences. As a result of PCR with these primers (Fig. 2), one type of POU domain sequence was obtained from the cDNA of the middle silk gland, which is named POU-M1. In these trials, POU domain sequences could not be detected in the cDNA of the posterior silk gland. Using this PCR fragment as a probe, longer cDNA clones were isolated from a middle silk gland cDNA library. From 240,000 plaques 23 POU-M1-derived clones were detected. However, other types of POU domain genes were not obtained by cross hybridization. Fig. 4 shows the nucleotide sequence of the longest clone and the deduced amino acid sequence of the putative protein. The sequence revealed one open reading frame of 351 amino acids with a deduced molecular mass of 38.6 kDa. The methionine residue at the nucleotide position 181 is designated as the initiator because it is the first methionine residue after many termination codons and the sequence around it matches with an optimal consensus sequence for eukaryotic translation initiation, -CCA/GCC-

ATG, as defined by Kozac (1984).

The POU-M1 protein contains a POU domain identical to that of the *Drosophila* Cf1-a protein which is a putative neuron-specific transcription factor (Johnson and Hirsh, 1990). This 170 amino acid region shows a homology of greater than 90% with those of the POU-III type proteins (He *et al.*, 1989), such as the human Brn-1 and Brn-2 proteins and the murine Oct-6 protein (Fig. 5). Considering the phylogenic divergence between insects and mammals, it is surprising to find such a high conservation. In the N-terminal region, repeats of proline-rich region alternated with histidine-rich region can be found (Fig. 4). Similar repeats are also found in the Oct-6 protein (Suzuki N. *et al.*, 1990). Proline-rich regions have been shown to function as an activation domain in several transcription factors (Mermod *et al.*, 1989; Mitchell and Tjian, 1989). It is possible that this region of the POU-M1 protein has a similar regulatory function.

The POU-M1 Protein and SGF-3 Bind Indistinguishably to the SC Region.

In vitro transcription/translation of the POU-M1 cDNA revealed a protein of about 38 kDa as deduced from the nucleotide sequence (data not shown). To know whether this POU-M1 protein is identical to SGF-3 or not, binding specificity was tested for the *in vitro* synthesized POU-M1 protein by mobility shift and nuclease protection assays. Three protein binding regions of the sericin-1 gene, the

SA, SB and SC regions, have been identified previously (Matsuno *et al.* 1989) and their schematic construction and sequences are described in Fig. 3A and B. Among them, the SC region is known to interact with SGF-3 with a high affinity while the SA region binds another silk gland specific factor, SGF-1 (Hui *et al.*, 1990; Matsuno *et al.*, 1990) which are also schematically described in Fig. 3A. As shown in Fig. 6A, the *in vitro* synthesized POU-M1 protein bound strongly to the SC region but not to the SA region. Interestingly, the POU-M1 protein also bound weakly to the SB region, which contains a 7/8 match of the canonical octamer sequence, ATGTAAAT (Matsuno *et al.*, 1989). The binding of the POU-M1 to the SC region is specific because an excess of the SC oligonucleotide, but not the SA oligonucleotide, competed the formation of the complex (Fig. 6B). This binding was affected by mutations in the SC region as shown by a diminished ability of the SCM oligonucleotide in competition analysis. It should be emphasized that the SCM oligonucleotide also showed a diminished ability to bind SGF-3 (Matsuno *et al.*, 1990).

Next, a mobility shift assay of the *in vitro* synthesized POU-M1 protein and SGF-3 in a middle silk gland extract was carried out in parallel. As shown in Fig. 7, they form complexes which migrate virtually with the same mobilities. Furthermore, the SC oligonucleotide competed these complexes essentially with the same kinetics. The SB and the SCM oligonucleotides were also shown to compete these complexes with similar kinetics,

though much weakly (data not shown). The intensities of the bands were measured and expressed as persent activities (Fig. 8), which indicate the competition patterns are similar. These observations suggest that the POU-M1 protein and SGF-3 are identical. To further support this hypothesis, DNaseI protected patterns were examined for the POU-M1 protein and SGF-3 on the sericin-1 gene promoter (Fig. 9). The POU-M1 protein gave a DNaseI footprint and hypersensitive sites on the SC region virtually the same as the SC region protection by the SGF-3 in middle silk gland extract, while the extract gave protection at SA and SB regions as well.

Antibodies Raised Against the Deduced POU-M1 Amino Acid Sequences Also Reacted to SGF-3 with the Same Specificity. Oligopeptide sequences corresponding to the deduced POU-M1 amino acid sequences N1, C1 and HC shown in Fig. 2 were synthesized and used for immunization by K. Matsuno. Matsuno further demonstrated that antiserums against the N1 and C1 peptides reacted with POU-M1 protein. Affinity purified IgGs from the serums were added to the gel mobility shift assay reaction mixtures. Both the N1 and C1 antibodies reacted with SGF-3/SC complex and POU-M1/SC complex and resulted in hyper-shifting the complexes (Fig. 10). Addition of the cognate antigens wiped out the antibody effect leaving the SGF-3/SC and POU-M1/SC complexes uneffected. Addition of unrelated antigens did not disturb the hyper-shift effect of the antibodies. These results clearly indicate that both antibodies are

very specific. It is concluded that the POU-M1 protein is identical with the SGF-3.

Developmentally Regulated Expression of the POU-M1 Gene in the Silk Gland.

To know the profile of POU-M1 transcript and protein during development of posterior and middle silk gland, RNA blot hybridization and Western blotting were performed. A 3.1 kb transcript was detected by Northern hybrydization (Fig. 11A) both in the posterior and middle silk glands during the fourth molting stage (lanes 1 and 5), and when the larvae entered the fifth intermolt the level in the middle silk gland was reduced and then increased again slightly (lanes 6 to 8) while the transcript became undetectable in the posterior silk gland (lanes 2 to 4). As shown in Fig. 11B the transcript continued to be detected in middle silk gland until the end of the fifth larval instar (Fig. 11B lane 6), along with active expression of sericin-1 gene. By mobility shift assays, SGF-3 has been found abundantly in the middle silk gland of 2-day-old fifth instar larvae while at a much lower level in the posterior silk gland of this stage (Hui *et al.*, 1990; Matsuno *et al.*, 1990) and found both in the middle and posterior slik glands during the fourth molting stage (Fig. 12). In Western blotting performed using nuclear extract proteins from silk gland (Fig. 13B), the POU-M1 C1 antibody detected an approximately 38 kDa band in both extracts from the posterior and middle silk gland of the fourth molt and only in the extract from the middle

silk gland of 2-day-old fifth intermolt. The POU-M1 protein analyzed in parallel showed the the same size band (Fig. 13A). This differential existence of POU-M1 transcript and protein during the fifth intermolt is consistent with the middle silk gland specific expression of the sericin-1 gene. These results indicate that the POU-M1 expression is differentially regulated in the posterior and middle silk glands during the fourth molt/fifth intermolt.

Discussion

It has been previously described that the silk gland factor SGF-3 is probably a key *trans*-acting factor for the regulation of the sericin-1 gene (Matsuno *et al.*, 1990). Based on the observation that the SGF-3 is an octamer-binding protein (Hui *et al.*, 1990) and that several mammalian octamer-binding proteins possess a POU domain for specific binding (Ruvkin and Finney, 1991; Herr *et al.*, 1988), it has been attempted to clone the cDNA for SGF-3 by isolating POU domain-containing cDNA from the silk gland. The present paper reports here the cDNA cloning and characterization of a POU domain gene, POU-M1. The POU-M1 protein was shown to be identical with the SGF-3 in DNA-binding and immunological studies. Northern blot hybridization and Western blotting analyses revealed that the POU-M1 was detected specifically in the middle silk gland during the fifth intermolt. The result is consistent with the hypothesis that the POU-M1 is SGF-3, since sericin-1 gene is expressed specifically in middle silk gland during intermolt. It is concluded that the POU-M1 gene encodes SGF-3 and postulated that its developmental regulation in the silk gland is probably involved in the transcriptional control of the silk protein genes. For the sake of clarity, SGF-3/POU-M1 will be used in place of the POU-M1 protein in the following discussions.

SGF-3/POU-M1 belongs to a family of evolutionarily

conserved proteins. It posesses a POU domain identical to that of the *Drosophila Cf1-a* protein, a putative transcription factor involved in the regulation of the dopa decarboxylase gene in selected dopaminergic neurons (Johnson and Hirsh, 1990), and closely related to those of other POU-III type proteins, such as the mammalian Brn-1, Brn-2 and Oct-6 proteins, which are likely involved in the developmental regulation of the central nervous system (He *et al.*, 1989; Suzuki, N. *et al.*, 1990). It has been further discussed that POU domain genes have met the increasing requirement for the development of newly acquired midbrain and forebrain in vertebrate (Rosenfeld, 1991; He and Rosenfeld, 1991). It is highly probable that SGF-3/POU-M1 might play a role in neurogenesis and that the insect POU domain gene have met the evolutional requirement and developmental maintenance for such highly specialized tissue as silk gland.

Since the POU domains are involved in DNA binding, it is likely that the recognition sequences of these evolutionarily conserved proteins are similar. Considering that the POU domain part of POU-M1 is identical with that of *Drosophila* Cf1-a, it is interesting to compare the binding sequences of the *Drosophila* Cf1-a, the Cf1 region of the dopa decarboxylase gene (Johnson and Hirsh, 1990), and the SGF-3/POU-M1 binding site, the SC region of the sericin-1 gene (Matsuno *et al.*, 1990). They matches only five bases in the octamer eight bases including TATNCA motif, where POU specific domain is

supposed to contact, but interestingly they matches as much as five bases in the six base A/T rich sequences 5' adjacent to the octamer sequence, where POU homeo domain is supposed to contact (Rosenfeld, 1991). However, no significant matches were observed around these regions. On the other hand it has been shown previously that, though different POU domain proteins can bind similar octamer-like sequences, each of them recognizes distinct high affinity sites (see Ruvkin and Finney, 1991 for a review). A relaxed binding specificity as well as other features like cooperative binding are probably important for functional interaction (see Struhl, 1991 for a review). As shown in Fig. 6A, SGF-3/POU-M1 can also bind to the SB region of the sericin-1 gene with a lower affinity and the mobility shift band formed by SB oligonucleotide and middle silk gland nuclear extract proteins were hyper-shifted by adding the POU-M1 antibodies (K. Matsuno unpublished), indicating that multiple SGF-3/POU-M1 binding sites might be involved in the precise control of the sericin-1 gene transcription.

Though the transcriptional function of SGF-3/POU-M1 has not been demonstrated, the proline-rich and histidine-rich regions found in the N-terminal region suggest that it might work as a transcriptional activator like other mammalian transcrition factors possessing similar structural features (Suzuki, N. *et al.*, 1990; Mermod *et al.*, 1989; Mitchell and Tjian, 1989). Actually on the promoter possessing multimerized SC sites in its

upstream SGF-3/POU-M1 worked as an activator (Matsuno *et al.*, 1990) tissue-specifically (K. Matsuno, unpublished). However, Western blotting and mobility shift analyses revealed that a lower amount of SGF-3/POU-M1 exists during the fourth molting stage when sericin-1 gene is almost repressed. To explain this contradiction it may be necessary to consider effects of post-translational modification, protein-protein interaction or difference in concentration that affects the function of SGF-3/POU-M1 during the fourth molt/fifth intermolt. But considering the role of SGF-3/POU-M1 during the fourth molting stage in posterior silk gland, it might have some other function commonly needed in this stage. As several POU domain proteins have been shown to be DNA replication factors (Verrijzer *et al.*, 1990) and the maternally expressed Oct-3/4 protein was found to be important for the first embryonic cell division in mice (Rosner *et al.*, 1991), SGF-3/POU-M1 might also play an additional role in DNA replication. This is of particular interest because multiple rounds of DNA replication are known to take place during the development of the silk gland (Tashiro *et al.*, 1968). Especially the last three rounds of replication are initiated at the fourth molting stage and finished by 3 days of the fifth instar. The active expression of the SGF-3/POU-M1 gene during the fourth molt in both the posterior and middle silk glands, when silk genes are repressed, might serve specially in this process. On the other hand it may be considered such an amount of

expression preceding the activation of its putative target gene might indicate the involvement of its product in the initiation of the target gene transcription. The specific expression of POU-M1 gene during the fifth intermolt might confer the strict discrimination of expression pattern between the posterior part and the middle part of the silk gland. However, further biochemical and genetic analyses are necessary to prove any function on the silk protein genes and the control on DNA replication during silk gland development.

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References

Amanai, K.,Sakai, M.,Sakurai, S.,Mori, T.,Nikaido, O., & Otaki, T. (1991). Occurrence of lectin in the silkgland of the silkmoth, *Bombyx mori*. *Develop. Growth & Differ.* **33**, 421-427.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, K. (1987). *Current Protocols in Molecular Biology* (John Wiley & Sons, New York).

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

He, X. and Rosenfeld, M. G. (1991). Mechanism of complex transcriptional regulation; Implication for brain development. *Neuron* **7**, 183-196.

He, X., Treacy, M.M., Simmons, D.M., Ingraham, H.A., Swanson, L.W. & Rosenfeld, M.G. (1989). Expression of a large family of POU-domain regulatory genes in mammalian brain development. *Nature* **340**, 35-42.

Herr, W., Sturm, H.A., Clerc, R.G., Corcoran,L.M., Baltimore, D., Sharp, P.A., Ingraham, H.A. & Rosenfeld, M.G. (1988). The POU domain; A large conserved region in the mammalian *pit-1, oct-1, oct-2* and *Caenorhabiditis elegans unc-86* gene products. *Genes & Dev.* **2**, 1513-1516.

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.

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

Kozak, M. (1984). Compilation and analysis of upstream from the translational start site in eukaryotic mRNAs. *Nucl. Acids Res.* **12**, 857-872.

Li, S., Crenshaw, E.B., Rawson, E.J., Simmons, D.M., Swanson, L.W. & Rosenfeld, M.G. (1990). Drawf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene *Pit-1. Nature* **347**, 528-533.

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, M., 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. *Nucl. Acids Res.* **18**, 1853-1857.

Mermod, N., O'Neill, E.A. Kelly, T.J. & Tjian, R. (1989). The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. *Cell* **58**, 741-753.

Mitchell, P.J. & Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**, 371-378.

Monuki, E.S., Kuhn, R., Weinmaster, G., Trapp, B. & Lemke, G. (1990). Expression and activity of the POU transcription factor SCIP. *Science* **249**, 1300-1303.

Okamoto, K., Okazawa, H., Okuda, A., Sakai, M., Muramatsu, M. & Hamada, H. (1990). A novel octamer transcription factor is differentially expressed in mouse embryonic cells. *Cell* **60**, 461-472.

Rosenfeld, M.G. (1991). POU-domain transcription factors; pou-er-ful developmental regulators. *Genes & Dev.* 5, 897-907.

Rosner, M.H., De Santo, R.J., Arnheiter, H. & Staudt, L.M. (1991). Oct-3 is a maternal factor required for the first mouse embryonic division. *Cell* **64**, 1103-1110

Rosner, M.H., Vigano, M.A., Ozato, K., Timmons, P.M. Poirier, F., Rigby, P.W.J. & Staudt, L.M. (1990). A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345**, 686-691.

Ruvkin, G. & Finney, M. (1991). Regulation of transcription and cell identity by POU-domain proteins. *Cell* **64**, 475-478.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning*; Laboratory Manual (Cold Spring Harbor, New York).

Scholer, H.R., Ruppert, S., Suzuki, N., Chowdhury, K. & Gruss, P. (1990). New type of POU domain in germline-specific protein Oct-4. *Nature* **344**, 435-439.

Struhl, K. (1991). Mechanism for diversity in gene expression patterns. *Neuron* **7**, 177-181.

Suzuki, N., Rohdewohld, H., Neuman, N., Gruss, P. & Scholer, H.R. (1990). Oct-6; A POU domain transcription factor expressed in embryonic and stem cells and in the developing brain. *EMBO.J.* **9**, 435-439.

Suzuki, Y., Takiya, S., Suzuki, T., Hui, C.-c., Matsuno, K., Fukuta, M., Nagata, T. & Ueno, K. (1990). Developmental regulation of silk gene expression. in *Molecular Iesect Science*, eds. Hagedorn, H.H., Hildebrand, J.G., Kidwell, M.G. & Law, J.H. (Plenum press, New York), pp83-89.

Suzuki, Y., Tsuda, M., Takiya, S., Hirose, S., Suzuki, E., Kameda, M. & Ninaki, O. (1986). Tissue-specific transcription enhancement of the fibroin gene characterized by cell-free systems. *Proc. Natl. Acad. Sci. USA* **83**, 9522-9526.

Tashiro, Y., Morimoto, T., Matsuura, S. & Nagata, S. (1968). Studies on the posterior silk gland of the silkworm, *Bombyx mori*. I. Growth of posterior silk gland cells and biosynthesis of fibroin during the fifth larval instar. *J. Cell Biol.* **38**, 574-588.

Tsuda, M. & Suzuki, Y. (1983). Transcription modulation in vitro of the fibroin gene exerted by a 200-base-pair region upstream from the TATA box. *Proc. Natl. Acad. Sci. USA* **80**, 7442-7446.

Verrijzer, C.P., Kal, A.J. & Van der Vliet, P.C. (1990). The DNA binding domain (POU domain) of transcription factor oct-1 suffices for stimulation of DNA replication. *EMBO J.* **9**, 1883-1888.

Fig. 1. Schematic representaion of four first identified POU domain proteins, Pit-1, Oct-1, Oct-2 and Unc-86, with the approximate location of the POU specific and POU homeodomains indicated. Two highly conserved amino acid regions represented as FK(V/Q)RRIKLG and RVWFCN(R/Q)RQ, on which oligonucleotide primers were designed and used for polymerase chain reaction (PCR), were also shown.

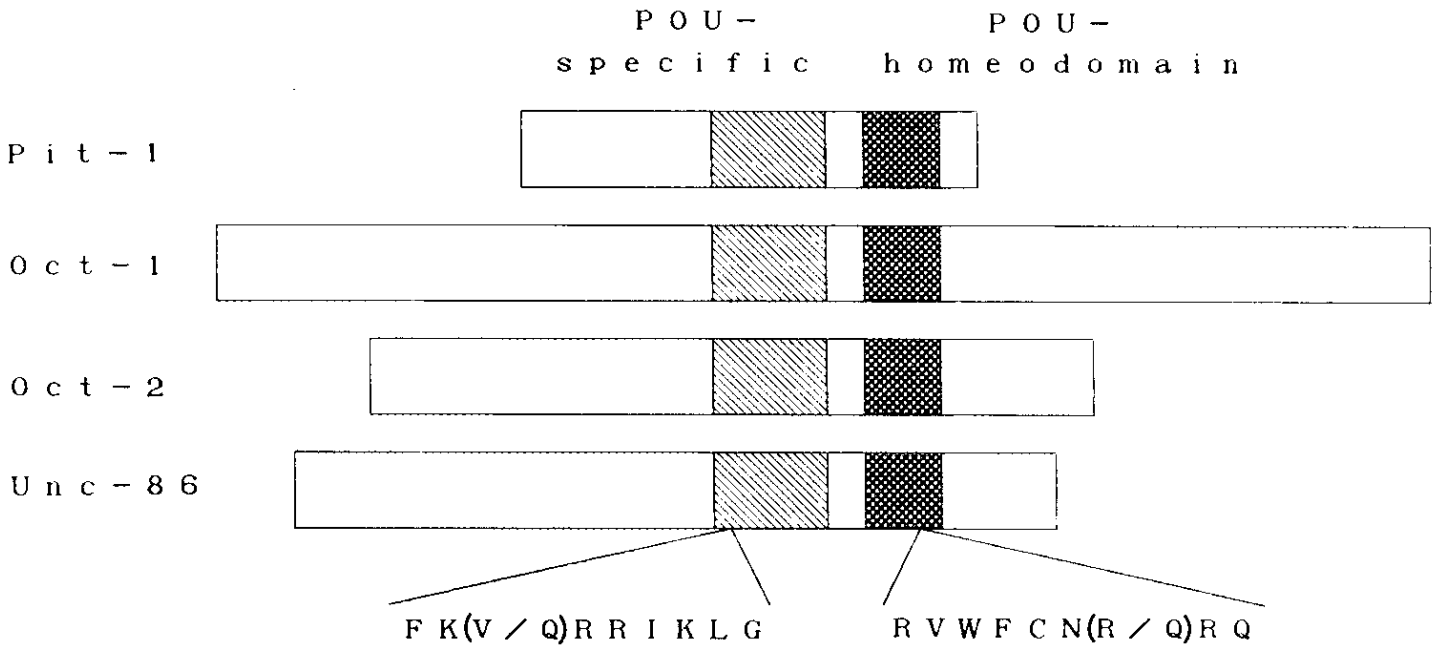

P O U –
s p e c i f i c
P O U –
h o m e o d o m a i n
P i t – 1
O c t – 1
O c t – 2
U n c – 8 6
F K(V / Q)R R I K L G
R V W F C N(R / Q)R Q

Fig. 2. The PCR products from cDNAs of the posterior and middle silk gland of 2-day-old fifth instar larvae. The amplified products from the posterior (lanes 2 and 3) and middle silk gland (lanes 4 and 5) cDNAs were visualized on a 2% Sea Plaque agarose gel (TAKARA SHUZO CO.,LTD.). The bands of expected size (350-400bp) indicated by the arrow were cut out and subjected to sequencing analysis. Lane 1 shows a size marker, pBR322 *Hin*fI digests.

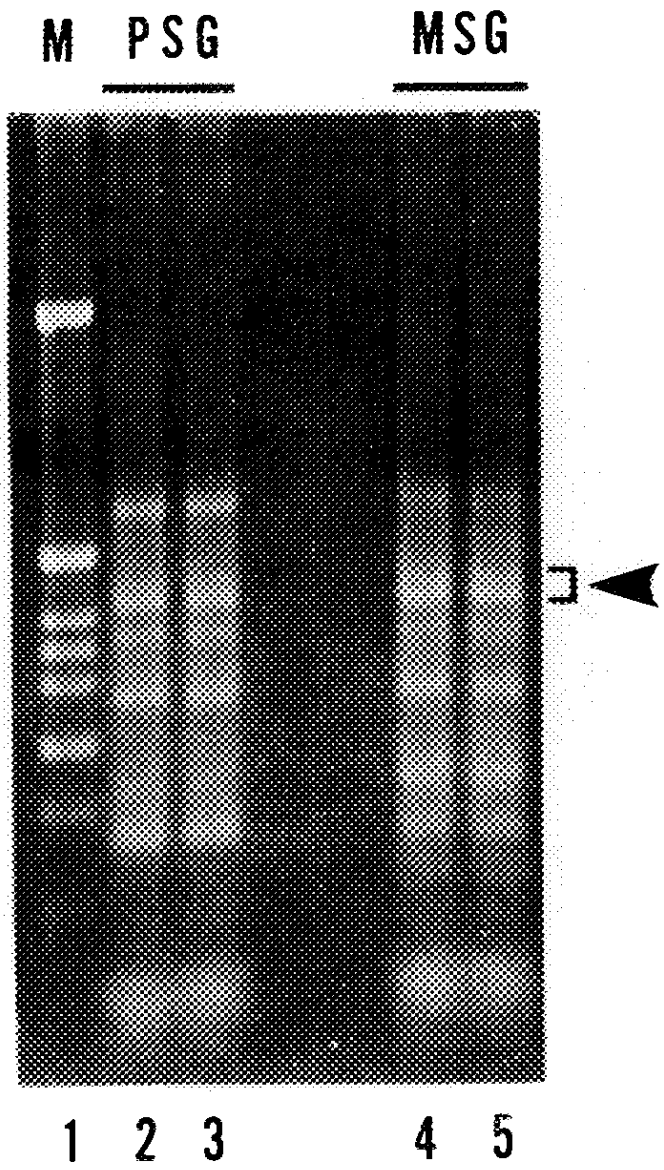
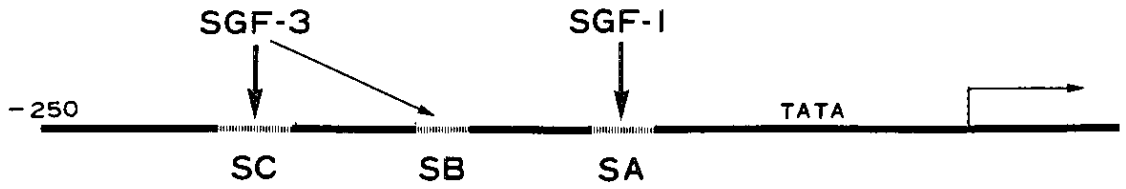

M
PSG
MSG
1
2
3
4
5

Fig. 3. (A) Schematic representation of sericin-1 gene promoter. Three *cis* elements, SA, SB and SC, have been identified on the promoter (Matsuno *et al.*, 1989). Putative transcription factor SGF-1 is known to interact with SA site, while SGF-3 interacts with SC strongly and SB with lower affinity. (B) The nucleotide sequences of double stranded oigonucleotides for SC, SCM, SB and SA sites. Nucleotide sequence of *Drosophila* Cf1 was aligned under the SC site sequence with identical nucleotide shown by dashes. Octamer-like regions were shaded. The mutated nucleotides in SCM were shown in bold letters.

A



B

SC
AATTCAACGAGCCATGAATAAATTAGAAATCAAT
GTTGCTCGGTACTTATTTAATCTTTAGTTATTAA

Cfl
ATTT--TTT----CC-TC-----TTG

SCM
AATTCAACGAGACATAGATAAATTAGAAATCAAT
GTTGCTCTGTATCTATTTAATCTTTAGTTATTAA

SB
AATTCCATTTACATAGAG
GGTAAATGTATCTCTTAA

SA
AATTTTGTATCTCATTGTTTGCACAAATGTTTG
AACGATATGTAACAAACGTGTTTACAAACTTAA

Fig. 4. Nucleotide and deduced amino acid sequences of POU-M1. POU-specific domain and POU-homeodomain are boxed. Except for those in the POU-domain, proline residues are underlined and histidine residues are circled. Proline-rich regions are marked with wavy lines. The amino acid sequences synthesized for immunization are shaded and named N1, HC and C1 respectively.

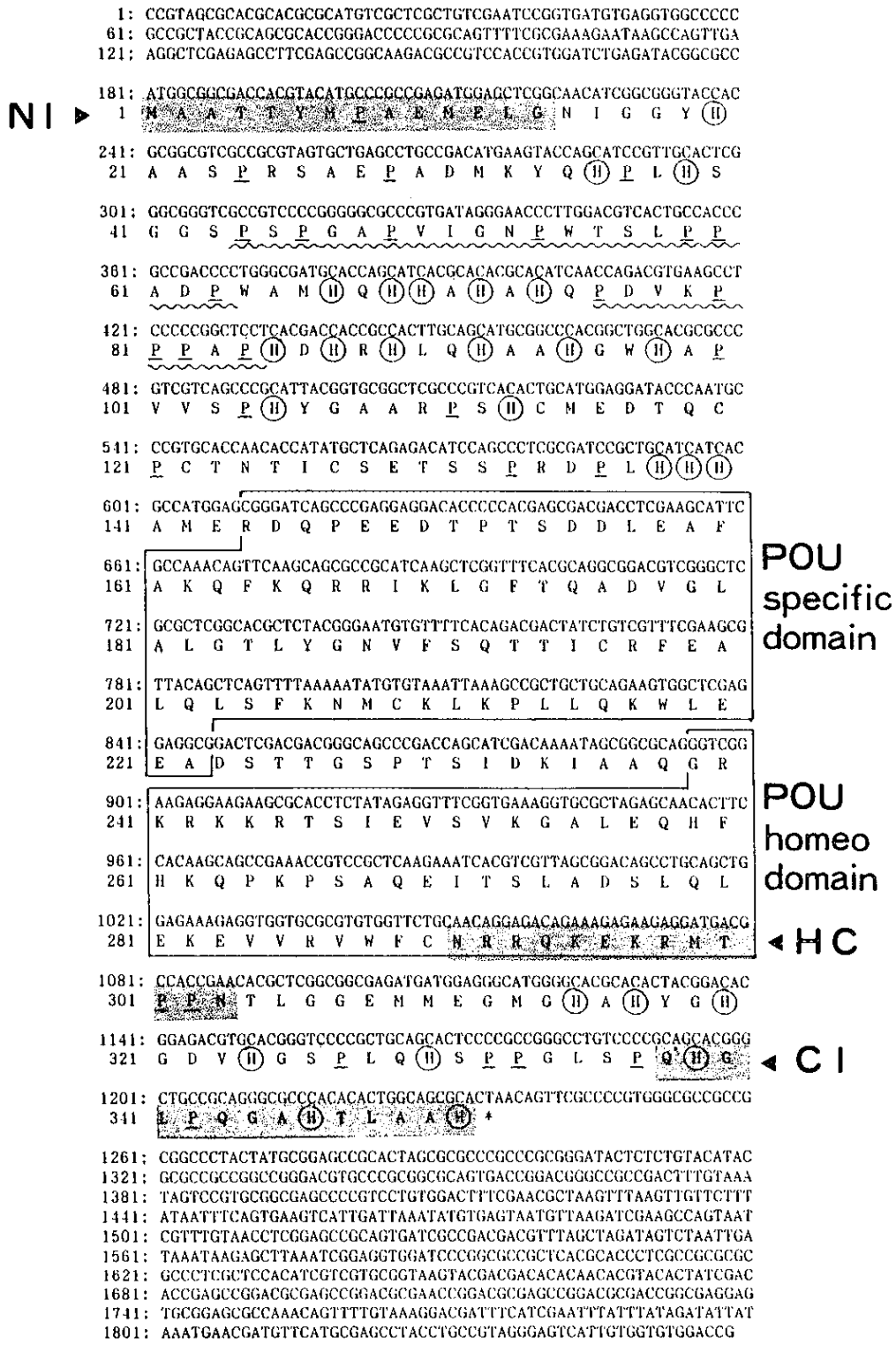

NI
POU specific domain
POU homeo domain
HC
CI

Fig. 5. Sequence comparison of the POU-domains of POU-M1, Cf1-a, Pit-1, Oct-1, Oct-2 and Oct-3. Identical amino acid residues are indicated by dashes and dots represent a gap in the aligned sequences.

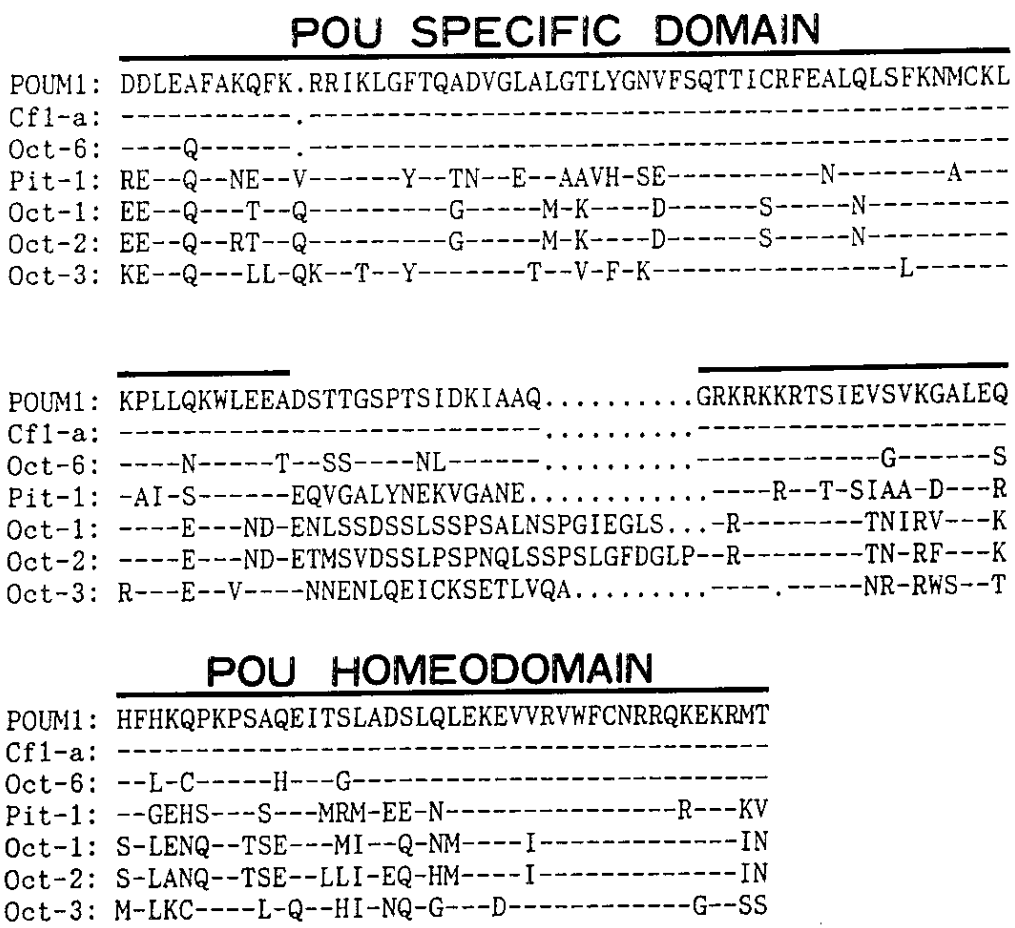

POU SPECIFIC DOMAIN
POUM1: DDLEAFAKQFK.RRIKLGFTQADVGLALGTLYGNVFSQTTICRFEALQLSFKNMCKL
Cf1-a: ----------.----------
Oct-6: ----Q------.----------
Pit-1: RE--Q--NE--V------Y--TN--E--AAVH-SE----------N-------A---
Oct-1: EE--Q---T--Q---------G-----M-K----D------S-----N---------
Oct-2: EE--Q--RT--Q---------G-----M-K----D------S-----N---------
Oct-3: KE--Q---LL-QK--T--Y-------T--V-F-K----------L------
POUM1: KPLLQKWLEEADSTTGSPTSIDKIAAQ.....GRKRKKRTSIEVSVKGALEQ
Cf1-a: ----------.----------
Oct-6: ----N-----T--SS----NL------.----------G------S
Pit-1: -AI-S------EQVGALYNEKVGANE.....----R--T-SIAA-D---R
Oct-1: ----E---ND-ENLSSDSSLSSPSALNSPGIEGLS...-R--------TNIRV---K
Oct-2: ----E---ND-ETMSVDSSLPSPNQLSSPSLGFDGLP--R--------TN-RF---K
Oct-3: R---E--V----NNENLQEICKSETLVQA.....----.-----NR-RWS--T
POU HOMEODOMAIN
POUM1: HFHKQPKPSAQEITSLADSLQLEKEVVRVWFCNRRQKEKRMT
Cf1-a: ----------
Oct-6: --L-C-----H---G----------
Pit-1: --GEHS---S---MRM-EE-N----------R---KV
Oct-1: S-LENQ--TSE---MI--Q-NM----I----------IN
Oct-2: S-LANQ--TSE--LLI-EQ-HM----I----------IN
Oct-3: M-LKC----L-Q--HI-NQ-G---D----------G--SS

Fig. 6. (A) Mobility shift assay with the SC, SB and SA oligonucleotide probes. Each probe was assayed with a rabbit reticulocyte lysate programmed with (+) or without (-) the POU-M1 mRNA. The arrow head shows the POU-M1 complex. (B) Competition analysis of the POU-M1/SC complex. The reaction mixtures were incubated with 50- or 200-fold molar excess of unlabelled SC, SCM, or SA oligonucleotide, or without competitor (-). The larger arrow head indicates the POU-M1/SC complex. The smaller arrow indicates a complex probably formed with single stranded probes.

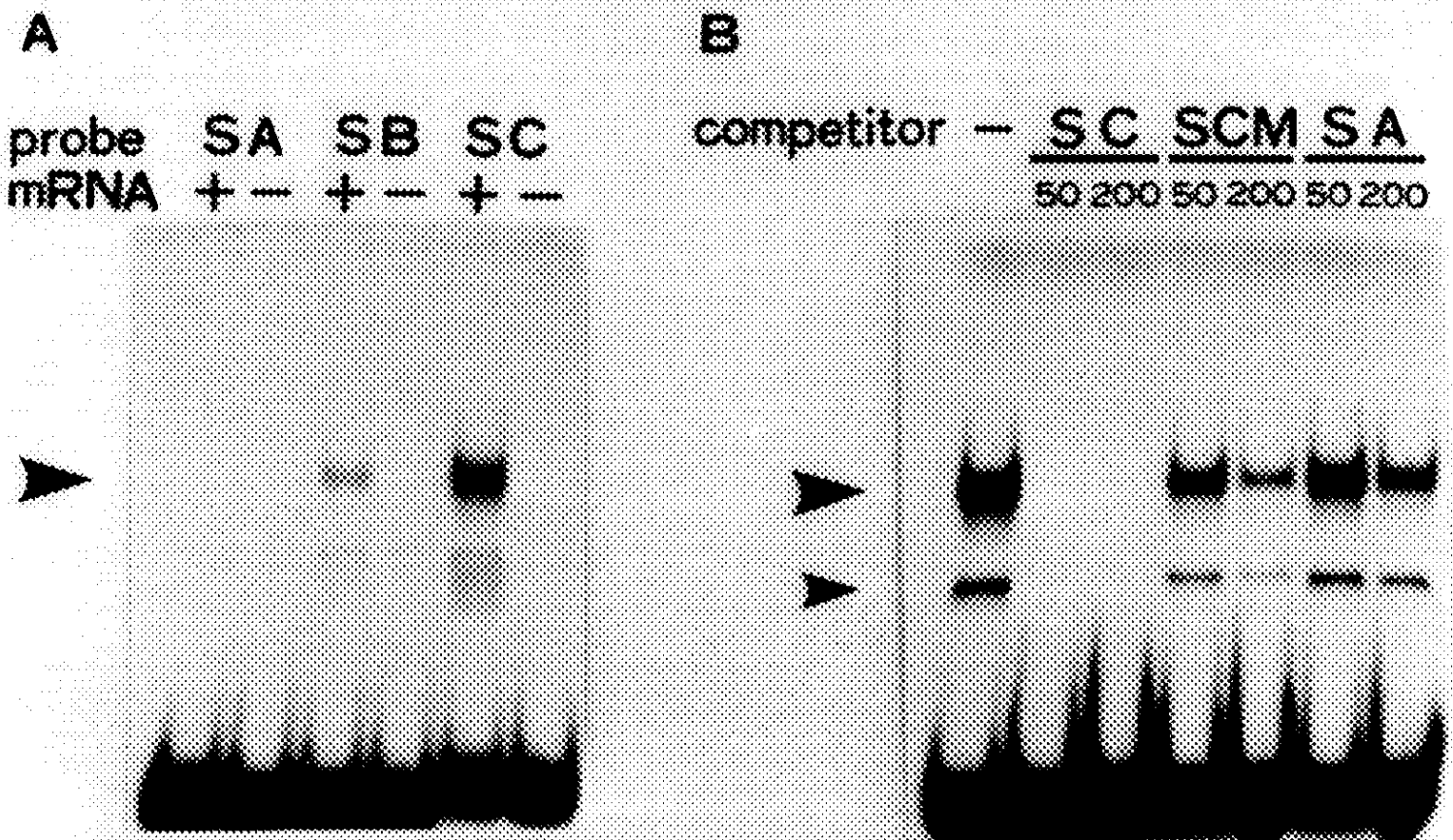

A
probe SA SB SC
mRNA + – + – + –
B
competitor – SC SCM SA
50 200 50 200 50 200

Fig. 7. Mobility shift assay of the POU-M1 protein and SGF-3. The POU-M1/SC and SGF-3/SC complexes were examined by competing with 5-, 25- and 50-fold molar excess of unlabelled SC oligonucleotide.

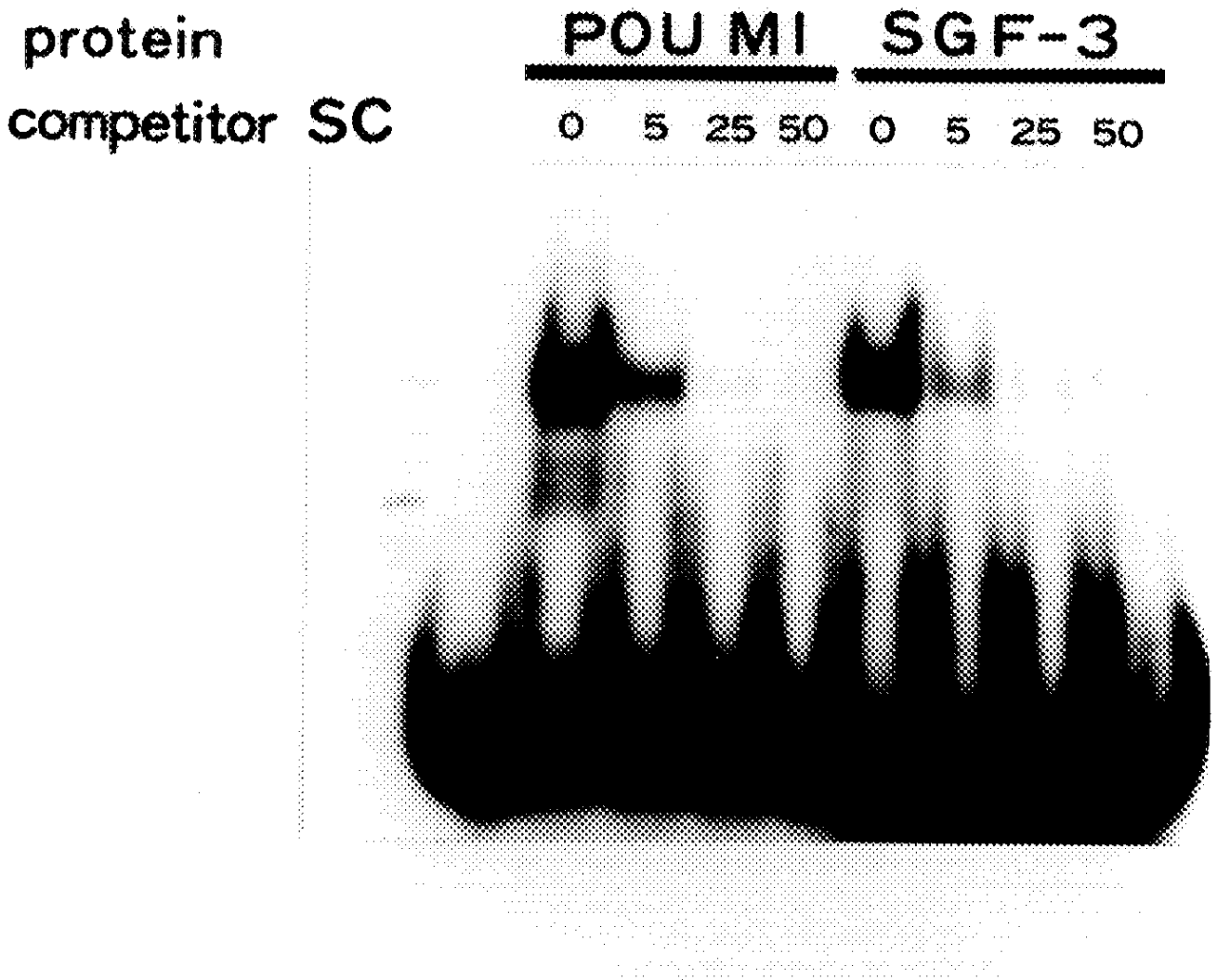
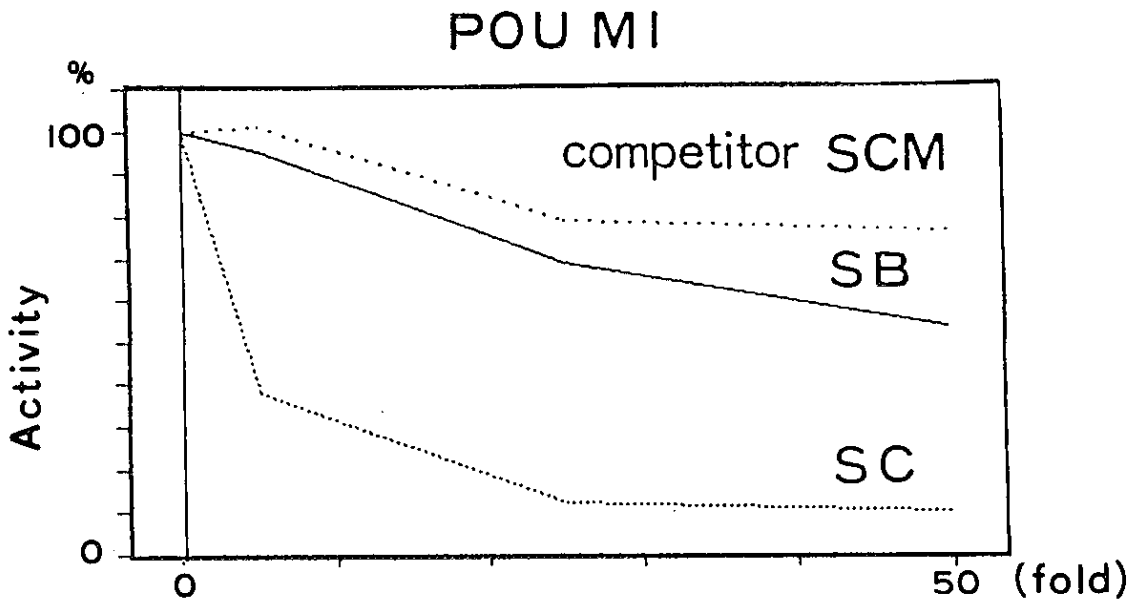

protein
competitor SC
POU MI
SGF-3
0 5 25 50 0 5 25 50

Fig. 8. A comparison of the competition kinetics expressed as percentage activities between the POU-M1/SC and the SGF-3/SC complexes against SC, SB and SCM competitors. Intensity of the bands was measured by Bio Image Analyzer (Fuji).


POU MI
%
100
0
Activity
competitor SCM
SB
SC
0
50
(fold)

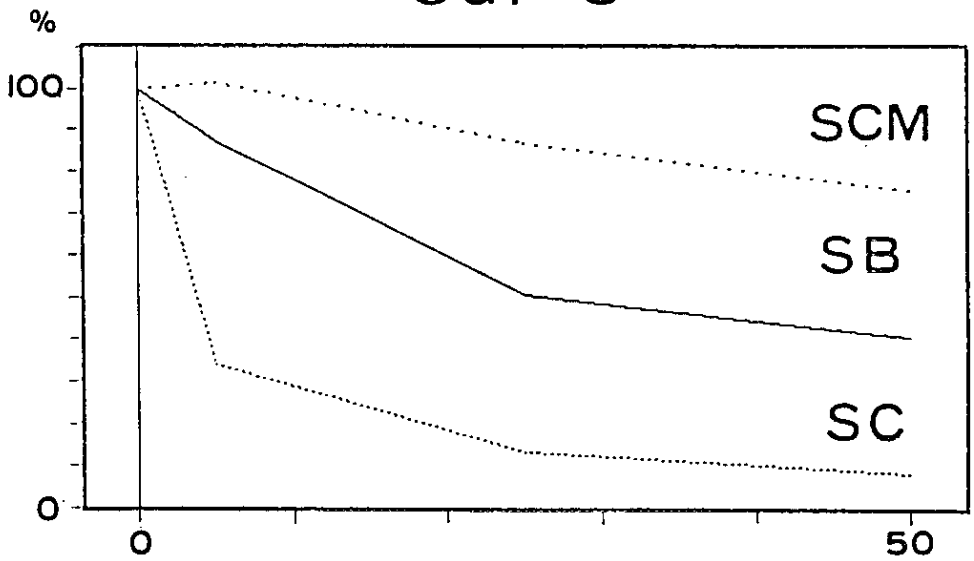

SGF-3
%
100
0
SCM
SB
SC
0
50

Fig. 9. A comparison of the DNaseI footprints by the POU-M1 protein and SGF-3. Footprint reactions were performed in the absence of protein (-), in the presence of rabbit reticulocyte lysate incubated without mRNA (Mock) or with POU-M1 mRNA (POUM1), and in the presence of a middle silk gland extract (Extract). Vertical lines indicate the protected regions referred to as the SC, SB and SA sites. Hypersensitive sites generated in common between POUM1 and Extract were indicated by arrows.

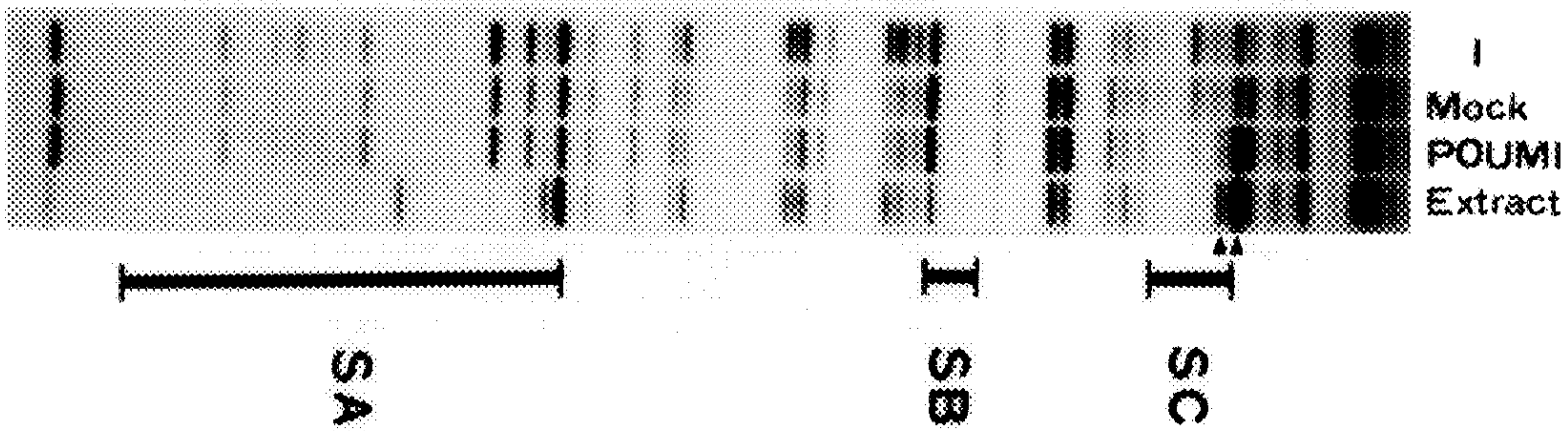

I
Mock
POUMI
Extract
SA
SB
SC

Fig. 10. Hyper-shift assay and peptide competition analysis to the POU-M1/SC and SGF-3/SC complexes. Two kinds of antibodies raised against the N1 and C1 oligopeptides, respectively, were added to the POU-M1/SC and SGF-3/SC complexes with or without 15 μg of competitor peptide N1, C1 or HC.

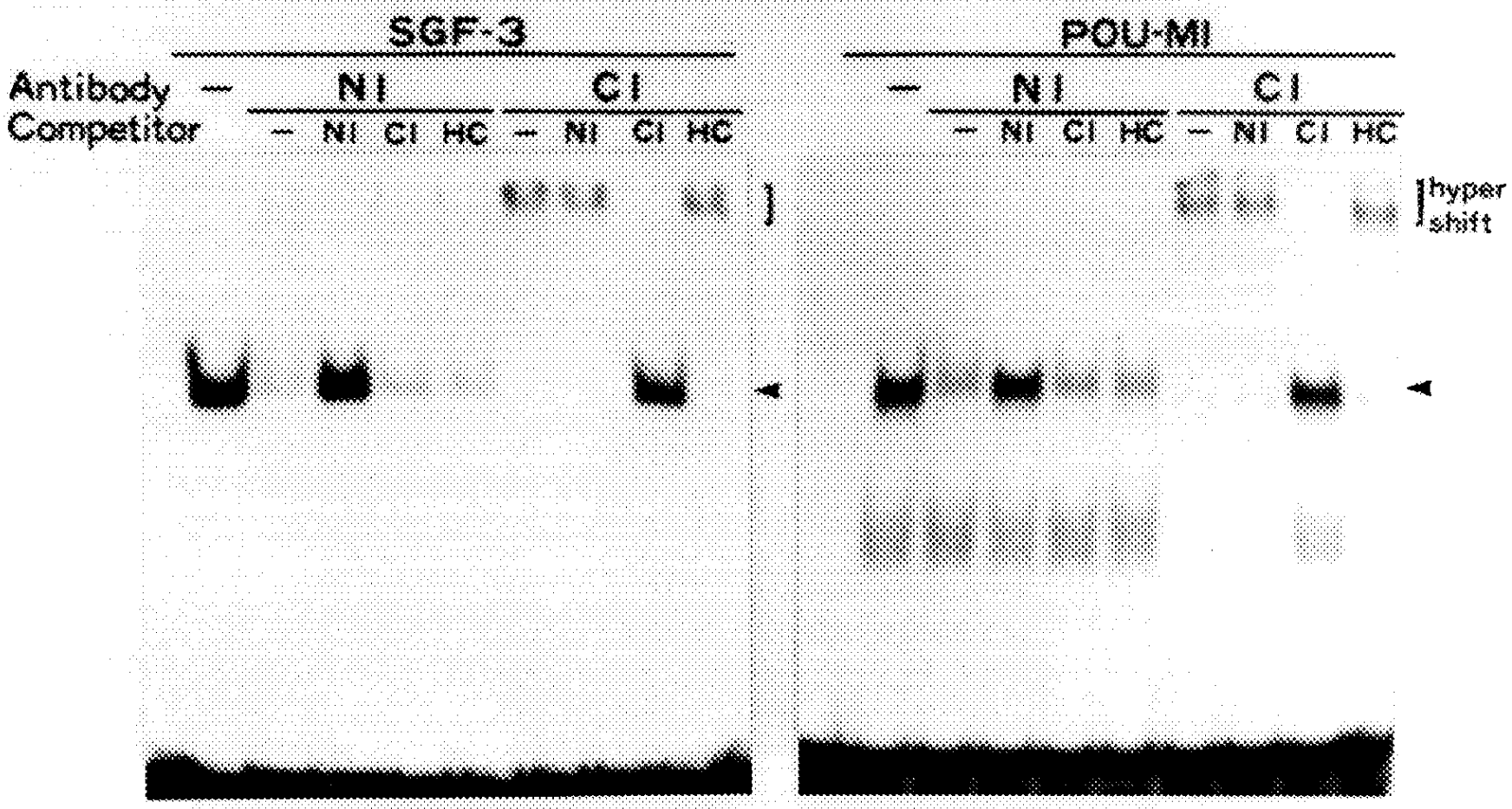

SGF-3
POU-MI
Antibody
Competitor
–
NI
CI
HC
hyper shift

Fig. 11. Expression of the POU-M1 gene in the middle and posterior silk gland during the fourth molting stage and the fifth larval instar. (A) A Northern blot of poly(A)$^+$ RNA (5 µg) isolated from the posterior (lanes 1-4) and the middle silk gland (lanes 5-8). Lanes: 1 and 5, 15 h after the fourth apolysis; 2 and 6, the fourth ecdysis; 3 and 7, 24 h after the fourth ecdysis; 4 and 8, 48 h after the fourth ecdysis. They were hybridized with the POU-M1 cDNA probe and a control probe (C). (B) Northern blot analysis of poly(A)$^+$ RNA (5 µg) isolated from the middle silk gland. Lanes: 1, 72 h after the third ecdysis; 2, the fourth ecdysis; 3, 4, 5 and 6, 24, 48, 72 and 144 h after the fourth ecdysis. They were hybridized as described in (A).

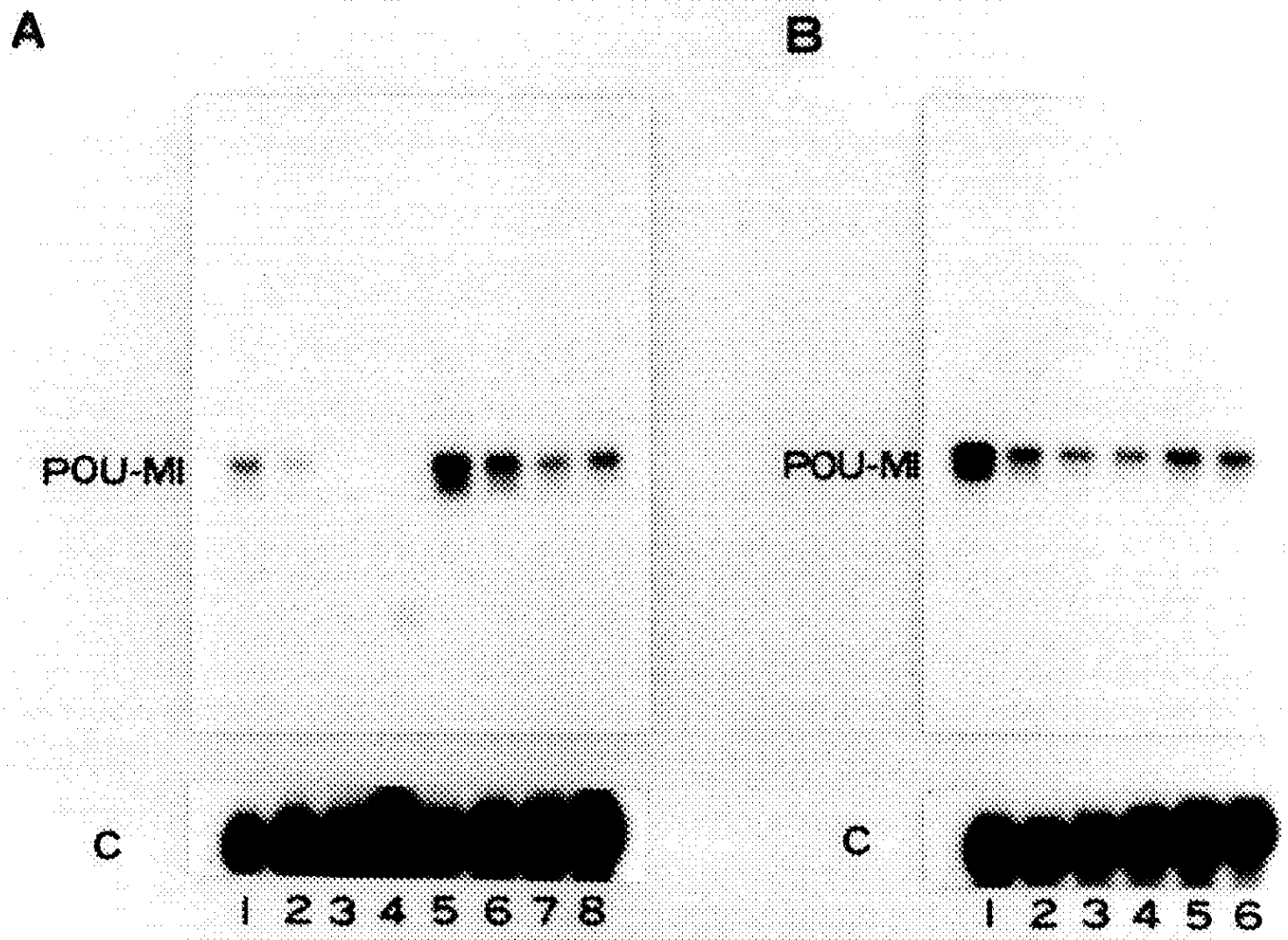

A
B
POU-MI
POU-MI
C
C
1 2 3 4 5 6 7 8
1 2 3 4 5 6

Fig. 12. Mobility shift assay of the SGF-3 in the middle or posterior silk gland of the fourth molting or fifth intermolt and the POU-M1 synthesized *in vitro*. Mobility shift assays with SC probe were performed using extract (10 µg) of the middle (MSG) and posterior (PSG) silk gland of the fourth molting stage (IVm) or 2-day-old fifth intermolt (V2) or using *in vitro* synthesized POU-M1. Identity of each shift band indicated by the arrow was confirmed by competition with 50-fold unlabeled SC oligonucleotide and hyper-shifting with the C1 antibody.

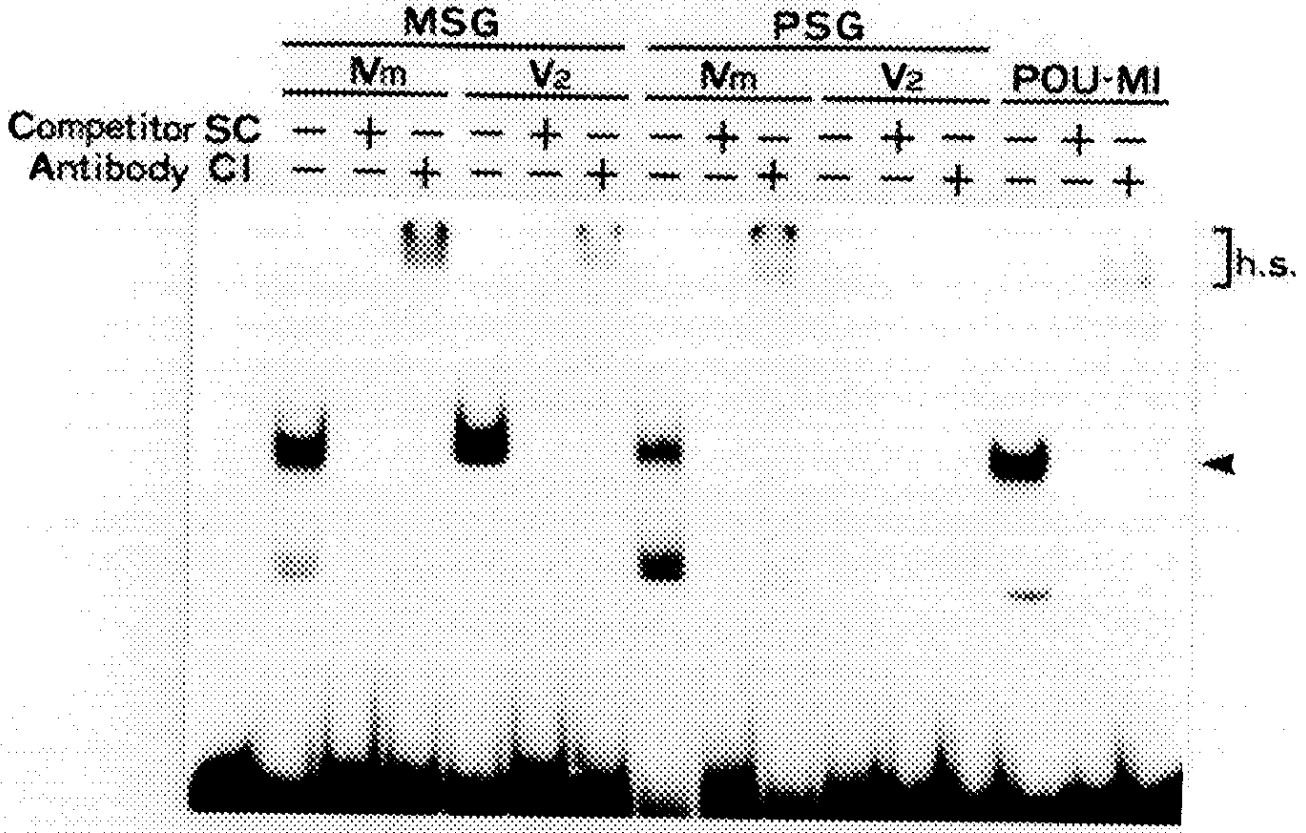
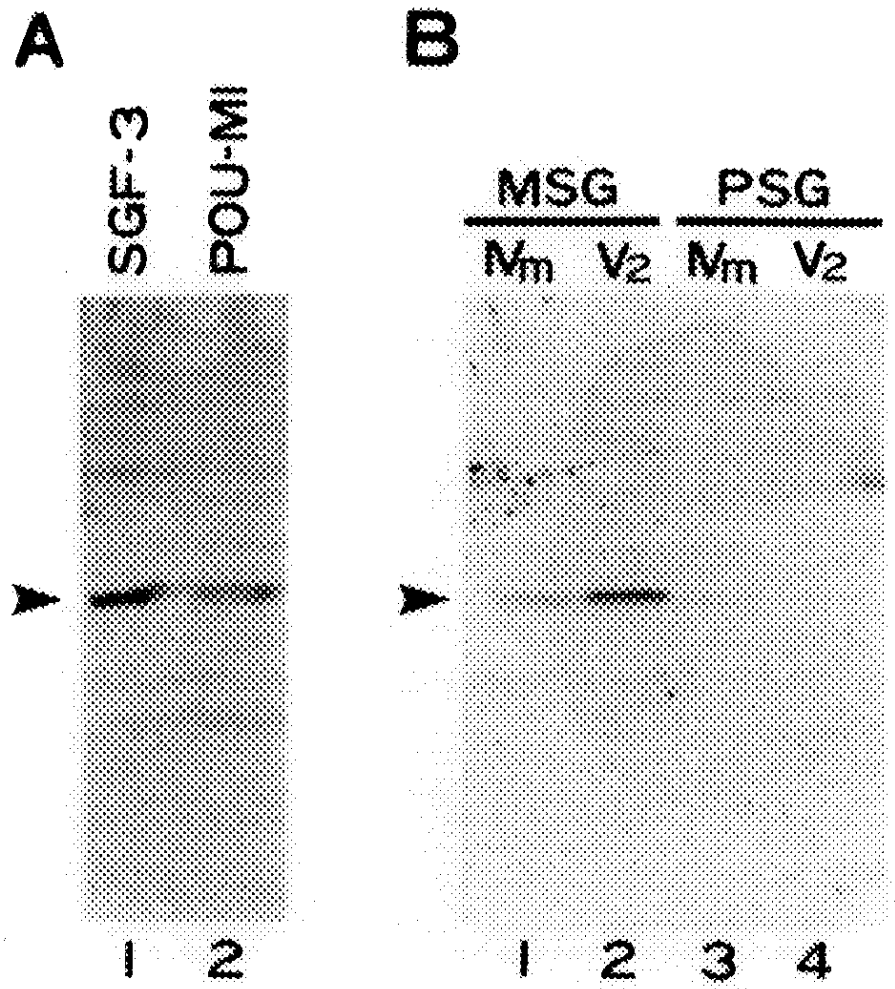

MSG
PSG
Vm
V2
Vm
V2
POU-MI
Competitor SC
Antibody CI
h.s.

Fig. 13. Western blotting with the C1 antibody against the middle and posterior silk gland nuclear extracts of the fourth molting stage and the fifth intermolt or *in vitro* synthesized POU-M1. (A) Western blotting of the middle silk gland nuclear extract (20 µg) from 2-day-old instar larvae (lane 1) and *in vitro* synthesized POU-M1 (10 µl) in rabbit reticulocyte lysate (lane 2). (B) Western blotting of middle (lanes 1 and 2) and posterior (lanes 1 and 2) silk gland nuclear extracts (20 µg). Lanes: 1 and 3, 15 h after the fourth ecdysis; 2 and 4, 48 h after the fourth ecdysis. The arrow indicates the band of SGF-3/SC and POU-M1/SC.


A
SGF-3
POU-Mi
1
2
B
MSG
PSG
Vm
V2
Vm
V2
1
2
3
4

List of publication

1. Fukuta, M., Meng, B. Y., Hayashida, N. & Sugiura, M. (1989) Nucleotide sequence of the psbK gene of the cyanobacterium, Anacystis nidulans 6301. Nucl. Acids Res. **17**, 7521.
2. Shimada, H., Fukuta, M., Ishikawa, M. & Sugiura, M. (1990). Rice chloroplast RNA polymerase genes: The absence of an intron in rpoC1 and the presence of an extra sequence in rpoC2. Mol. Gen. Genet. **221**, 395-402.
3. Matsuno, K., Takiya, S., Hui, C,-c., Suzuki, T., Fukuta, M., 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. Nucl. Acids Res. **18**, 1853-1858.
4. Chen, J. C., Meng, B. Y., Fukuta, M. & Sugiura, M. (1990). Nucleotide sequence of the psbI gene of the cyanobacterium, Anacystis nidulans 6301. Nucl. Acids Res. **18**, 4017.
5. Suzuki, Y., Takiya, S., Suzuki, T., Hui, C,-c., Matsuno, K., Fukuta, M., Nagata, T., & Ueno, K. (1990). Developmental regulation of silk gene expression. In Molecular Insect Science, Hagedorn, H. H., Hildebrand, J. H., eds., Plenum publ. Corp. (New York), pp. 83-89.
6. Suzuki, Y. Takiya, S., Suzuki, T. Hui, C,-c., Matsuno, K., Fukuta, M., Xu, P,-x., Nagata, T., Lou, Y,-h., Ohno, K. & Ueno, K. (1991). Developmental regulation of the silk gene expression. In Gene Expression, Mochida Memorial Foundation for Medical and Pharmaceutical Research, Tokyo, pp. 85-100.
7. 福田雅一、松野健治、滝谷重治、鈴木義昭 (1991). 絹糸腺特異的なセリシン遺伝子の転写制御 実験医学 **7**, 679-685.
8. Ueno, K., Hui, C,-c., Fukuta, M. & Suzuki, Y. (1991). Molecular analysis of the deletion mutants in the E homeotic complex of the silkworm Bombyx mori. Development, in press

9. Fukuta, M., Matsuno, K., Hui, C,-c., Nagata, T., Takiya, S., Xu, P.-X. & Suzuki, Y. (1992). Molecular cloning of a POU domain transcription factor involved in regulation of Bombyx sericin-1 gene. in preparation
10. Matsuno, K., Xu, P.-X., Fukuta, M., Nagata, T., Ueno, K., & Suzuki, Y. (1992). Cloning of a cDNA encoding a protein binding to the regulatory element SC of Bombyx sericin-1 gene. in preparation