Promoter of the POU-M1/SGF-3 Gene Involved in the Expression of Bombyx Silk Genes*

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To characterize the transcriptional regulation of the POU-M1/SGF-3 gene, we have cloned a genomic DNA fragment encompassing the whole coding region and its flanking sequences. This gene does not contain any intron. The 5'-flanking region of the gene contains several interesting motifs, such as homeodomain-binding motifs, sequences resembling the transcriptional factor Spl1-binding site, and TGTIT motifs, but lacks some of the typical transcriptional regulatory sequences, such as TATA and CCAAT boxes. Transcriptional analysis of a series of deletion mutants of the gene in the nuclear extracts prepared from the middle silk gland of 2-day-old fifth instar larvae revealed the presence of multiple cis-regulatory elements located both upstream and downstream of the initiation site. One of these elements, the homeodomain-binding element, was identified to mediate negative regulation. By mobility shift assay using the POU-M1 specific antibodies, we found that this negative element interacts with the POU-M1/SGF-3. Transcription analysis in vitro using templates mutagenized in the PB region and one of the POU-M1 antibodies indicated that the PB region is an autoregulatory element responsible for SGF-3-dependent transcriptional repression.

We have been studying the regulation of silk protein gene expression in Bombyx mori (reviewed in Ref. 1). The sericin-1 gene is expressed exclusively in the middle silk gland (MSG) while the fibroin gene is specific to the posterior silk gland (PSG). Both genes are actively expressed in the feeding stages but repressed during the molting stages. Several silk gland factors (SGF-1-4) involved in controlling the silk genes have been identified (2-4). Among them, the SGF-3 has been proposed to be an important factor for the regulation of silk genes (3, 4). The SGF-3 binds to the SC region, a key element for the sericin-1 gene transcription, and also binds to the distal upstream region of the fibroin gene. Since the SC and fibroin distal upstream region contain octamer-like sequences, it has been proposed that SGF-3 is an octamer-binding protein (4).

Based on this assumption, a POU domain-containing cDNA, POU-M1 has been cloned (5). Its POU domain part is identical with that of Drosophila CI-1 protein (6). The POU-M1 protein was shown to be identical with SGF-3 (5). The POU-M1 transcript and protein are subject to dramatic changes during silk gland development (5). The transcript was observed at a high concentration in the fourth molting stage and became lower in the fifth larval instar in the MSG. In the PSG, the transcript was detected only in the fourth molting stage. By Western blotting, the POU-M1 protein is estimated to have a molecular mass of about 38 kDa and exists at a low concentration in the fourth molting stage and becomes higher in the 2-day-old fifth instar larvae in the MSG (5). In the PSG, the protein was detected only in the fourth molting stage. This pattern suggests that the POU-M1/SGF-3 gene expression is temporally programmed and spatially restricted.

The highly conserved POU domain was initially recognized after the simultaneous cloning of three mammalian transcription factors and a Caenorhabditis elegans developmental regulator (7). Many additional POU domain proteins have been identified subsequently, and they are expressed in distinct temporal and spatial patterns during development (reviewed in Ref. 8). POU domain transcription factors appear to exert critical functions in proliferation of specific cell types, as well as in the activation of specific programs of gene expression that define specific cell phenotypes within an organ (9, 10). Several POU domain regulators appear in early embryogenesis and are important in the early developmental regulation of gene transcription (11-16). Since the POU domain exerts critical developmental actions, it is very important to study the roles of the POU domain in regulating organogenesis. POU domain proteins have been described to exert either positive or negative transcriptional effects by binding to recognition elements as monomer or as homodimers formed as a consequence of DNA-dependent cooperative interactions (17-19). On the basis of precedents in Drosophila for other classes of developmental regulators (20, 21), it is also important to understand the mechanisms that mediate the regulated expression of POU domain genes. Analysis of mammalian Oct-3/4, Tst-1/SCIP/Oct-6 and pit-1/GHF-1 genes suggested that the molecular mechanisms of activation and maturation of POU domain proteins are likely to involve the actions of numerous other classes of transcriptional regulators (22-25).

As a first step toward understanding the molecular basis of the regulatory hierarchy during silk gland development and probing the mechanisms of transcriptional regulation in generating the control patterns of POU-M1/SGF-3 gene expression, we have analyzed the expression of the POU-M1/SGF-3 promoter in vitro. In this work, by studying the POU-M1/SGF-3 gene transcription using mutated promoters, we have detected transcriptional important cis-acting elements within the 700-bp region around the transcription start site. We report
here that POU-M1/SGF-3 can auto-repress its own promoter activity as a consequence of binding to the negative element, a homodomain-binding element.

**EXPERIMENTAL PROCEDURES**

**Isolation of Genomic and cDNA Clones—**A Bombyx genomic library was prepared in EMBL3A vector. A 200-bp fragment (+17 to +216, see Ref. 5) of POU-M1 cDNA insert containing an internal XhoI site was labeled with [α-32P]dCTP by multiprimer and used for plaque hybridization screening of the genomic library. Two cDNA libraries in gt11 vector were used for screen for full-length POU-M1 cDNA. One library was constructed using poly(A)+ RNA from MSG of the fourth molting stage larvae and oligo(dT) primer. The other library was prepared from poly(A)+ RNA isolated from the 2-day-old fifth instar larval and oligo(dT) primer. The 200-bp fragment of 5'-cDNA insert and a 135-bp fragment of 3'-untranslated cDNA region (+1621 to +1755, see Ref. 5) were labeled with [α-32P]dCTP by multiprimer and used for screening of the cDNA libraries.

The positive genomic and cDNA clones were purified and λ phages were isolated by a standard method. 6-kb EcoRI and 11-kb HindIII genomic fragments as well as cDNA inserts were subcloned into pBluescript II KS(−) (Stratagene) for restriction site mapping and sequencing.

**Southern Blotting—**The genomic DNA was extracted from FSG of the Sho-Wa strain of *B. mori* from the Kanebo Co. The filters were hybridized to the 5'-labeled DNA probe isolated from the MSG of the 2-day-old fifth instar larvae and oligo(dT) primer. The 200-bp fragment of 5'-cDNA insert and a 135-bp fragment of 3'-untranslated cDNA region (+1621 to +1755, see Ref. 5) were labeled with [α-32P]dCTP by multiprimer and used for screening of the cDNA libraries.

**DNA Sequencing—**Genomic DNA and cDNA fragments were subcloned into pBluescript II KS(−). Both strands of DNA were sequenced by the dideoxynucleotide chain-termination method using the Sequenase 2.0 DNA sequencing kit (U. S. Biochemical Corp.).

**S1 Mapping—**For S1 nuclease mapping the protocol of Favaloro et al. (21) was used. A 225-bp fragment was used in some modifications. The probe used for S1 mapping was a double-stranded DNA. A 5'-deletion mutant plasmid in pBluescript II containing 366-bp upstream region was used as a template DNA. The probe was prepared by digestion with XhoI, which cleaves at position +144 on the antisense strand, 5'-end-labeling with [γ-32P]ATP, digestion with BamHI, and gel purification of the 510-bp fragment by using a low melting temperature agarose gel. Total RNA was extracted from the MSG of 2-day-old fifth instar larvae by using an acid guanidinium thiocyanate/phenol/chloroform method (27). The RNAs in different amount were coprecipitated with 1 × 10⁶ cpm of the denatured 5'-end-labeled fragment, hybridized at 70 °C for 16 h, digested with nuclease S1, and electrophoresed on 6% polyacrylamide, 7 M urea sequencing gels.

**Primer Extension—**Primer extension was performed according to the protocol described elsewhere (28, 29). A synthetic oligonucleotide primer (+136 to +98, 5'-TCAACTGGTCTATTCCTTCCCAGAAAACTGGCCGGGGTTGCGT-3') was 32P-labeled at the 5'-end. Total RNAs were extracted from different stages larvae using the acid guanidinium thiocyanate/phenol/chloroform method (27). About 1 × 10⁶ cpm of the primer were coprecipitated with 50 μg of the RNAs. Hybridization was carried out at 50 °C for 16 h. The samples were then precipitated with ethanol. The hybrids were dissolved in 20 μl of 50 μg Tris-HCl (pH 8.3), 75 mM KC1, 3 mM MgCl₂, 10 mM dithiothreitol, 1 mM of each dNTP, 2 units/μl RNasin, and 200 units (1 μl) of reverse transcriptase from Moloney murine leukemia virus were added to each sample. Then the samples were incubated for 5 h at 42 °C. The extended products were electrophoresed on 6% polyacrylamide, 7 M urea sequencing gels.

**Construction of Deletion Mutants—**Deletion mutants of the 5'-flanking region were constructed from pPomx. This plasmid was constructed by cloning the 1449-bp Clal-XhoI fragment of plasmid pPom19 into pBluescript II vector. The pPom19 plasmid was prepared by cloning a 3-kb EcoRI fragment into pBluescript II KS(−). The pPomx was cut with PstI and EcoRI, and processed by Klenow-deletion deletion kit using exonuclease III and mung bean nuclease as previously described (2). The boundaries of the upstream contained in these plasmids were determined by dideoxynucleotide sequencing. A 5'-deletion mutant pPomx T4A was made with KpnI and XhoI to generate 3'-deletion mutants as described above.

**Mutagenesis of the PB Region in POU-M1 Promoter—**Oligonucleotides (upper strand covering +485 to +387 and lower strand covering +384 to +485 with blunt 5'-end and HindIII linker at 3'-end) corresponding to wild type as well as mutagenized PB region (see Fig. 7A) were synthesized, annealed, phosphorylated, and ligated with HindIII-XhoI-digested promoter fragment (−386 to +144), respectively. The resulted fragments were then ligated with another oligonucleotide located further upstream (−486 to −542, with XbaI linker “ctag” at 5'-end and blunt 3'-end). The resulted longer fragments were then subcloned into pBluescript II vector upon XhoI-XhoI digestion. The promoter regions of the wild type and mutants were confirmed by sequencing.

**DNA 1 Footprint Analysis—**Three DNA fragments containing upstream regions of the POU-M1/SGF-3 promoter, −301 to +34 and −34 to +34 and −680 to −282 were used for footprinting assay. DNA 1 footprint experiments were carried out in 25-μl reaction mixtures containing 2 mg (1 × 10⁶ cpm) of 5'-end-labeled DNA fragments in the presence or absence of 100-150 μg of protein of nuclear extracts under the conditions previously described (2). The reaction mixture was treated with 1–2 μg of DNase I for 1 min on ice. A sample of 5'-end-labeled DNA probe was cleaved following the MAXAM and Gilbert “G+A”-reaction sequence procedure to give a molecular size marker.

**Gel Retardation and Hypershift Analysis—**The oligonucleotide probes used for mobility shift and competition assays are shown in Table I. Binding reaction was carried out in 10-μl mixes containing 25 μg HEPES-NaOH (pH 7.9), 25 mM NaCl, 5 mM MgCl₂, 0.7% glycerol, 1 μg of poly(dI-dC)-poly(dI-dC), 1 μg of sonicated salmon testis DNA, 0.1 μg of oligonucleotide probe labeled by filling-in reaction with Klenow enzyme, and 10 μg of protein and incubated for 30 min on ice. Protein-DNA complexes were visualized on 5% polyacrylamide gels (3).

The oligopeptides N1, C1, and HC were synthesized according to the deduced POU-M1 amino acid sequence by a peptide synthesizer, and the antisera N1 and C1 were raised against the peptides N1 and C1, respectively (5). Affinity-purified IgGs were added to the shift reaction mixes with or without competitor peptides N1, C1, and HC, and the mixes were incubated further for 30 min on ice and tested for mobility shift as described elsewhere (3).

**Preparation of Nuclear Extracts—**Nuclear extracts from the MSG of 2-day-old fifth instar larvae of *B. mori* were prepared according to the method previously described (2), except for the use of 0.07 volume of saturated (NH₄)₂SO₄. Nuclear extracts from the MSG of fourth molting stage larvae and the FSG of fourth molting stage larvae and 2-day-old fifth instar larvae were prepared according to the method previously described (30).

**In Vitro Transcription Analysis of the POU-M1/SGF-3 Promoter—**In vitro transcription was performed as described previously (31). The MSG extracts of 2-day-old fifth instar larvae and covalently closed circular DNA templates were used. The transcript was detected by primer extension as described above.

**Immunodepletion—**To 100 μl (−1500 μg of protein) of the nuclear extracts prepared from the MSG of 2-day-old fifth instar larvae, 25 μl (−25μg) of affinity-purified POU-M1 anti-C1 IgG were added, and the mix was incubated on ice for 1 h. In the control incubation, the nuclear extracts were preincubated with 25 μl (−25 μg) of normal IgG on ice for 1 h. Protein A-Sepharose resin (100 μl of packed bed volume) was then added to each, and the mix was shaken on ice for 1 h with occasional tapping at 5-min intervals. The resin was centrifuged off at 12,000 rpm (10,000 g) (centrifuge) for 2 min at 4 °C. Upon checking by gel shift assay, the SGF-3 was almost completely removed. The depleted extract was used for transcription assay.

**RESULTS**

**Structure of the POU-M1/SGF-3 Gene—**By Southern blots, a 200-bp POU-M1 cDNA probe was hybridized to the Bombyx genomic DNA digested with various restriction enzymes (Fig. 1A). Every digest except the XhoI digest revealed a single hy-
broadization band (lanes 1, 2, 4, and 5). Because the cDNA probe region contains a XhoI restriction site, the probe hybridized to two XhoI-digested genomic fragments (lane 3). These simple Southern blot patterns and the intensity of hybridization suggested that the *Bombyx* haploid genome contains a single copy of the gene for POU-M1/SGF-3. Upon screening of a genomic library, a positive clone was isolated. The clone contained an insert of about 20 kb. Southern blot analysis of the cloned DNA with the 200-bp probe showed restriction digest profiles identical with those of the genomic DNA described above, indicating that the phage contained the entire or most region for the authentic copy of the POU-M1/SGF-3 gene. Thus, the organization of the POU-M1/SGF-3 gene was determined through analysis of this clone. A 6-kb EcoRI fragment and an 11-kb HindIII fragment from the cloned phage were subcloned and used for detailed mapping sequencing of the genomic DNA. The entire 6-kb EcoRI fragment was sequenced.

To determine exon-intron boundaries, full-length POU-M1 cDNA was obtained and sequenced. Upon comparison of the POU-M1/SGF-3 genomic sequences with its cDNA sequences, we concluded that the POU-M1/SGF-3 gene does not contain any intron (Fig. 1B). Other examples of POU-family genes that do not contain introns were reported (39).

**Localization of the 5′-End of the POU-M1/SGF-3 Gene**—To map the transcription initiation site of the POU-M1/SGF-3 gene we performed S1 nuclease mapping with an end-labeled 510-bp fragment originating from a XhoI site (+144 in Fig. 2C, also see “Experimental Procedures”) and an RNA sample prepared from the MSG of 2-day-old fifth instar larvae. A protected fragment of 144 bp was detected (Fig. 2A). To further confirm the start site we performed primer extension experiments with a 39-nt primer encompassing +136 to +98 and RNA samples prepared from the MSG and PSG of fourth molting stage and 2-day-old fifth instar larvae. An extended product of 136 nt was obtained (Fig. 2B). The signal was stronger in the MSG sample from fourth molting stage (lane 1), and weaker in the MSG of 2-day-old fifth instar larvae (lane 2). It was detected weakly in the PSG of fourth molting stage (lane 3) but not detectable in the PSG of 2-day-old fifth instar larvae (lane 4). This result coincides with the expression pattern of POU-M1/SGF-3 as observed by Northern blot (5). The extension product was also sequenced by the chemical method (data not shown), and the result confirmed the above data. From these results we conclude that the transcription initiation site of the POU-M1/SGF-3 gene is located 197 bp upstream of the translation initiation codon, ATG (Fig. 2C; see also Fig. 3).

**DNA Sequence of the 5′-Flanking Region of the POU-M1/SGF-3 Gene**—The putative promoter region of the POU-M1/SGF-3 gene was sequenced and reveals several interesting features (Fig. 3). First, the region up to −240 is highly GC-rich and includes multiple GC boxes (from nucleotide position −102 to −38) (34), as well as a 32-nt region composed of repeat sequences (−194 to −163). Second, there are two TGGTTT motifs (PA and PC elements). These motifs were previously found in the SA, FA, and FB sites of the silk genes interacting with the SGF-1, another important factor for the transcription (4, 35). Third, there were three potential Pit-1-binding sites (PD, PE, and PF elements) with a sequence similarity to the consensus sequence of ATGNAT(A/T)(A/T) (36, 37). Fourth, there is a stretch of sequences which accommodates homeodomain-binding element (element FB). Finally, however, the putative promoter region has neither a TATA-like motif nor a CCAAT element, sequences found in many eukaryotic promoters and thought to be important for RNA polymerase II recognition (38). Moreover, consensus sequence for the transcription initiation element (39) is not present.

**DNase I Footprint Analysis of the Presumed Promoter Region**—To identify sequence-specific DNA-binding factors that might be involved in the regulation of POU-M1/SGF-3 transcription, we have carried out DNase I footprinting analysis using several probes. The first probe covered from position −301 to +34 and was labeled at position +34. Increasing amounts of the MSG nuclear extracts from 2-day-old fifth instar larvae were incubated with this probe prior to partial degradation with DNase I. One obvious protected region was detected (Fig. 4A, lanes 2–4; labeled as GC boxes), mapping to position −93 to −50 of the noncoding strand. The complementing result was obtained on the coding strand (lanes 7–9). We also tested tissue and stage specificity of protection at this region, using four kinds of extracts from MSG and PSG. The same amount of extract protein was added to the binding reactions. As shown in Fig. 4B, all four extracts conferred protec-
Fig. 2. Mapping of the 5'-end of the POU-M1/SGF-3 transcript. A, S1 nuclease mapping. A 510-bp probe was end-labeled at a XhoI site (+144). RNA samples: lane 1, 50 μg of Yeast tRNA; lanes 2-4 contained 50, 100, and 150 μg of the MSG total RNA from 2-day-old fifth instar larvae, respectively. Arrowhead indicates the protected fragment. Lane M represents a size marker. B, primer extension. A 35-nt primer (+36 to +60) was used for this assay. RNA samples: lane 1, 50 μg of total RNA from the MSG of fourth molting stage larvae; lane 2, 50 μg of total RNA from the MSG of 2-day-old fifth instar larvae; lane 3, 50 μg of total RNA from the PSG of fourth molting stage larvae; lane 4, 50 μg of total RNA from the PSG of 2-day-old fifth instar larvse. Arrowhead indicates the extension product. C, schematic representation of S1 mapping and primer extension.

Using another DNA fragment that extended further upstream, from −282 to −680, three protected regions (termed as PA, PB, and PC) were detected (Fig. 4, C and D). For the PA region, the MSG extracts from fourth molting stage and 2-day-old fifth instar larvae and the PSE extract from fourth molting stage gave a strong protection (lanes 2–4 and 8–10), while the PSE extract of 2-day-old fifth instar larvae gave a very weak (lane 5) or a weak protection (lane 11). For the 32-nt repeat region and the possible Pit-1 binding elements, only very weak protection was observed (data not shown). These DNase I footprinting analyses indicate that several regions upstream of the POU-M1/SGF-3 promoter are recognized by sequence-specific DNA-binding proteins present in the silk gland.

Analysis of POU-M1/SGF-3 cis-Acting Control Elements—To test whether POU-M1/SGF-3 and other factors binding to the POU-M1/SGF-3 promoter resulted in gene activation or repression, we first showed that the POU-M1/SGF-3 promoter was active in the MSG nuclear extracts from 2-day-old fifth instar larvae. The MSG extract of 2-day-old fifth instar larvae is an appropriate material source for studying the transcription regulation in vitro since active and faithful transcription of the sercin-1 gene was previously shown in the cell-free system (2). Using a primer extension assay, as shown in Fig. 5A, the transcript corresponding to the faithful transcription of the POU-M1/SGF-3 gene (indicated by arrowhead) was detected. This transcript was not detected in the absence of the POU-M1/SGF-3 gene template or in the presence of 1 μg/ml α-amanitin (data not shown). An additional signal was also detected in this experiment but was nonspecific product, because it could be detected even in the presence of 1 μg/ml α-amanitin (data not shown).

Transcription analysis of a series of 5'-deletion mutants revealed the presence of at least four important transcriptional control regions (Fig. 5A). For quantification of the transcription levels of the mutants, an internal control (plasmid pFbCP2 DNA) was included, and densitometric scanning of the autoradiographs of three experiments was carried out. Deletion beyond −510 gave only a slight effect on transcription (lanes 1–5). Further deletion of sequences between −509 and −484 (element PC) resulted in about 40% reduction in the level of transcription (lane 6), suggesting that this region is a positive control element that contributes to a high level of transcription. We conclude that element PC is involved in activation of transcription from the POU-M1/SGF-3 start site. Deletion of sequences between −483 and −448 had no effect on transcription (lane 7). In contrast, deletion of sequences between −447 to −386 resulted in an increase in transcription (approximately 2-fold, lane 8), suggesting that the element PB is a negative control element.

Deletion from −385 to −255 resulted in a decrease in activity (lane 9), but further deletion from −254 to −82 gave only a slight effect on transcription (lanes 10 and 11). Deletion from −81 to −5 gave an additional drop to about 30% of the full activity (lane 12), and a template ending at +34 had no activity (lane 13). These data indicate that element PA and the GC boxes are also the activation elements for the transcription of the POU-M1/SGF-3 gene.

We have further investigated whether there were sequences between −4 and +34 important for in vitro transcription. As shown in Fig. 5C, deletion from −4 to +2 (lane 3) and −4 to +7
The POU-M1/SGF-3 promoter, +1 marks the start of the POU-M1/SGF-3 mRNA. Various sequence motifs discussed in the text are denoted. PA and PC elements contain the consensus sequence ATGNAT(\theta)(T/A). (lane 8) obliterated transcriptional activity.

The DNA sequences that lie downstream of the POU-M1/SGF-3 start site (within the untranslated leader) also appear to have influence on the levels of RNA synthesis directed in vitro. Deletion from +118 to +77 resulted in a decrease of 77%; POU-M1/SGF-3 did not recognize any of the possible Pit-1-binding sites.

We measured the activity of a series of 3'-deletion mutants discussed in the text are denoted. To characterize what kind of protein(s) was responsible for the binding to PB region, we performed competition experiments using PB elements mutagenized by its own gene product as a consequence of binding to this element. In addition to the SGF-3/PB complex, there is another faster-migrating PB-specific complex (Fig. 6A, indicated by a filled triangle; also see Fig. 6B, right panel). We presently do not know the nature of this complex and further experiments are required to identify it.

To determine which motif in natural promoter is bound with SGF-3 and responsible for observed transcriptional repression, we carried out transcriptional assay. As shown in Fig. 7A, mutation in the first AT-rich motif (mutant 1) resulted in a stimulatory effect on promoter activity by 173% (lane 2), whereas mutation in the second motif (mutant 2) did not significantly alter promoter activity (lane 3). A combined mutation of both motifs (mutant 3) also resulted in an increase of promoter activity (lane 4). These results indicate that motif 1 within the PB region is critical for SGF-3-dependent repression.

The same result was also obtained by using POU-M1 protein synthesized in vitro in a reticulocyte lysate (data not shown). These results clearly indicate that SGF-3 binds to this homeodomain-binding element and suggest that the POU-M1/SGF-3 gene might be autoregulated by its own gene product as a consequence of binding to this element. In addition to the SGF-3/PB complex, there is another faster-migrating PB-specific complex (Fig. 6A, indicated by a filled triangle; also see Fig. 6B, right panel). We presently do not know the nature of this complex and further experiments are required to identify it.
FIG. 4. DNase I footprint analysis of the POU-M1/SGF-3 promoter. A, two end-labeled DNA probes containing 344 and 172 bp of the POU-M1/SGF-3 promoter (+34 to −310, for noncoding strand; −138 to +34, for coding strand) were subject to DNase I footprinting using different amount of the MSG nuclear extracts from 2-day-old fifth instar larvae. The amounts of DNase I used are 1.0 (lanes 1, 5, 6, and 10) and 2.0 (lanes 2-4 and 7-9) µg, respectively. The protein concentrations: lanes 2 and 7, 100 µg; lanes 3 and 8, 120 µg; lanes 4 and 9, 150 µg. Control experiments
In the present study, we have analyzed the cis-acting elements both in the upstream and downstream regions of the transcription initiation site of POU-M1/SFG-3 gene and factors interacting with them using cell-free transcription systems and DNase I as well as gel shift assays. The POU-M1/SFG-3 promoter consists of several distinct types of regulatory elements including positive as well as negative elements that are clustered in the region surrounding the initiation site, and these elements respond efficiently to specific Bombyx silk gland DNA-binding factors in cell-free transcription reactions. Thus, it is likely that there are various synergistic and antagonistic interactions that are combined to regulate POU-M1/SFG-3 gene. Therefore, this promoter provides an ideal system with which to analyze the interplay of multiple transcription factors and how they contribute to complex patterns of gene expression during silk gland development.

To begin the analysis, we have demonstrated that in vitro RNA synthesis from POU-M1 promoter is accurate and efficient in Bombyx MSG nuclear extract of 2-day-old fifth instar larvae. The presence of only one initiation site on the POU-M1 promoter is intriguing. The POU-M1 promoter lacks an upstream TATA sequence which is thought partly to be responsible for fixing the transcription initiation site (40, 41). The Drosophila Antennapedia P2 (Antp P2), engrafted (en) and Deformed (Dfd) genes also lack TATA boxes (42–44). In vitro transcriptional analysis of Antp P2 and en promoters has revealed that they utilize multiple transcription initiation sites. The possibility exists that the POU-M1 gene contains other sequences that function to position the start site. In vitro transcription of a series of 5’- and 3’-deletions has defined the minimal sequences (−4 to +48) needed to direct POU-M1/SFG-3 transcription from the correct site position (Fig. 5). Deletions upstream from −4 and downstream from +48 have no apparent effect on the POU-M1 initiation site (Fig. 5). Deletion of sequences between +48 to +20 completely inactivates transcription (Fig. 5). Thus, it is possible that there were sequences lying within the 29-bp region to have an effect on positioning the start site of transcription, but further experiments are required to establish this point.

It has been shown previously that different POU-domain proteins can bind similar octamer-like sequences, each of them recognizing distinct high affinity sites (reviewed in Ref. 45). The SC region of the sericin-1 gene contains octamer-like sequences. As shown in Fig. 6, POU-M1/SFG-3 can bind to the AT-rich PB region of POU-M1 gene, but can not recognize any of the octamer-like sequences in the upstream region of POU-M1 promoter (data not shown). Such a binding specificity of POU-M1 is probably important for functional interaction. The PB region is a negative element of POU-M1 gene (Fig. 5), and our experiments demonstrated that the POU-M1/SFG-3 gene is autorepressed by its own gene product as a consequence of binding to the homeodomain PB element (Fig. 7). The SC region is an activation element of sericin-1 gene (2). We speculate that the POU-M1/SFG-3 could act as both activator and repressor during certain stages of development or in different cell types. We also speculate that the PB element interacts not only with the POU-M1/SFG-3 but also with other homeodomain proteins, because many homeodomain-encoding genes have been shown to function by regulating the spatial and temporal patterns of expression of many genes, including other homeodomain genes (6, 20, 46). To date, we have cloned the middle silk gland expressed Bm en, Bm in (4) and Bm Antp. Further experiments are required to elucidate the role, if any, of the Bm en, in and Antp in the POU-M1/SFG-3 transcription.

Regulation of gene expression is a complex process that can be achieved in multiple steps (47). The generation of complex spatial and temporal patterns of transcription in eukaryotes involves the coordinated interaction of multiple factors bound to promoter and distinct enhancer/silencer elements. What is the initial activation mechanism and how do the multiple factors contribute to the complex pattern of POU-M1/SFG-3 gene expression during silk gland development? While it is clear that additional studies will be required to answer this question fully, several points merit discussion. We have described that footprints gave almost the same protection patterns for the three activation elements, the GC boxes, PA and PC elements in the MSG and PSG of fourth molting stage and 2-day-old fifth instar larvae (Fig. 4). These results indicate that the factors interacting with these elements are present commonly in the four extracts, although their concentration could be variable. For the negative element PB, a weaker protection was observed in the PSG extract of 2-day-old fifth instar larvae (Fig. 4) and SFG-3 interacts with this element (Figs. 6 and 7). SFG-3 is present most abundantly in the MSG extract of 2-day-old fifth instar larvae, and is less abundant in the MSG and PSG extracts of fourth molting stage (5). In the 2-day-old fifth instar larvae, the concentration of SFG-3 of the PSG is about 3% of the MSG. The POU-M1 transcript was observed at a high concentration in the fourth molting stage and became lower in the fifth instar larvae in the MSG, while in the PSG, it is only expressed in the fourth molting stage (5). These observations suggest current in vitro experiments may not detect all the factors that control the complex pattern of POU-M1/SFG-3 gene expression. It is likely that there are additional activator(s) and repressor(s) involved. In the MSG, the multiple factors together with the additional activator(s) could initiate the high level expression. By increase of SFG-3, occupancy of SFG-3 binding site in the POU-M1 promoter together with the additional repressor(s) will lead the negative feedback controls. Even in the PSG of 2-day-old fifth instar larvae, it is unlikely that SFG-3 only is enough to switch off the gene expression.

By mobility shift assay, we have found proteins interacting with the possible Pit-1-binding sites and the 32-nt repeat region, respectively (data not shown). However, the transcriptional analysis of 5'-deletion mutants reported here indicates that deletion of these sequences has no apparent effect on the POU-M1 transcription (Fig. 5A). The possibility remains that these sequences play a role in the in vivo expression of POU-M1 gene. It is also possible that there are tissue- and stage-specific cis-acting elements for the POU-M1/SFG-3 gene. It will be interesting to identify such control elements in the POU-M1/

(no extract, lanes 1, 5, 6, and 10) and markers are shown. The protected regions are labeled as GC boxes, B, DNase I protection in various extracts. The coding and noncoding strands were the same as described in (A). The DNA binding activities of four extracts were analyzed by DNase I footprinting. The coding (lanes 1–6) or noncoding (lanes 7–12) strand probe was incubated with 106 µg of extracts. The amounts of DNase I used are 1.0 (lanes 1, 6, 7, and 12) and 2.0 (lanes 2–5, 8–11) µg, respectively. The extracts: lanes 2 and 8, MSG extracts of fourth molting stage larvae (D2P); lanes 3 and 9, MSG extracts of 2-day-old fifth instar larvae (V2M); lanes 4 and 10, PSG extracts of fourth molting stage larvae (D2P); lanes 5 and 11, PSG extracts of 2-day-old fifth instar larvae (V2P). Control experiments (no extracts, lanes 1, 6, 7, and 12) and markers are shown. C, DNase I protection of the distal upstream region (−698 to −202) of the POU-M1/SFG-3 promoter in various extracts. Lanes 1–6, noncoding strand; lanes 7–12, coding strand. This experiment was performed as described in (B). The protected regions are labeled as PA, PB, and PC, D, schematic representation of DNase I footprints.
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Fig. 5. In vitro transcription of the POU-M1/SGF-3 promoter deletion mutants. A, upstream deletion. Each reaction contained 30 ng of internal control template and equivalent molar (100 fmol) of test plasmid. The 39-mer POU-M1-specific primer (+136 to +98) was used to detect the in vitro POU-M1 transcript. The faithful transcript should be 136 nt. The in vitro transcriptional activity relative to pPom 5' -3100 was measured by densitometric scanning of the autoradiographs. The mean of three quantification experiments performed with the MG extracts is shown as percent activity together with a standard error in the parentheses. The downstream ending point is +144. B, downstream deletion. Each reaction contained 40 ng of internal control template and equivalent molar (150 fmol) of test plasmid. A 5'-end-labeled 40-mer of vector pBluescript II primer (5'-TGGATACGCGCCGCAATTAACCCTCACTAACAGGGAA-3') was used to detect the POU-M1 transcript. The transcriptional activity was measured as described in (A) for three experiments. C, each reaction contained 30 ng of internal control template and equivalent molar (150 fmol) of test plasmid. Lane 1, pPom 5' -254; lane 2, pPom 5' -4; lane 3, pPom 5' +3; lane 4, pPom 5' +8; lane 5, pPom 4 -48 (contained promoter region -4 to +48). The 40-mer of vector pBluescript II primer was used to detect the transcript for internal control. For (A), a 5'-end-labeled 30-mer of vector pBR322 primer (5'-AATGGTGCATGCAAGGAGATGGCGCCCAAC-3') was used as an internal control. For (A), a 5'-end-labeled 30-mer of vector pBR322 primer (5'-AATGGTGCATGCAAGGAGATGGCGCCCAAC-3') was used to detect the transcript for internal control (205 nt); for (B) and (C), another 5'-end-labeled 35-mer of vector pBR322 primer (5'-AAGGAGCTGACCTGGGTGAAGGCTCTCAAGGGCAT-3') was used to detect the transcript for internal control (325 nt). Arrowheads indicate the product.
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Fig. 6. Binding analysis of POU-M1/SGF-3 to the upstream element PB. The extracts were prepared from the MSG of 2-day-old fifth instar larvae. A, hyper-shift assay and peptide competition analysis to the SGF-3/PB complex. Two kinds of antibodies raised against the N1 (lanes 3-6) and C1 (lanes 7-10) oligopeptides, respectively, were added to the SGF-3/PB complex without (lanes 3 and 7) or with 15 µg of competitor peptide N1 (lanes 4 and 8), C1 (lanes 5 and 9) or HC (lanes 6 and 10). Lane 1, no extract; lane 2, no antibody. The arrowhead represents SGF-3 complex; the filled triangle represents another shift complex with unknown protein. B, relative affinity of the PB binding to the SC site of sericin-1 gene. The assay was performed with the SC probe (lanes 1-8) and PB probe (lanes 9-16) in 10 µg of the MSG nuclear extracts. Lanes 1 and 9, no extract; lanes 2 and 10, no competitor. Lanes 3, 5, 7, 11, 13, and 15 and lanes 4, 6, 8, 12, 14, and 16 contained 50- and 200-fold molar excess of the unlabeled oligonucleotides, respectively. Arrowheads represent SGF-3 complex; the filled triangle represents an unknown PB-specific protein.

Fig. 7. Effect of SGF-3/PB interaction on the POU-M1/SGF-3 gene transcription. A, In vitro transcription analysis with mutagenized PB regions in the natural promoter. Respective plasmid contains the PB region of wild type sequence (lane 1), mutagenized in motif 1 (Mutant 1, lane 2), in motif 2 (Mutant 2, lane 3) or in both motifs (Mutant 3, lane 4). Each 25 µl of reaction contained 400 ng of test template plasmid, 50 ng of internal control plasmid, and 200 µg of the extract protein. The 40-mer of vector pBluescript II and 35-mer of pBR322 primers were used to detect the transcripts for POU-M1 and internal control, respectively. The percent activity was measured as described in Fig. 5A. B, Immunodepletion. The MSG nuclear extract of 2-day-old fifth instar larvae was incubated with the affinity purified POU-M1 anti-C1 IgG (lanes 2, 4, 6, and 8) or normal IgG (lanes 1, 3, 5, and 7). Protein A-Sepharose was then added and mixed intermittently for 1 h on ice. The mix was centrifuged at 12,000 rpm (microcentrifuge) for 2 min. The supernatant was assayed for transcription as described under “Experimental Procedures.” Plasmid pPom 5'Δ -483 (lanes 1 and 2), pPom 5'Δ -385 (lanes 3 and 4), pPom 5'Δ -385 (lanes 5 and 6) and pPom 5'Δ -594 were used for assay. Each 25-µl reaction contained 400 ng of test template plasmid, 40 ng of internal control plasmid, and 200 µg of the extract protein. The 39-mer of POU-M1-specific and 30-mer of pBR322 primers were used to detect the transcripts for POU-M1 and internal control, respectively. Lanes 1-6 and lanes 7 and 8 are two independent experiments. The percent activity was measured as described in Fig. 5A. Arrowheads indicate the product.

SGF-3 promoter by using the MSG and PSG extracts of the fourth molting stage larvae. It is also important to analyze the factors that interact with the downstream region. These experiments may give a more complete understanding of how the POU-M1/SGF-3 gene is controlled during silk gland development.
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