Transcriptional regulatory elements in the upstream and intron of the fibroin gene bind three specific factors POU-M1, Bm Fkh and FMBP-1

Shigeharu TAKIYA*, Hiroki KOKUBO and Yoshiaki SUZUKI†

National Institute for Basic Biology and Graduate University for Advanced Studies, Myodaiji, Okazaki 444, Japan

The transcriptional modulator in the fibroin gene intron is composed of multiple octamer-like AT-rich elements, to which several specific DNA-binding proteins named fibroin-modulator-binding proteins (FMBPs) bind. Three major FMBPs in the silk gland were characterized. Two of them (FMBP-2 and -3) were identified as a Fork head homologue (Bm Fkh) and a POU-domain protein (POU-M1) respectively. These factors were expressed in the silk gland with distinct temporal- and spatial-specificities during late larval development as well as during embryogenesis, and did not correlate directly with fibroin gene

expression. The other (FMBP-1) appeared to correlate with the expression of the fibroin gene for temporal- and spatial-specificity. These FMBPs also bind to the elements in the upstream modulator. Transcriptional enhancement by both modulators was inhibited by binding competition for these factors with oligonucleotides. These results suggest that expression of the fibroin gene is controlled by co-ordination of these factors with distinct specificities during silk-gland development.

INTRODUCTION

The identification and characterization of transcription factors of tissue-specific terminal genes would be a key step in the analysis of the molecular mechanism regulating the expression of these genes during development. The silk gland of *Bombyx mori* is an ideal system with which to analyse how the regulation mechanisms of expression of tissue-specific genes and those of the development of the tissue are associated (for a review see [1]). Development of the silk gland starts at stage 19 of embryogenesis as a pair of invaginations of the ventral ectoderm cells in the labial segment [2,3]. The invaginated glands are elongated by rapid growth and are morphologically complete as a pair of silk glands by stage 25, about 60 h after the invagination. Expression of the fibroin (H-chain) gene begins after the silk glands are fully developed [4]. The fibroin gene is expressed specifically in the posterior part of the larval silk gland, and the sericin-1 gene, which encodes a glue protein, is expressed in the middle of the larval gland (see [5] and references therein). Genes encoding small subunits of fibroin are also expressed in concert with the fibroin H-chain gene [6–8] in the posterior silk gland (psg), and the sericin-2 gene is expressed concomitantly with the sericin-1 gene but with a different sublocal specificity [9] in the middle silk gland (msg). These silk genes are expressed and suppressed during feeding and moulting stages respectively during larval development.

Promoter elements of the fibroin and sericin-1 genes have been analysed in a cell-free transcription system prepared from silk glands [10–15] as well as in a reconstructed system made up of its fractions [16]. At least three parts of the fibroin promoter exert efficient and specific transcription. The fibroin core promoter, which covers only the TATA box and the initiation region, is preferentially transcribed in psg extracts [13,17]. Both the upstream and intronic elements enhance transcription from the cognate core promoter and also the heterologous adenovirus major late promoter (AdMLP), when those elements are placed

upstream of these promoters [13,18]. The intronic transcriptional modulator has been found in the proximal half of the fibroin gene intron [13]. Here we further dissected the intronic modulator and analysed its binding proteins. We identified and characterized three specific binding factors named fibroin-modulator-binding proteins 1, 2 and 3 (FMBP-1, -2 and -3), which also bound the upstream transcriptional modulator of the fibroin gene and were expressed with distinct temporal- and spatial-specificities.

EXPERIMENTAL

Preparation of the extracts

Embryos, larvae and pupae were staged as described previously [19–21]. Psg extracts were prepared from larvae at 72 h after the third ecdysis (the fourth instar, IV), D2 (mD) and E2 (mE) stages of the fourth moulting, and 2 days of the fifth instar (V) as previously described [13,21]. Msg extracts were prepared from larvae at the E2 stage of the fourth moulting and at 2 days of the fifth instar as described [14], and proteins were extracted with 0.42 M (NH₄)₂SO₄. Extracts of whole embryos at stage 24 and larval carcasses at 2 days of the fifth instar were prepared by crushing with a mortar and pestle under liquid nitrogen. Extracts of fat-body at 2 days of the fifth instar and whole ovarian tissues of 4-day-old pupae were prepared as described [21]. Extracts of the cell line Bm e21 were prepared as described by Manley et al. [22] with minor modifications.

In vitro transcription

Circular DNA templates (150 ng in 12.5 µl of reaction mixture) were transcribed with psg extract prepared at 2 days of the fifth instar at 30 °C for 60 min as described [13]. RNA was labelled with [α -³²P]UTP and analysed with a modified S1 nuclease assay [23]. The single-stranded DNA probes M13CP [17] and M13Ad4

Abbreviations used: Bm Fkh, *Bombyx mori* Fork head; FMBP, fibroin-modulator-binding protein; psg, posterior silk gland; msg, middle silk gland; EMSA, electrophoresis mobility shift assay; AdMLP, adenovirus major late promoter.

* Present address: Research Center for Molecular Genetics and Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060, Japan.

† To whom correspondence should be addressed.

Table 1 Sequences of oligonucleotide probes and competitors

Only nucleotide sequences of the upper strand are shown. For annealed probes, double-stranded oligonucleotides were used after filling in the sticky ends generated by aatt, but as competitors, they were used directly without the fill-in.

Oligonucleotide	Sequence
	+200 +227
+220	aattGGAATAAACTACATTTATTTTAAAA
+220M	-----TGT-----
+220M2	-----A--A--T-A-----
	+280 +307
+290	aattGATGAATCTATGTAAATACTGGGCAGAC
+290M	-----TACCT-----
	+361 +398
+370	aattTTCAACAATATGTAAACAATTCTGTAAGCATCATTAG
	+404 +441
+420	aattAGACGAAAGAAATTGCATAAAATTATAACCGCATTATT
	-71 -44
-60	aattTCTCTATCGAAAGTAAATACGTCAAAACT
	-93 -66
-80	aattCAATACAATGTGTAGATGTTTATTCTAT
	-115 -88
-110	aattTTTCTATGTAATTGTATCTGTACAATAC
	-124 -97
-120	aattAAAGATTAATTTCTATGTAATTGTATCT
	-143 -116
-130	aattGTATTGTTATGTAAATAAAAAGATTAA
	-152 -125
-140	aattTCATACGTTGTATTGTTATGTTAAATAA
	-168 -141
-160	aattCACAATTTAATTTACTTCATACGTTGTA
	-193 -166
-180	aattCATAATTAATCACATTGTTTCATGATCAC
	-208 -181
-200	aattTAAGATCAATTAAATCATAATTAATCAC
	-220 -193
-210	aattAAGTAAGAACAATAAGATCAATTAAATC
	-238 -211
-230	aattAATTCTTTAAATATTAAAGTAAGAAC
	-109 -81
SA	aattCTGTATACATTGTTTGCACAAATGTTTG
SAM	-----AA-A-----
	-209 -179
SC	aattCAACGAGCCATGAATAAATTAGAAATCAATG

[23] were used to detect transcripts from the fibroin and AdMLP promoters respectively. To assess the stimulating effect of the subfragments from the intronic modulator, pFb3'Δ-31 (10 ng) was added as an internal control and transcripts from the pFb3'Δ-31 were detected with the f1Fb38 probe [23]. The basal template pFbCP2 was prepared by cloning the fibroin core promoter region from -37 to +10 between the *HindIII* and *BamHI* sites of pBR322 [13] and see Figure 1a). pIECP2 and pI.IECP2 were constructed by inserting the whole intronic modulator from +156 to +454 into the *ClaI* site at -47 of pFbCP2 [13]. Other reconstructed fibroin templates were made by inserting a subfragment of the intronic modulator prepared by digestion with restriction enzymes into the *ClaI* site as shown in Figure 1(b). Each fragment was filled with T4 DNA polymerase before the ligation with pFbCP2. The structure of the plasmids was determined by digestion with restriction enzymes, and orientation of the inserted fragment was confirmed by sequencing. In the chimaeric templates of the fibroin modulators and AdMLP, the fibroin upstream modulator from -238 to -66 or the intronic modulator from +156 to +454 was inserted into the *SmaI* site at -52 of an AdMLP plasmid pM7 [13].

DNase I footprinting of circular DNA

Modified DNase I footprinting of circular DNA was performed with primer extension to detect cleavage sites by DNase I [17]. Poly(dI-dC)·poly(dI-dC) was used as a carrier instead of poly(dA-dT)·poly(dA-dT) and the amounts of DNase I were empirically determined.

Electrophoresis mobility-shift assay (EMSA)

EMSA was performed as described previously [15] with several modifications. Poly(dI-dC)·poly(dI-dC) was used as a carrier, but tRNA and single-stranded DNA were omitted. An oligonucleotide probe (0.1–0.2 ng) was incubated with the extract (12 µg of protein), and protein–DNA complexes were resolved on 7% (w/v) polyacrylamide gels. In the competition studies, 1 µl of 10 mM Tris/HCl, pH 7.9, containing 1 mM EDTA, 200 mM NaCl and 0–20 ng of unlabelled double-stranded competitor was added. In the hypershift assay of FMBP-2 and -3, purified IgG against the synthesized peptide C1 of POU-M1 [24], or serum against a fusion protein of the C-terminal 101 amino acids of Bm Fkh and glutathione S-transferase [25], was added to

the EMSA reaction mixtures in the presence or absence of competing peptide.

Cloning of *Bm fkh* cDNA

PCR was performed with degenerate primers synthesized according to the amino acid sequence of *Drosophila* Fkh using cDNA synthesized from polyadenylated RNA of the psg at 2 days of the fifth instar. The PCR products were cloned into pBluescript and the nucleotide sequence was confirmed as *Bm fkh*. An oligonucleotide was synthesized according to the sequence of the fkh domain of the *Bm fkh* obtained by PCR and used as a probe to screen an embryonic cDNA library and a psg cDNA library. A clone covering the entire open reading frame of *Bm fkh* from the embryonic library was sequenced and used as a probe for Northern blotting. One major signal of about 5 kb was obtained with polyadenylated RNAs from embryos, psg and msg. The nucleotide sequence of *Bm fkh* cDNA is described elsewhere [26].

Probes and competitors

The nucleotide sequences of probes and competitors used for EMSA are shown in Table 1.

RESULTS

Major element responsible for transcription enhancement by the intronic modulator of the fibroin gene

The intronic modulator is the region spanning +156 to +454 ([13]; also see Figure 10). We dissected the modulator more precisely to map the elements responsible for the enhancement. Various restriction fragments from the modulator were inserted upstream of the fibroin core promoter and the extent of the transcriptional enhancement by each fragment was compared in the psg extract (Figure 1b). Transcription from the core promoter

was stimulated by the independent fragments, suggesting redundancy of the elements responsible for the enhancement. Clones 1-2, 1-3 and 4-107, which have the 35 bp region from *Hpa*II (+188) to *Aha*III (+223) in common, enhanced the transcription as efficiently as the entire intronic modulator. Clone 5-27, which carries only the 35 bp region, significantly enhanced the transcription. However, clones 3-10, 5-17 and 6-37, which lack the 35 bp region, showed less transcriptional enhancement.

Footprinting studies of the psg extract using the circular DNA of clones 1-3 and 3-10 showed that three regions of the intronic modulator at around +220, +290 and +370 were protected from nuclease digestion (Figure 2). Protein binding to the modulator was further analysed with extracts from msg, whole embryos and the cultured cell line Bm e21. The footprints of these extracts differed from that of the psg extract and also from each other. In the msg extract, the +420 region was protected as well as the +220, +290 and +370 regions common to the psg extract. The region around +250 was protected only in extract prepared from the cultured cells. The region around +290 was protected in all the extracts, but with distinctive profiles.

We superimposed the footprints of the protected regions on the nucleotide sequence of the intronic modulator and found that all regions contain octamer-like AT-rich sequences (Figure 1b and Table 2). All subfragments of the modulator used for the transcription assay carried more than one octamer-like element, correlating with the redundancy of the transcription-stimulation ability. The octamer-like sequences in the +220 and +290 elements are actually within the target sequences of specific binding proteins (see below). We name these binding proteins fibroin-modulator-binding proteins (FMBPs). Although transcription enhancement and protein binding of the border between the regions covered by clones 1-3 and 3-10 at around +327 (Figure 1b) were not examined, we concluded that the +220 region contains the major element responsible for the transcription enhancement by the intronic modulator.

Figure 1 Transcriptional enhancement by the intronic modulator of the fibroin gene

(a) Structure of relevant templates. Details for the construction are described in the Experimental section and [13]. The filled box shows the fibroin core promoter and the arrowhead in the circle of pFbCP2 shows the direction of transcription. **(b)** Each fragment from the modulator was inserted upstream of the fibroin core promoter as shown in **(a)** and the extent of the transcriptional enhancement in the psq extract from 2-day-old larvae of the fifth instar was compared in three independent experiments with reference to an internal control. EV, Hp, Ah and Hf indicate sites of cleavage by *EcoRV*, *HpaII*, *AhaIII* and *HinfI* respectively. The position number shows the 5' end of each fragment except for +454 which is the 3' end of the intronic modulator. The box indicates the approximate position of the octamer-like elements. The arrowhead shows the orientation of the fragment inserted.

Figure 2 Footprints of the intronic modulator of the fibroin gene in various extracts

Circular DNA of clones 1-3 (**a** and **b**) and 3-10 (**c**) was incubated with extracts from the psg and msg at 2 days of the fifth instar, whole embryo at stage 24 (Embryo) and the cell line Bm e21 (Cell line), and analysed by footprinting. — means the same treatment of the DNA incubated without the extract. Thick bars show the approximate protected regions. A and T are adenine and thymine ladders obtained by sequencing the same DNA with the same end-labelled primers.

Table 2 Sequences of octamer-like elements in the protected regions of the intronic modulator

Element	Sequence
	+ 213 + 206
+ 220	ATGTAGTT
	+ 243 + 250
+ 250	ATGTAAC
	+ 289 + 296
+ 290	ATGTAAAT
	+ 370 + 377
+ 370	ATGTAAAA
	+ 421 + 414
+ 420	ATGCAATT

The major element responsible of the intronic modulator responsible for transcription enhancement binds FMBP-1

Factors binding to the major element responsible for the transcription enhancement by the intronic modulator were analysed by EMSA. With an oligonucleotide probe covering the + 220 region, a complex produced by the factor (FMBP-1) in the psg extract was detected among several complexes that were not competed for efficiently by the + 220 element (Figure 3a). Competition experiments with oligonucleotides corresponding to the four octamer-like elements at around + 220, + 290, + 370 and + 420 (Table 1) showed that binding of FMBP-1 to the + 220 probe was competed for by the + 290 element as well as the + 220 element itself. Only slight competition was observed for the + 370 and + 420 elements at a high dose. FMBP-1 was also detected with the + 290 probe and competed for by the + 220 and + 290 elements (Figure 3b). Oligonucleotides + 220M

and + 290M, which were mutated at the octamer-like sequence (see Table 1), were used to examine the sequence specificity of the FMBP-1 binding. The + 290M oligonucleotide had lost the ability to compete for the binding, and + 220M had reduced ability (results not shown but see Figure 5b for + 290M). Thus the octamer-like elements around + 220 and + 290 are recognized by FMBP-1.

Tissue- and stage-specificity of FMBP-1 activity

The fibroin gene is expressed only in psg at the larval feeding stages [5]. We examined with the + 290 probe whether FMBP-1 activity correlated with fibroin gene expression. In EMSA using staged extracts from psg and msg, FMBP-1 was detected intrinsically only in the psg at the feeding stages (Figure 4a). A faint signal was observed at the same position as FMBP-1 with msg extracts and psg extracts at the moulting stages, but the binding specificity of the signal was not examined. Cell-type-specificity of the FMBP-1 was analysed with extracts from the fat-body, carcass, whole ovarian tissues and whole embryos in addition to the silk-gland extracts. As shown in Figure 4(b), a faint or negligible signal for FMBP-1 was detected in extracts from the fat-body, carcass and stage-24 embryos, as in msg. In the extract from whole ovarian tissue, there was a significant level of FMBP-1 activity, but the activity in the psg extract was by far the highest. Thus FMBP-1 activity correlated well with fibroin gene expression both temporally and spatially.

FMBP-1 also binds to the upstream modulator and is necessary for the transcriptional enhancement by both upstream and intronic modulators

The upstream transcriptional modulator of the fibroin gene also contains octamer-like AT-rich elements. We examined whether

Figure 3 EMSA of the psg extract with octamer-like elements in the intronic modulator

The oligonucleotide probe covering the protected region +220 (a) or +290 (b) was incubated with psg extract from 2-day-old larvae of the fifth instar in the presence or absence of unlabelled oligonucleotides as competitors. Triangles on the top of the panels show increasing (10-, 50- and 200-fold) amounts of competitors against the probe. Complexes indicated by a small circle were not fully characterized but they were competed for by either single-stranded DNA or high concentrations of poly(dI-dC)·poly(dI-dC).

Figure 4 Tissue- and temporal-specificity of FMBP-1, -2 and -3

The +290 probe was incubated with psg and msg extracts prepared from staged larvae (a) or extracts from psg, msg, fat-body, carcass, whole ovarian tissues and whole embryo at stage 24 (b), and then evaluated by EMSA. The silk-gland extracts were prepared from larvae at 72 h after the third ecdysis (IV), D2 (mD) and E2 (mE) stages of the fourth moulting, and at 2 days of the fifth instar (V).

FMBP-1 can bind to the upstream modulator. Since the upstream modulator exhibited complex footprints induced by the factors SGF-1–4 in the psg extract [27], we used a series of oligonucleotides that covered whole upstream modulator to detect FMBP-1 binding. Eleven overlapping 32-mers (Table 1) were used in EMSA as competitors, and representative results are shown in Figure 5(a). Of the 11 oligonucleotides, only the –130 element competed significantly for FMBP-1 binding to the +290 probe. FMBP-1 was also detected by the –130 element as a probe and competed for by the +220 and +290 elements, but

not by the mutant oligonucleotides +220M2 and +290M (Figure 5b). +220M2 was disrupted at the ATTT repeats in the +220 region (see Table 1). This mutation was more effective than that at the octamer-like sequence (+220M, results not shown), demonstrating that FMBP-1 recognized the ATTT repeats.

To assess the role of FMBP-1 in transcriptional enhancement, chimaeric templates of AdMLP with the upstream or intronic modulator of the fibroin gene were transcribed in the presence of a competitor or non-competitor oligonucleotide of FMBP-1

Figure 5 Binding of FMBP-1 and -2 to the upstream modulator of the fibroin gene

(a) The +290 probe was incubated with the psg extract from 2-day-old larvae of the fifth instar in the presence or absence of unlabelled oligonucleotides of the upstream modulator and evaluated by EMSA. A 10-, 50- or 200-fold excess of competitor was added. (b) The -130 probe was incubated with the psg extract in the presence or absence of various competitors.

Figure 6 Inhibition of the transcriptional enhancement by competition for the binding of FMBPs

An AdMLP template (pM7; -), its chimaeric template with the fibroin upstream modulator (UE) or the intronic modulator (IE) was transcribed in the psg extract from 2-day-old larvae of the fifth instar in the presence of the mutant (+290M) or wild-type (+290) competitor. The fibroin core promoter template pFbCP2 was transcribed simultaneously as an internal control. Transcripts from AdMLP and the fibroin promoter are indicated by Ad and Fb respectively.

binding (Figure 6). Psg extract was incubated with the wild-type or mutant competitor on ice for 20 min, and then transcription was started by adding the template; it was allowed to proceed for

Figure 7 Identification of FMBP-2 as Bm Fkh/SGF-1

(a) Hypershift of FMBP-2 by a Bm Fkh-specific antibody in EMSA. Bm Fkh-specific antiserum or control serum was added to EMSA reaction mixtures with psg or msg extract from 2-day-old larvae of the fifth instar. The large arrowhead shows hypershift products at the top of the gel. (b) Competition for FMBP-2 binding to the +290 region by the SGF-1-specific binding site, SA. A 50-fold excess of either wild-type (SA) or mutant (SAM) oligonucleotide against the probe was added as a competitor to the EMSA reaction mixtures with the psg extract.

a further 60 min at 30 °C. In the presence of +290M, transcription from AdMLP was enhanced by both upstream and intronic modulators, whereas in the presence of the wild-type +290 oligonucleotide, the enhancement was significantly reduced. There were no differences between the +290 and +290M competitors in terms of the effect on transcription from either AdMLP itself or the internal control fibroin core promoter. Although the +290 element can bind FMBP-1, -2 and -3 (see below), the results mainly reflect the effects of competition by FMBP-1 binding (see the Discussion).

Bm Fork head binds to the intronic modulator

Besides FMBP-1, we detected another specific binding protein (FMBP-2) in the psg extract from 2-day-old larvae of the fifth instar with the +290 probe (Figure 3b). FMBP-2 binding was competed for by the +290 and +220, but not by the +370 and +420, elements. The binding specificity of FMBP-2 was similar to that of FMBP-1, but the ability of the +220 element to outcompete FMBP-2 binding to the +290 probe was significantly lower than that of the +290 element, and the complex between FMBP-2 and the +220 probe was scarcely detectable under our conditions (Figure 3a). FMBP-2 activity was surveyed in silk-gland extracts during larval development and also in extracts prepared from other tissues and cells. Although the electrophoretic location of the FMBP-2 complex was close to that of FMBP-3 (see below), FMBP-2 migrated more slowly than FMBP-3, and the FMBP-2 complex can be visualized by depletion of the FMBP-3 complex by using the FMBP-3-specific binding element, SC, as a competitor (see Figure 8) or the FMBP-3-specific antibody (see Figure 9). Throughout these experiments FMBP-2 was found in psg and msg at all stages tested, but was undetectable in other tissues and cells. Representative results are shown in Figures 4(b) and 9.

In *Drosophila* the *fkh* gene is expressed in the salivary gland, which is derived by invagination of the labial segment, and it is indispensable for salivary-gland development [28,29]. We obtained an *fkh* homologue of *Bombyx* (*Bm fkh*) and Northern blots showed that *Bm fkh* was expressed in both psg and msg

Figure 8 Binding of FMBP-3 to the octamer-like elements of the intronic modulator

(a) The +290 probe was incubated with msg extract from 2-day-old larvae of the fifth instar in the presence or absence of a 10-, 50- or 200-fold excess of unlabelled oligonucleotide as competitor. (b) The +420 probe or the SC region of the sericin-1 gene was incubated with psg and msg extracts prepared from staged larvae.

(results not shown, but see [26]). Since the binding sequences of FMBP-2 are similar to the consensus binding sequence of the mammalian members of *fkh* family HNF3s [30,31], a specific antibody against Bm Fkh was prepared and used to examine the identity of FMBP-2. The addition of the antibody to the EMSA reaction mixtures hypershifted the FMBP-2 complex in both psg and msg extracts, whereas the FMBP-1 and -3 complexes were not affected (Figure 7a).

Competition experiments with various oligonucleotides covering the upstream transcriptional modulator indicated that FMBP-2 also bound to the proximal upstream region of the fibroin gene (Figure 5a). A factor that binds to the proximal regions of the fibroin and sericin-1 genes has been detected and named SGF-1 [27]. Binding of FMBP-2 to the +290 probe was competed for by the SGF-1-specific element, SA, of the sericin-1 gene [14,15] but not by the mutant SA (SAM) which did not bind to SGF-1 (Figure 7b). From these results we conclude that

Figure 9 Identification of FMBP-3 as POU-M1/SGF-3

The +290 probe was incubated with staged silk-gland extracts in the presence or absence of an antibody against the C1 peptide of POU-M1. The C1 peptide was also added as a competitor to the antibody. The larger arrowhead shows hypershifted products at the top of the gel.

FMBP-2 is the same as SGF-1. We purified SGF-1 and confirmed that FMBP-2/SGF-1 is the Fork head homologue of *Bombyx* (Bm Fkh) [26]. The affinity of FMBP-2/Bm Fkh for the +290 element of the fibroin gene intron was higher than that for the proximal region of the upstream modulator (see a summary in Figure 10).

The POU-domain protein POU-M1 binds to the intronic modulator

In the msg extract at 2 days of the fifth instar, we found another factor (FMBP-3) besides FMBP-2 that bound to the +290 element (Figure 7a). FMBP-3 migrated somewhat faster than FMBP-2 on EMSA. FMBP-3 activity was also high in the extracts from carcass, but only barely detectable in the whole ovarian tissues and whole embryos, and undetectable in the fat-body (Figure 4b). In the psg extract at 2 days of the fifth instar, FMBP-3 was detectable but the concentration was very low compared with that in the msg extract (see Figures 7a and 8b). The binding specificity of FMBP-3 was examined in competition experiments with the msg extract (Figure 8a). The +220 and +290 elements competed for both the more slowly (FMBP-2) and more rapidly (FMBP-3) migrating complexes, but the +370 and +420 elements competed for the faster one (FMBP-3) alone, and the mutant +290M competed for neither. FMBP-3 was therefore competed for by every oligonucleotide containing octamer-like sequences, but not by +290M, which has a mutation at the octamer sequence. FMBP-3 activity in the silk gland was traced from the fourth to the fifth feeding stages with the +420 element used as probe (Figure 8b). FMBP-3 activity similar to that in the msg extracts was detected in the psg extract except at 2 days of the fifth instar.

Binding of FMBP-3 to the +290 probe was competed for also by the SC element of the sericin-1 gene (Figure 8a) which contains an octamer-like sequence and enhances transcription in the msg extract [14,15]. When the SC element was used as a

Figure 10 Binding sites of FMBPs in the fibroin gene promoter

Binding sites of FMBP-1, -2 and -3 are shown by arrows and marked with the name of oligonucleotides with which the binding of FMBPs was detected. Larger arrows show high-affinity sites and smaller arrows show lower-affinity sites, but the binding affinity between various factors was not considered, although the affinities are shown with the same size of arrow. Differences in the binding affinity between the weak sites were also disregarded. UE, upstream transcriptional modulator element; CP, core promoter; IE, intronic transcriptional modulator element. A cloned upstream element (enhancer I) covers the region from -234 to -66 of the fibroin gene.

probe, only one major complex was obtained in the psg and msg extracts and the EMSA profile was the same as that with the +420 probe (Figure 8b). Because POU-M1/SGF-3 is the only major binding protein to the SC element [15], FMBP-3 is probably POU-M1/SGF-3. To confirm that FMBP-3 is the same as POU-M1, an antibody against a C-terminal peptide of POU-M1 [24] was used in EMSA. Except for the psg extract from 2-day-old larvae of the fifth instar, the FMBP-3 complex was hypershifted by the POU-M1 antibody and appeared at the top of the gel (Figure 9). The hypershifted products disappeared when the C-terminal peptide was added together with the antibody, indicating the specific interaction of the antibody with FMBP-3. In the psg extracts at the fourth and fifth feeding stages, the complex with FMBP-2 disappeared when the C-terminal peptide was added, but the reason for this phenomenon is not known.

The binding affinity of FMBP-3 for the octamer-like elements of the fibroin gene modulators was assessed by competition studies using the +290 probe or the SC probe. All octamer-like elements of the intronic modulator competed for FMBP-3 binding to the +290 element (Figure 8a), but only the +420 element competed for that to the SC element (results not shown). Similar studies showed that all octamer-like elements of the upstream modulator were weak binding sites for FMBP-3 (results not shown). Accordingly, FMBP-3 is the same as POU-M1/SGF-3 and binds to the octamer-like elements in the fibroin promoter both upstream and in the intronic modulator (Figure 10). The +420 element had the highest affinity for POU-M1 in the fibroin promoter.

DISCUSSION

Of the five octamer-like AT-rich elements in the intronic modulator of the fibroin gene, the +220 region is the major element responsible for transcriptional enhancement. There are similar AT-rich sequences in the upstream region and intron of other silk genes of *Bombyx* and also in the promoter of the salivary-gland-specific genes of *Drosophila* [13,32,33]. We analysed binding factors to these AT-rich elements, and identified three major factors named FMBP-1, -2 and -3. Since they all bound to the +220 and +290 elements and to other elements independently (Figure 10), their recognition sequences seem to overlap simply

Table 3 Summary of tissue- and stage-specificity of FMBP activities and expression of the fibroin and P25 genes

Data for P25 gene are from ref. [6]. (—), A trace level band was observed. (+), The FMBP-3 activity seemed to decrease during the fourth moulting stage. NT, not tested.

Genes or FMBPs	Psg			Msg		
	IV	IVm	V	IV	IVm	V
Fibroin	+	—	+	—	—	—
P25	+	—	+	—	—	—
FMBP-1	+	(—)	+	NT	(—)	(—)
FMBP-2/Bm Fkh	+	+	+	+	+	+
FMBP-3/POU-M1	+	(+)	(—)	+	+	+

* Results from Northern-blotting experiments and immunohistochemistry analyses ([25]; S. Takiya, H. Kokubo and Y. Suzuki, unpublished work), but EMSA was not carried out.

at the elements around +220 and +290. Although we cannot exclude the possibility that these factors interact, co-ordination is not necessary for their DNA binding.

Two other factors that bind to promoter elements of the P25 gene, a member of the silk protein gene family, have been identified and named BMFA and SGFB [32]. The fibroin (H-chain) and P25 genes are expressed with the same tissue- and stage-specificity (Table 3), but neither BMFA nor SGFB appears to correspond to the FMBPs. BMFA is ubiquitous, but FMBP-1, -2 and -3 are cell-type-specific. SGFB binds to the promoter element of the P25 gene, which contains ATTT repeats and is similar to the binding sequence of FMBP-1 in the transcriptional modulators of the fibroin (H-chain) gene. However, SGFB has been reported to bind to the -160 region of the fibroin gene upstream modulator [32], whereas FMBP-1 binds to the -130 region and not to the -160 region. The stage-specificities are also different between FMBP-1 and SGFB. The fibroin and P25 genes may be controlled by parallel but separate mechanisms.

FMBP-1 activity is specific or highly enriched only in psg with the exception of significant activity in whole ovarian tissues. Moreover, the activity in psg changes in concert with fibroin gene expression from the fourth to the fifth instar. Competition for the

FMBP-1 binding to the upstream modulator or the intronic modulator inhibited the transcription enhanced by these modulators. The +290 element also competed for the binding of FMBP-3/POU-M1 and FMBP-2/Bm Fkh, but POU-M1 is found only at a low concentration in the psg extract from 2-day-old larvae of the fifth instar (Figure 8b), which was used for the transcription assay. Furthermore Bm Fkh and POU-M1 are abundant in the msg where the fibroin gene is not expressed. Although the upstream modulator enhanced the transcription from AdMLP more than the intronic modulator in the psg extract (Figure 6), POU-M1 and Bm Fkh bound to the upstream modulator with a lower affinity than to the intronic modulator. Only FMBP-1 bound these modulators with similar affinities (Figure 10). These results indicate that FMBP-1 is an important and specific component of the activator for fibroin gene transcription.

Five factors that bind to the upstream transcriptional modulator have been found: SGF-1–4 [27] and a composite factor of FF-1 and FF-2 [34,35]. SGF-1, identified recently as Bm Fkh [26], was first found as a factor that bound the proximal upstream elements of both the fibroin and sericin-1 genes and was detected in psg and msg. SGF-2, -3 and -4 bound three distal regions of the upstream modulator of the fibroin gene, which is composed of AT-rich repeats, with different affinities. SGF-3 was identified as POU-M1 [24]. SGF-2 was detected only in the psg extract, but SGF-2 and -4 have not yet been cloned. FF-1 and FF-2 bound to the region around –200 of the upstream modulator, and gave a psg-specific complex, MBC I. Here we have shown that the intronic modulator-binding factors FMBP-1–3 also bind to the upstream modulator, and that FMBP-2 and -3 are identical with Bm Fkh/SGF-1 and POU-M1/SGF-3 respectively. These factors have distinct temporal- and spatial-specificities during silk-gland development (Table 3).

Genes of the *fkh* family are sometimes expressed during tissue-invasion processes and are involved in the regulation of the specific genes in tissues that originated from invagination [29,36,37]. Silk gland is invaginated from the labial segment and expression of *Bm fkh* is detectable at the time of its invagination [25]. Bm Fkh should regulate both fibroin gene expression and silk-gland development. POU-M1 binds to the +420 element in the intronic modulator of the fibroin gene with a high affinity (Figure 10). POU-M1 suppresses the *POU-M1* gene itself [38], and the SC element of the sericin-1 gene functions as an activation element [14,15]. The function of the +420 element of the fibroin gene remains ambiguous, because it is located in the distal half of the intronic modulator and the transcriptional stimulation effect of the distal half is weak. The proximal half stimulates transcription as efficiently as the whole modulator. Some results suggest a suppressive function of the distal half of the intronic modulator (S. Takiya, H. Kokubo and Y. Suzuki, unpublished work). Furthermore, the major element (+220) responsible for transcriptional enhancement is the weakest binding site for POU-M1. Fibroin gene expression may also be controlled by POU-M1 through its suppressive function, and its disappearance from the psg at the fifth instar may be necessary for full activation of fibroin gene expression. The transcriptional regulatory elements of the fibroin gene are composed of multiple binding sites for Bm Fkh, POU-M1 and FMBP-1. Although the function of these factors in transcription remains to be elucidated, expression of the fibroin gene seems to be controlled directly by the co-ordination of these factors, which are differentially expressed during silk-gland development.

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