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Loss of Posterior Silk Gland Transcription Specificity of Fibroin Light Chain Promoter due to Absence of 41 bp Sequence Containing Possible Inhibitor Binding Sites

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Abstract The gene encoding fibroin light chain protein (FibL) is specifically expressed in the posterior silk gland of silkworm and repressed in other tissues. The binding sites of several transcription factors involved in the silk gland transcription specificity of *fibl* promoter have been recognized, including SGFB, PSGF and BMFA. Here we report the leak expression of the enhanced green fluorescent protein (*EGFP*) reporter gene in tissues other than the posterior silk gland *in vivo* when under the control of a shortened *fibl* promoter with deletion of the 5′ terminal 41 bp sequence, which is located at –650 nt to –610 nt upstream of the *fibl* transcription starting site. Assay of silk gland specificity of the promoters was performed by observation of green fluorescence in tissues of silkworm larvae following inter-haemocoelic injection of recombinant *Autographa californica* multiple nuclear polyhedrosis virus carrying the *EGFP* reporter gene controlled by different lengths of *fibl* promoters. Our results indicated that availability of the binding sites of several known factors, including SGFB, PSGF and BMFA, is not sufficient for intact silk gland transcription specificity of *fibl* promoter, and there are possible inhibitor binding sites in the 41 bp sequence (–650 nt to –610 nt) upstream of the transcription starting site which may be required to repress the activity of *fibl* promoter in other tissues.

Key words promoter specificity; fibroin light chain; silkworm; *Bombyx mori*; recombinant AcMNPV

The silk fiber spun by the silkworm *Bombyx mori* is a mixture of sericins and fibroin, the latter composed of three kinds of major silk proteins. During the insect's fifth larval instar, fibroin proteins are synthesized in the cells of a pair of posterior silk glands (PSG), secreted into the lumen of PSG and transported to the lumen of the middle silk gland (MSG). Here the fibroin is coated with sericin secreted by cells of MSG, then transported toward the anterior silk gland to form and spin the silk fiber. All three kinds of fibroin proteins, fibroin heavy chain protein (FibH), fibroin light chain protein (FibL) and fibrohexamerin/p25 protein (FHX/P25), are expressed in PSG of *Bombyx mori* with strict territorial and developmental specificities [1,2]. The developmental regulation of silk genes is mainly hormone-mediated transcription regulation and related to chromatin topology [3–5]. Many genes have been identified to be involved in the regulation of silk genes [1,6–8]. By nuclease protection and mobility shift assays, three PSG-expressed genes were shown to share similarity in upstream and intron sequences. The *cis*-elements and *trans*-activators of silk genes have been identified [9–14].

Understanding the mechanisms underlying the tissue-specific expression of fibroin genes would shed light on the transcription regulation and development of the silk gland. So far, many efforts have been made to identify the *cis*-regulatory elements and *trans*-acting factors responsible

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for the silk gland-specific activity of these fibroin promoters, especially for the expression of the *fhx*/*p25* gene. Of the elements identified, the binding sites of BMFA, SGFB and PSGF are considered to be responsible for silk gland specificity. BMFA is a ubiquitous protein proposed to be involved in the repression of fibroin genes at molting [3]. SGFB is a silk gland-specific regulatory protein expressed in both PSG and MSG, but has access to its target sequence only in PSG cells [15]. PSGF is a factor deduced from DNase I protection assay, supposed to be expressed only in PSG and to facilitate the recruitment of SGFB [15]. The control mechanism by which the expression of the *fhx*/*p25* gene is restricted in PSG but not in MSG was illustrated *in vivo* using organ transplantation and transgenic methods [15–17]. It was shown that the proximal 254 bp sequence of *fhx/p25* promoter contains all sequences required for its specific expression in PSG. In order to understand the PSG-specific expression of the *fhx/p25* gene, the binding sites of SGFB and PSGF were combined with a synthetic TATA box of the A3 gene and this synthetic promoter was sufficient to drive PSG-specific expression of the reporter gene. It was also hypothesized that inactivation of fibroin promoters in tissues other than PSG may be due to the combination of the absence of PSGF and the attachment of BMFA to the promoters [15].

Among silk genes, the fibroin light chain (*fibl*) gene is relatively less studied on the regulation of gene expression. The nucleotide sequence of the *fibl* gene was determined [18] and the possible elements were analyzed [19]. Imamura *et al.* [17] has reported a 691 bp (–650 nt to +41 nt) functional *fibl* promoter with PSG transcriptional specificity, in which the binding sites of SGFB, BMFA, and PSGF could be recognized. However, it has not yet been determined whether the availability of these binding sites is sufficient for PSG specificity of *fibl* promoter.

Autographa californica nucleopolyhedrovirus (AcNPV), whose permissive host is *Trichoplusia ni*, is commonly applied for large-scale expression of eukaryotic proteins in permissive cell lines or insects [20]. In our previous studies, recombinant AcNPV has been used as a highly efficient transient *in vivo* gene delivery vector to some strains of silkworm larvae by haemocoel injection [21,22]. Recombinant AcNPV vector has also been demonstrated to facilitate the study of silk gland-specific protein expression and secretion, using the enhanced green fluorescent protein (*EGFP*) reporter gene fused to the sericin signal peptide coding sequence and under the control of the promoter of the sericin 1 gene [23].

In this study, we delivered recombinant AcNPVs harboring the *EGFP* cassettes controlled by different lengths of *fibl* promoter into Sf9 cells and silkworm larvae, and found that a shortened *fibl* promoter with the deletion of a 41 bp sequence (–650 nt to –610 nt) at its 5′ terminal could be activated in Sf9 cells, hemocytes, MSG and fat body in addition to PSG, as revealed by expression of *EGFP* reporter. The binding sites of SGFB, BMFA, and PSGF remained intact. Our results suggested the existence of binding sites of other unrecognized factors necessary for repressing the activity of *fibl* promoter in other tissues but not in PSG within the 41 bp (–650 nt to –610 nt) sequence.

Materials and Methods

Silkworm strains and cell lines

Silkworm *B. mori* strain 54A, which is AcNPV permissive, was provided by the Sericultural Research Institute, Chinese Academy of Agricultural Sciences (Zhenjiang, China). The Sf9 cells were maintained in Grace's medium (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 27 ºC. The *Escherichia coli* DH10BacΔEGT cell line was established in our laboratory [21].

Construction of recombinant baculoviruses

Fibroin light chain promoters FL1 (–650 nt to +41 nt) and FL2 (–609 nt to +41 nt) were amplified by polymerase chain reaction (PCR) using genomic DNA extracted from the silk gland of fifth instar silkworm larvae as the template. Primers for amplification of FL1 were: U1, 5′-gagctcTG-CATATTGGACATCC-3′ (*Sac*I site in lowercase) and L1, 5′-gaattcTTTAGTGGTCTGTTA-3′ (*Eco*RI site in lowercase). Primers for amplification of FL2 were: U2, 5′-gagctcGTAATTCTCGGTACGGTTCG-3′ (*Sac*I site in lowercase) and L1. The PCR products were cloned into pGEM-T vector (Promega, Madison, USA) to generate plasmids pTV-FL1 and pTV-FL2. They were confirmed by sequencing. Then the promoters were digested out with *Sac*I/*Eco*RI and ligated into the same sites in plasmid pEGFP-N3 (Clontech, Palo Alto, USA) to produce plasmids pFL1-EGFP and pFL2-EGFP, which were then digested by *Sac*I/*Pst*I and inserted into plasmid pFFa2 [21] to produce donor plasmid pFFa2FL1-EGFP and pFFa2FL2-EGFP, respectively. The restriction maps showed the clones were correct (data not shown). pFFa2FL1-EGFP and pFFa2FL2-EGFP were transformed into *E. coli* DH10BacΔEGT cells to make recombinant bacmid

AcFL1egfpΔEGT and AcFL2egfpΔEGT.

Recombinant baculovirus preparation

Generation and large-scale harvest of recombinant baculoviruses followed the instruction manual "Bac to Bac baculovirus expression systems" (Invitrogen) using the Sf9 cell line. Stocks of virus were concentrated by centrifugation at 35,000 *g* for 60 min, and pelleted virus was resuspended in phosphate buffered saline (PBS, pH 7.5) supplemented with 1% (*V*/*V*) FBS before being stored at –70 ºC or subjecting to insect injection. Virus titer was determined by the Tissue Culture Infectious Dose 50 method, which is based on end-point dilution.

Insect inoculation and dissection

Larvae of silkworm *B. mori* strain 54A were routinely reared on mulberry leaves. Recombinant baculoviruses were injected into the haemocoel of newly-ecdysed fifth instar larvae. For analysis of *EGFP* reporter expression in various kinds of tissues, larvae were dissected at appropriate time points. Tissues of hemocytes, fat body, MSG and PSG were collected after they were rinsed in PBS (pH 7.5).

Observation of EGFP fluorescence

Fluorescence of EGFP in cultured cells and silkworm tissues was observed with fluorescence microscopes BX50 (Olympus, New York, USA) or MZ FL III (Leica, Wetzlar, Germany).

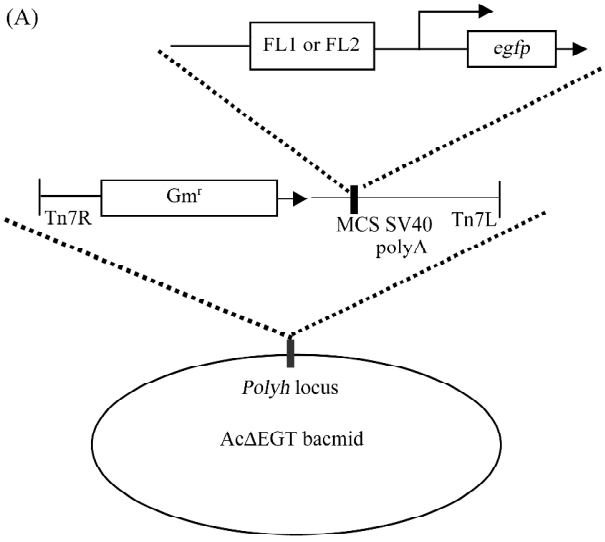

Fig. 1 Illustration of the construction of recombinant AcFL1egfpΔEGT and AcFL2egfpΔEGT

(A) Illustration of the construction of recombinant *Autographa californica* multiple nuclear polyhedrosis virus bacmids AcFL1egfpΔEGT and AcFL2egfpΔEGT. Enhanced green fluorescent protein (EGFP) cassettes controlled by FL1 or FL2 promoters were inserted into the multiple cloning site (MCS) of pFFa2 to produce donor plasmid pFFa2FL1-EGFP and pFFa2FL2-EGFP. The resulting plasmids were then transferred into the mini-*att*Tn7 site (indicated by right and left insertion sites, Tn7R and Tn7L) in the *polyhedrin* locus of AcΔEGT bacmid by Tn7-based transposition and produced recombinant bacmids AcFL1egfpΔEGT and AcFL2egfpΔEGT, respectively. (B) The sequence present in FL1 promoter and absent in FL2 promoter with its relative location to the transcription start site of the fibroin light chain protein gene.

Results

Recombinant AcNPVs harboring *EGFP* reporter controlled by different *fibl* promoters

Fibroin light chain promoters FL1 and FL2 were cloned and sequenced before they were used to drive *EGFP* expression. Then FL1- and FL2-driven *EGFP* cassettes were transferred to the bacmid AcΔEGT to generate the recombinant bacmids AcFL1egfpΔEGT and AcFL2egfpΔEGT (**Fig. 1**). Recombinant bacmids were confirmed by PCR analysis using wild-type bacmid-specific M13 reverse oligo and FL1 or FL2 specific oligo (U1 or U2) as primers. The PCR products were approximately 2 kb in length (**Fig. 2**, lanes 3 and 4). Another PCR assay using M13 reverse oligo and *EGFP*-specific primer EGFP-1 (5′-aagcttgtcgacagatctgcatgcatggtgagc-3′) produced a band of approximately 1.3 kb (**Fig. 2**, lanes 1 and 2). The purity of bacmid was assayed by PCR using wild-type bacmid-specific M13 reverse oligo and M13 forward oligo primers, with AcFFa2ΔEGT as the template, and a 2.2 kb band was observed (**Fig. 2**, lane 5). With AcFL1egfpΔEGT or AcFL2egfpΔEGT as the template, no 2.2 kb band was observed. Purified bacmids AcFL1egfpΔEGT and AcFL2egfpΔEGT were used to transfect cultured Sf9 cells with Cellfectin (Invitrogen) to produce recombinant viruses.

Leak expression of EGFP in Sf9 cells under control of the shortened *fibl* promoter

Baculoviruses of AcFL1egfpΔEGT and AcFL2egfpΔEGT were incubated with Sf9 cells at a multiplicity of infection of 10, and productive infection of baculovirus was achieved. In Sf9 cells infected by vAcFL1egfpΔEGT, no green fluorescence derived from expression of *EGFP* was

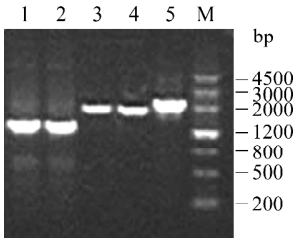

Fig. 2 Polymerase chain reaction (PCR) assay for bacmids AcFL1egfpΔEGT and AcFL2egfpΔEGT

1 and 2, PCR products were approximately 1.3 kb when bacmids was assayed by PCR using enhanced green fluorescent protein (EGFP) specific primer and M13 reverse oligo with AcFL1egfpΔEGT and AcFL2egfpΔEGT as the templates, respectively; 3 and 4, PCR products were approximately 2 kb when bacmids was assayed by PCR using FL-1 or FL-2 specific primer and M13 reverse oligo with AcFL1egfpΔEGT and AcFL2egfpΔEGT as the templates; 5, PCR products were approximately 2.2 kb when bacmids were assayed by PCR using M13 forward and M13 reverse oligo primers with AcFFa2ΔEGT as the template; M, DNA molecular weight marker.

observed even 3 d after incubation, when infectious symptoms were obvious [**Fig. 3(B)**]. This was in accordance with the fact that FL1 promoter could only be activated in PSG, not in Sf9 cells. However, green fluorescence could be observed in Sf9 cells infected with vAcFL2egfpΔEGT [**Fig. 3(A)**], where *EGFP* was controlled by FL2 promoter, which is 41 bp shorter at the 5′ terminal compared with FL1 promoter. The activation of FL2 promoter in Sf9 cells strongly suggested that the missing sequence of 41 bp contains information necessary to silence *fibl* promoter in Sf9 cells.

Leak expression of EGFP in tissues of silkworm larvae under control of the shortened *fibl* promoter

Leak expression of FL2 promoter-controlled *EGFP* in Sf9 cells intrigued us to ask whether deletion of the 41 bp sequence would also cause the loss of PSG transcriptional specificity of *fibl* promoter *in vivo*. Budded virus of vAcFL1egfpΔEGT or vAcFL2egfpΔEGT was injected into the haemocoel of newly-ecdysed fifth instar larvae of silkworm 54A at the amount of 2×10^5 pfu per larva. Between day 3 and 7 after injection, green fluorescence derived from expression of *EGFP* reporter was gradually observed in PSG cells, but not other tissues, when the reporter was controlled by FL1 promoter [**Fig. 4(A)**]. However, when *EGFP* expression was controlled by FL2 promoter, green fluorescence could be observed in fat body [**Fig. 4(C)**] and hemocytes (data not shown) in addition to PSG; green fluorescence could also be observed in MSG [**Fig. 4(B)**]. Expansion of the reporter expression pattern caused by the deletion of the 41 bp sequence from FL1 promoter indicated that the 41 bp sequence contains functional elements necessary for repression of *fibl* promoter in fat body.

Discussion

In this study, we addressed the necessary length of *fibl* promoter for its PSG transcriptional specificity in a "loss of function" manner. Expression of *EGFP* reporter was restricted in PSG cells under the control of FL1 promoter, indicating FL1 promoter does contain *cis*-elements sufficient for PSG transcriptional specificity, which is consistent with a previous report [17]. However, when driven by FL2 promoter, which is 41 bp (–650 nt to –610 nt) shorter than FL1 promoter, leak expression of *EGFP* reporter was observed in MSG, fat body, hemocytes and even Sf9 cells, in addition to PSG. Our results strongly suggested the 41 bp sequence contains possible elements required for binding of unrecognized inhibitory factors necessary for repressing the activity of *fibl* promoter in other tissues but not in PSG. Previous research performed by Horard *et al*. [15] revealed a synthetic promoter containing binding sites of SGFB and PSGF, located upstream of the TATA box from *Bombyx* cytoplasmic actin 3 promoter, has strict PSG transcriptional specificity. They further suggested that inactivation of fibroin promoters in tissues other than PSG might be due to the combination of the absence of PSGF and the attachment of BMFA to the promoters [15]. However, this is not the case in *fibl* promoter. In the sequence of FL2 promoter, which could be activated in MSG, fat body, hemocytes and Sf9 cells in addition to PSG, binding sites of SGFB, PSGF and BMFA were recognized (**Table 1**). As shown in **Table 1**, binding sites of SGFB, PSGF or BMFA are located from –366 nt to –15 nt, so deletion of the 41 bp sequence (–650 nt to –610 nt) did not remove or destroy any binding sites of the three known factors, but rather of some unrecognized factors. Previous studies have focused on the intact *cis*-regulatory elements responsible for the PSG-specific activity of the three silk promoters, but no inhibitory elements or factors for repressing expression of silk genes in other tissues have yet been reported. Our results implied that there are two kinds of mechanisms involved in controlling the expression of silk genes: one activates expression of silk genes in PSG, and another represses the expression of silk genes in other tissues. What is the inhibitory element? It needs to be further investigated.

In the 41 bp (–650 nt to –610 nt) sequence, we noticed 5′-TATAAA-3′ (–619 nt to –614 nt), which is a TATA box-

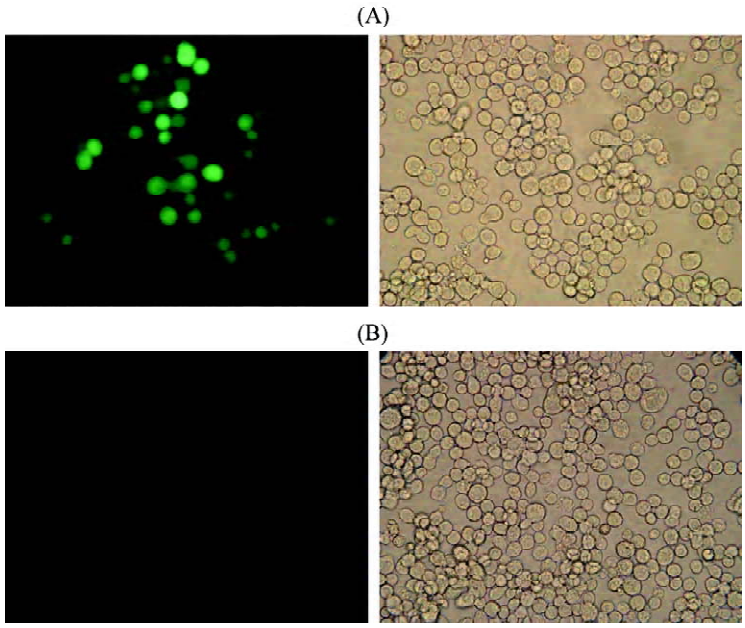

Fig. 3 Leak expression of enhanced green fluorescent protein (EGFP) in Sf9 cells under control of FL2 promoter but not FL1 promoter

Sf9 cells were incubated with budded virus of either vAcFL1egfpΔEGT or vAcFL2egfpΔEGT at a multiplicity of infection of 10. Photographs in the left panels showed fluorescent images, those in the right panels showed bright light images. (A) Sf9 cells incubated with vAcFL2egfpΔEGT for 3 d. (B) Sf9 cells incubated with vAcFL1egfpΔEGT for 3 d. Observed with an Olympus BX50 fluorescence microscope. Magnification, 200×.

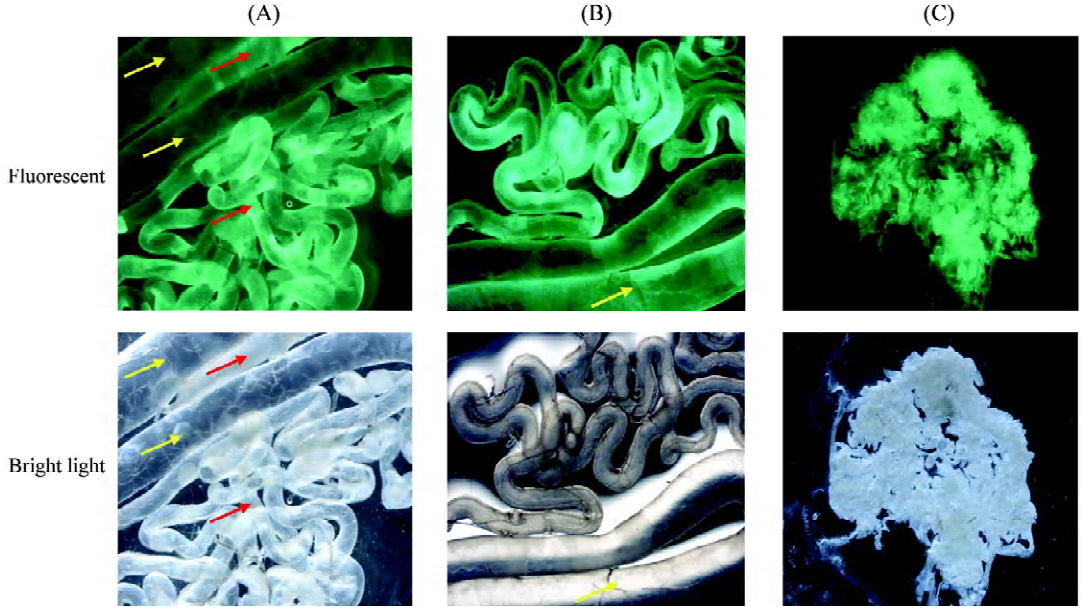

Fig. 4 Leak expression of enhanced green fluorescent protein (EGFP) *in vivo* under control of FL2 promoter but not FL1 promoter

Budded virus of vAcFL1egfpΔEGT or vAcFL2egfpΔEGT was injected into the haemocoel of newly-ecdysed fifth instar larvae of silkworm 54A and EGFP observation was taken at the indicated time points. Photographs in the upper panels show fluorescent images, those in the lower panels show bright light images. (A) Posterior silk gland (PSG)-specific expression of EGFP when controlled by FL1 promoter, 5 d post-injection of vAcFL1egfpΔEGT. (B) Expression of EGFP in PSG and middle silk gland (MSG) when controlled by FL2 promoter, 5 d post-injection of vAcFL2egfpΔEGT. (C) Expression of EGFP in fat body when controlled by FL2 promoter, 3 d post-injection of vAcFL2egfpΔEGT. PSG is indicated by red arrows and MSG indicated by yellow arrows. Observed by fluorescence microscope (Leica MZ FL III) with GFP plus fluorescence filter set (excitation filter 480/40 nm and barrier filter 510 nm).

Table 1 Distribution of possible binding sites of silk gland-specific transcription factors

Factor	*fibl* promoter		*fhx*/*p25* promoter	
	Binding location (nt)	Binding sequence	Binding location (nt)	Binding sequence
SGFB	–51 to –45	TATTTATTTTAA	–71 to –62	TATTTATTTAA
PSGF	–21 to –15	GGAACAT	–46 to –40	GGAACAA
	–217 to –211	AGAACAA		
	–240 to –234	GGAACAA		
	–249 to –243	GGATCAA		
	–261 to –255	CGAACAA		
BMFA	–366 to –360	AAAATGTCAT	–133 to –124	AAAATGGCGT

fibl, fibroin light chain protein gene; *fhx*/*p25*, fibrohexamerin/p25 protein gene.

like sequence. Srinivasan *et al*. reported a TATA box binding factor (TBF), which they designated P43 TATA box binding factor, that could inhibit *in vitro* transcription of the *tRNA*^{Gly} multi-gene family and RNA polymerase II transcription from *actin5C* promoter when binding to motifs including TATATAA and TATAAA [24]. P43 TBF was purified from PSG nuclear extracts of *B. mori*, and proposed to negatively modulate the transcription of *tRNA*^{Gly} genes. No evidence shows that P43 TBF is involved in transcription regulation of the *fibl* gene, but the regulation mechanism that binding of some TBF to TATA box-like elements helps to inhibit transcription may also apply to *fibl* promoter.

For many years, *in vitro* transcription assay and chromatin footprinting have proved to be valuable tools in helping to determine factors and their binding sites involved in the activation of silk gland-specific genes. Our knowledge of SGFB, BMFA, PSGF and many other factors associated with silk gland transcription regulation is either partially or totally derived from these *in vitro* assays [9,13]. However, it also means that many of these conclusions have not yet been tested *in vivo*, due to the absence of appropriate *in vivo* gene delivery and reporter assay systems for silkworm. In recent years, PSG specificity of fibroin promoters such as *fhx*/*p25* and *fibl* promoters has been readdressed, utilizing newly-established transgenic techniques [17] or transient *in vivo* gene delivery systems, like the ballistic method [15,25]. Organ transplantation needs skilled workers, whereas obtaining a transgenic silkworm strain takes a long time, therefore results from *in vivo* experiments were limited. In this report, we delivered *in vivo* the reporter cassettes using recombinant AcNPV bacmid as the vector. High efficiency of gene transfer, demonstrated by the expression of *EGFP* reporter *in vivo*, indicates recombinant AcNPV bacmid could serve as a vector for transcription regulation research in silkworm [21–23].

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