The expression of GFP under the control of fibroin promotor in primary ovarian cells of *Antheraea pernyi*

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The fibroin promoter can stably express foreign gene in lepidopteran cells. Total RNA was extracted from the gland of silkworm, *Antheraea pernyi* and the transcription initiation site of fibroin gene of *A. pernyi* was identified by RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE). The expression vector (pGFP-N2/Fib) was constructed by use of replacing the CMV promoter with the fibroin promoter. The results of visual screening under a fluorescent inverted microscope and Western blot analysis indicated that the GFP gene was expressed in the primary cells of ovary origins from *A. pernyi*.

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1. Introduction

Lepidopteran insects and their cells have been increasingly utilized in the past few years for the expression of heterologous proteins by baculovirus expression vectors (Luckow and Summers 1988; Miller 1988; Ramachandran *et al* 2001). Nuclear polyhedrosis virus (NPV) promoters allow high level expression of foreign genes after infection of host cells, but the expression is transient because the host cells die from virus infection, and continuous expression of the recombinant protein can not be obtained at late phase of infection. So, new promoters, which can be utilized for stable transformation of lepidopteran cells and expression of foreign gene would be useful (Jarvis *et al* 1990; Jarvis 1993; Giraud *et al* 1992; Yamao *et al* 1999; Hua *et al* 1994; Zhang *et al* 1999).

Larva of *Antheraea pernyi*, a *Lepidopteran* insect, produces a number of fibroin proteins during its 5th stage in the posterior section of silk gland. In the past, we have shown that 5′-flanking region of the fibroin gene contains promoter sequences including elements such as TATA and CAAT box (Li *et al* 2002).

In this report, the transcription initiation site of fibroin gene of *A. pernyi* is identified and the GFP is expressed in the primary cultures of ovary cells of *A. pernyi* using the cloned fibroin promoter.

2. Materials and methods

2.1 *Preparation of the total RNA from silk gland*

The silk gland of the silkworm, *A. pernyi*, was dissected, homogenized by adding Trizol (Gibco BRL, USA) immediately after removal and 4 μg total RNA (figure 1a) was extracted according to the method described by the manufacturer.

2.2 *Determination of the transcription initiation site of fibroin gene*

Freshly prepared total RNA (1 μg) was incubated at 65°C for 10 min and then transferred on ice immediately. A FirstChoice™ RLM-RACE Kit (Ambion, USA) was used for determining the transcription initiation site according to manufacturer's protocol. Briefly, full-length mRNAs are selected by treating total RNA with calf intestinal phosphatase (CIP) to remove the 5′-phosphate from all molecules which contain free 5′-phosphates. The RNA is then treated with tobacco acid pyrophosphatase (TAP) to remove the cap structure from the capped mRNA and leave a terminal 5′-monophosphate. A synthetic RNA adapter is ligated to the RNA population and only molecules containing a 5′-phosphate (the uncapped, full-length

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mRNAs) can accept the adapter. After reverse transcription reactions, the cDNA was obtained by nested PCR using the following two specific reverse primers (AF1): 5′-CGG ATC CTC TTC GAG GAC AAT AGT-3′; (AF2): 5′-GCC CCT GGT ACT CTC TTG ATA ACA ACT C-3′ and two forward primers from the kit respectively. The PCR production was sequenced after being cloned into T-vector.

2.3 *Construction of expression vector*

The 1·1 kb fragment containing some important elements, such as TATA box, CAAT box and promoter region was amplified by PCR by placing the *Sma*I site at its 5′ terminal and 3′ terminal. This fragment was used to replace CMV promoter of expression vector pGFP-N2 (Clontech, USA).

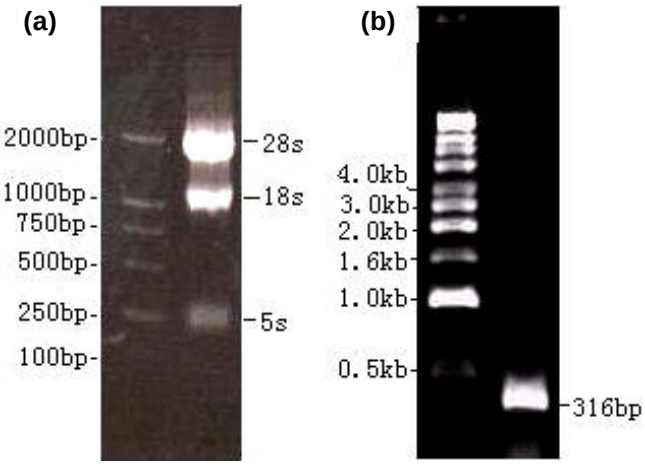

Figure 1. Determination of transcripts site with RLM-RACE. (**a**) The template RNA was prepared from the posterior section of silk glands taken from the last instar larvae 8 day. (**b**) RLM-RACE of the transcripts start are as follows: Full-length mRNAs are selected by treating total RNA with calf intestinal phosphatase (CIP) to remove the 5′-phosphate from all molecules which contained free 5′-phosphates. The RNA was then treated with tobacco acid pyrophosphatase (TAP) to remove the cap structure from the full-length mRNA leaving a 5′-monophosphate. A synthetic RNA adapter was ligated to the RNA population, only molecules containing a 5′-phosphate (the uncapped, full-length mRNAs) accepted the adapter. After reverse transcription reactions and nested PCR the production of 316 bp (line 2) was sequenced.

2.4 *Transient transfection*

The primary cells derived from ovary of *A. pernyi* were washed with PBS(-) and adjusted to 5×10^5 cells/well, and were transfected with linear DNA templates (150 ng in 12·5 µl of reaction mixture of Cellfectin reagent) according to the procedure (Gibco BRL). The transfected cells were cultivated in MGM448 medium at 27°C for one week. The cultures were observed periodically 6–72 h after the transfection under fluorescence microscope at 499 nM wavelength.

2.5 *SDS-PAGE assay and Western blot*

SDS-PAGE assay was performed following the manual of Sambrook *et al* (1989). After electrophoresis, the proteins were transferred onto a HybondC Nitrocellulose filter. The Western blot analysis was performed according to the protocol of ECL Kit (Amersham Pharmacia Biotech, Sweden) with anti-GFP antibody (Clonetech, USA).

3. Results and discussion

3.1 *Identification of transcription initiation site and analysis of 5′-flanking region of fibroin gene*

The FirstChoice RLM-RACE procedure was used to detect transcription initiation site of fibroin gene. After reverse transcription reactions and nested PCR, a band of about 300 bases was observed by agarose gel electrophoresis and the RT-PCR product was subjected directly to sequence analysis (figure 1b). Comparing with primary transcript sequence of L-chain gene of *Bombyx mori* (Kikuchi *et al* 1992) and *A. yamamai* (Meng *et al* 1997), we found that nucleotide at – 26 bp upstream from initiating ATG codon seemed to be used as a major transcription start point (tsp) in *A. pernyi* fibroin gene. Our results indicated that the sequence of the primary transcript was highly conserved with that of *A. yamamai* and the L-chain gene of *B. mori* (figure 2).

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B. mori      ATCAGTTCGG TTCCAACTCT CA------AGATG AGA GTC AAA ACC
A. yamamai   ATCAGTTCGG TTCCAGCTCT CATAA CCATG AGA GTA ACA GC
A. pernyi    ATCAGTTCGG TTCCAGCTCT CATAA CCATG AGA GTA ATA GCC TCC
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Figure 2. Alignment of transcript sequences of fibroin gene from *A. pernyi*, *A. yamamai* and *B. mori*. The start code is underlined gray indicates differences between them. Missing nucleotides are indicated by - - - -.

The nucleotide sequence of the 5′-flanking region of fibroin gene has been reported for *A. yamamai* and the L-chain gene of *B. mori*, but not for *A. pernyi* (Tsujimoto *et al* 1979; Meng *et al* 1997). In order to study the molecular basis of transcriptional regulation of fibroin gene, we isolated the *A. pernyi* genomic fibroin gene 1·1 kb fragment, and aligned its sequence with the L-chain gene of *B. mori*. Two important elements, CAAT box and TATA box, were identified at – 97 bp and – 52 bp upstream of the transcription initiation site of the fibroin gene, identical to the mammalian sequence, as shown in figure 3. In addition, three TAAT motifs were observed at the 5′-end of the 300 bp region upstream from the TATA box, which were considered to be binding sites for some proteins, i.e. two *Drosophila* homeodomain proteins, ZEN (*zerkniillt*) and EVE (*even-skipped*) (Hui *et al* 1990; Takiya and Suzuki 1989). The specific regions, – 164 to – 151 and – 101 to – 88, recognize common silk gland-specific factors (SGF-1).

3.2 *Transient expression and reporter gene assay*

We constructed a recombinant pGFP-N2/Fib to express the GFP reporter gene under the control of fibroin promoter (figure 4). The promoter activity of the 5′-flanking region of the fibroin gene was examined using the GFP gene as a reporter in cultured *A. pernyi* ovary primary cells. The expression of GFP was checked under fluorescence microscope at 499 nM excitation wavelength after transient transfection. Green fluorescent cells were seen 24 h after transfection (figure 5). Western blot analysis showed that a band of the same molecular weight as the positive control GFP was expressed in transfected cells

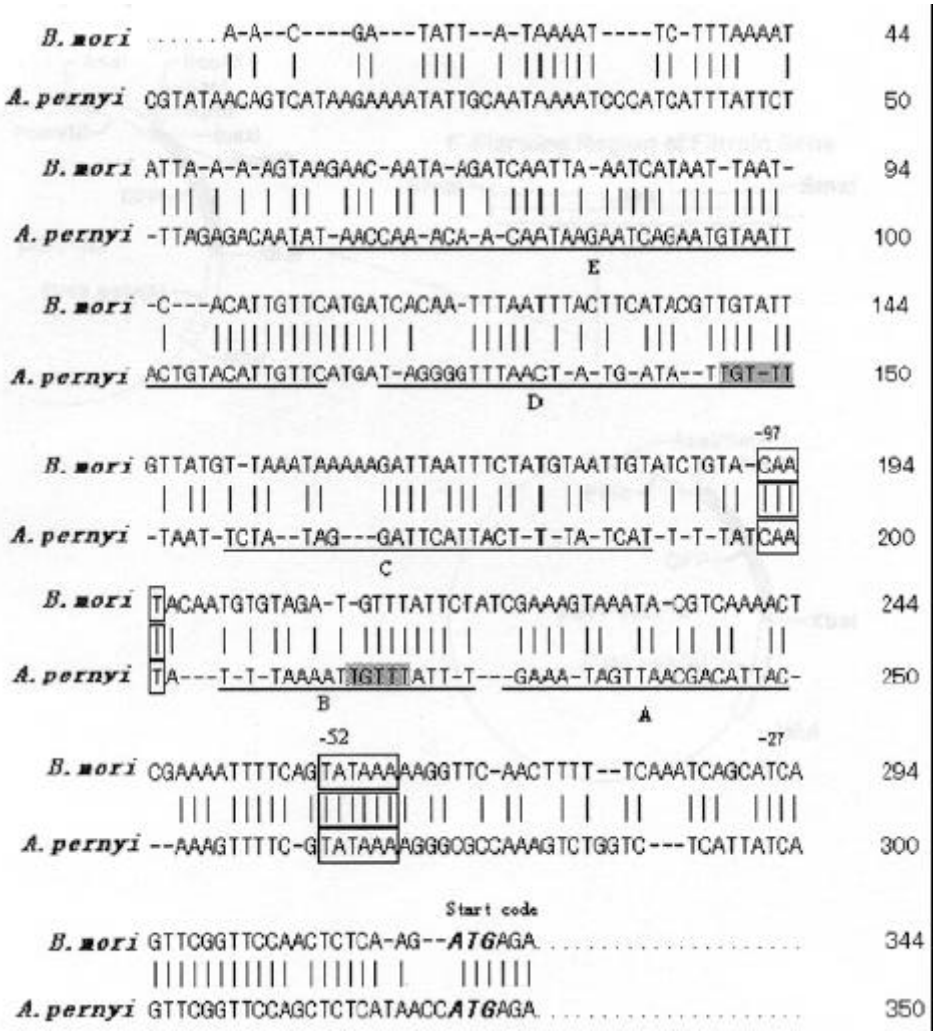

Figure 3. Nucleotide sequences of several regions of cloned fibroin DNA. The numbers denote the distance from the 5′end of the mRNA coding part of the gene. The CAAT and the TATA box are marked by box. The A + T-rich stretches are underlined. The TGTTT motifs (silk gland factor, SGF) are marked by the shadow. Start code ATG is in italic.

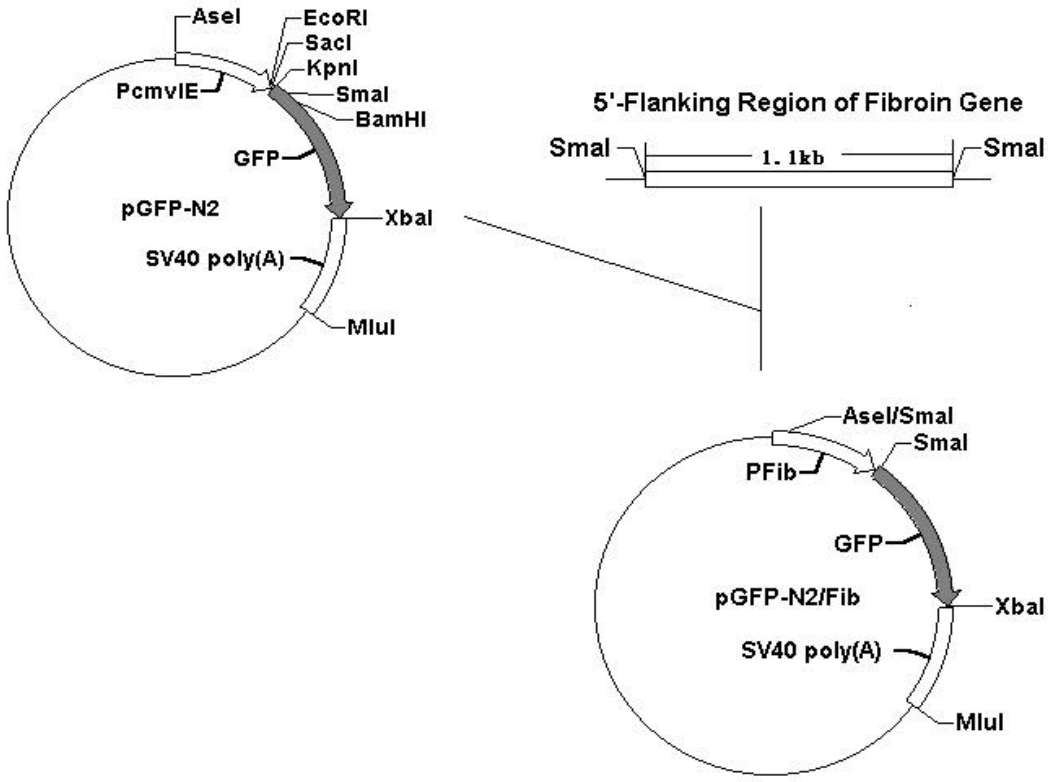

Figure 4. Construction of pGFP-N2/Fib. The 1·1 kb fragment containing promoter region was amplified by PCR by placing the *Sma*I site at its 5′ terminal and 3′ terminal. This fragment was inserted into the vector pGFP-N2.

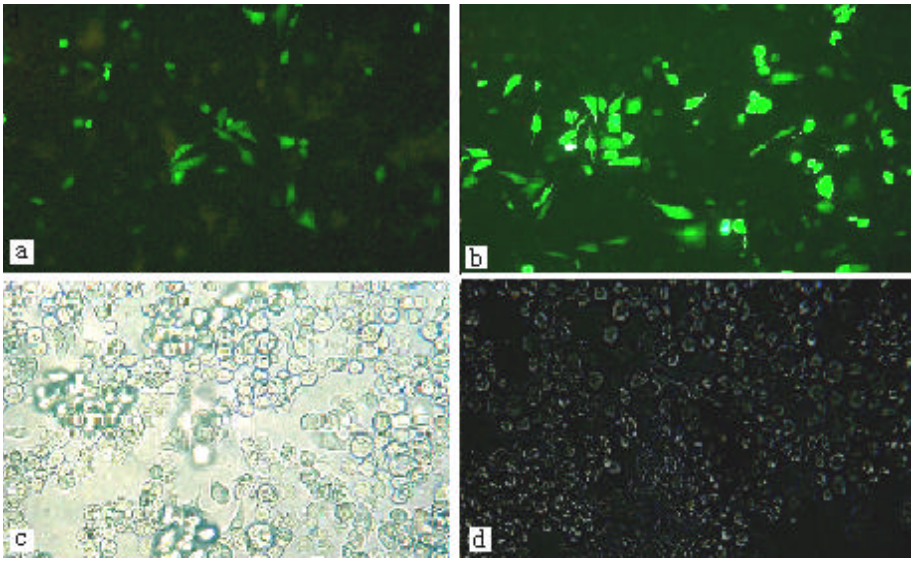

Figure 5. Expression of green fluorescent protein in primary cells of *A. pernyi*. Screening for gene expression under the fluorescent inverse microscope after transfected 60 h by use of (**a**) pGFP-N2/Fib, (**b**) pGFP-N2 and (**d**) pGFP-N2¯. Photo of pGFP-N2/Fib transfected after 60 h under visible light (**c**).

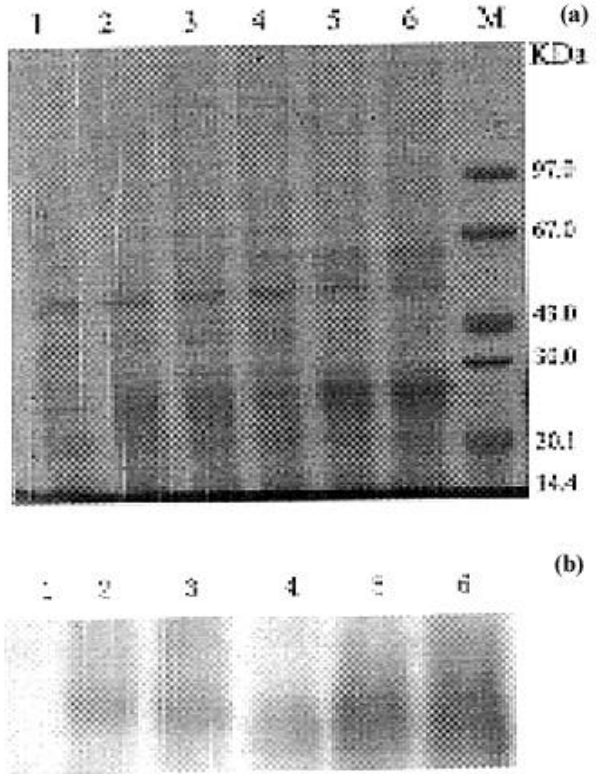

Figure 6. SDS-PAGE and Western blot analysis of GFP. (**a**) SDS-PAGE analysis of expression of GFP, and (**b**) Western blot analysis. Line 1 control; lines 2–5 transfected with pGFP-N2/Fib after 24, 36, 48 and 60 h; line 6, transfected with pGFP-N2 after 60 h.

but not in control cells (figure 6). All these results suggest that upstream of the 5′-flanking region of the fibroin gene contains elements which can promote and regulate the expression of GFP gene in the *A. pernyi* ovary primary cells. A silk gland transcription factor (SGF-1) binding site was found in the 5′-flanking DNA of fibroin and this interaction may play a general role in regulation of silk gland genes (Hui *et al* 1990). It is interesting to note that sequences with similarity to the SGF-1 site are present in *Drosophila* salivary gland genes (Harshman *et al* 1998). It is known that fibroin promoter activity is restricted to the silk gland of *B. mori* from *in vivo* studies using a variety of cell extracts (Suzuki *et al* 1986). This tissue-specificity probably is the result of the presence or absence of certain proteins in these extracts, which can bind to the fibroin promoter. We consider that ovary primary cells are not highly differentiated, and probably there are some other factors to recognize and activate the fibroin promoter and promote the process of expression of the reporter gene.

In summary, we describe a functional fibroin promoter from *A. pernyi* which can be used for expression of heterologous gene in primary ovarian cells.

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