Characterization of *CIb1* Gene Promoter from Silkworm, *Bombyx mori*

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The hemolymph chymotrypsin inhibitor b1 (CIb1) of silkworm, *Bombyx mori*, plays an important role in innate immunity. In order to study its encoding gene *CIb1*, five heterogeneous promoter fragments of 844 bp, 682 bp, 516 bp, 312 bp and 82 bp in length were cloned from genomic DNA of the p50 silkworm strain. Characterization of the *CIb1* promoter was performed *in vitro* using the firefly *luciferase* gene as reporter. The results showed that *CIb1* promoter fragments have transcription activities in the *B. mori* ovary-derived BmN cell line. The 82 bp fragment (−72 to +10 nt) containing the eukaryotic core promoter elements revealed a basic transcription activity. The Bm1 element, upstream the transcription initiation site, showed a positive regulation function to the *CIb1* promoter. *CIb1* promoter-like fragments from the genomic DNA of the tetra hybrid silkworm Suju×Minghu provided a natural deletion model for the study of the *CIb1* promoter. *In vitro* analysis indicated that the 132 bp fragment from −517 nt to −386 nt upstream of the transcription initiation site strongly suppressed the transcription activity of the *CIb1* promoter, suggesting that the 132 bp fragment harbours strong negative *cis*-acting elements. Infection of *Bombyx mori* nucleopolyhedrovirus (BmNPV) increased the activity of the *CIb1* promoter, having provided another evidence to the function of CIb1 in the innate immunity of silkworm.

Key words: Bombyx mori, *CIb1* Gene and Protein, Promoter, Deletion Assay

Introduction

The Kunitz-type inhibitor family is widely existent in various tissues of animals and plants. Because of its multifunction in physiology and antimicrobial peptides/proteins, it has been extensively studied (reviewed by He *et al*., 2003). Members of this inhibitor family have also been identified from the insect hemolymph of *Bombyx mori* (Sasaki, 1978, 1984, 1988), *Manduca sexta* (Ramesh *et al*., 1988), and *Sarcophaga bullata* (Papayannopoulos and Biemann, 1992; Sugumaran *et al*., 1985). In the hemolymph of silkworm, *B. mori*, nine kinds of this type of peptides were found to have inhibitory activity towards chymotrypsin including CIb1, b2, 1, 2, 2V, 9, 10, 13, 13V (Eguchi *et al*., 1986; Fujii *et al*., 1989, 1996a, b). CIb1 is an immunological response-related lipopolysaccharide (LPS)-binding protein and possibly participates in the melanization (Sasaki, 1988; He *et al*., 2004). Its encoding gene *CIb1* (also called *Ict-H* gene) has been cloned and expressed and the protein has been studied as well (He *et al*., 2003). But its function has to be further studied, since the characteristic of the CIb1 remains unknown as yet. To characterize the *CIb1* promoter, promoter deletion assay was carried out in the BmN cell line by using the firefly *luciferase* (*luc*) gene as reporter. This experiment will benefit to finally understand the function of hemolymph CIb1 of silkworm, *B. mori*.

Materials and Methods

Silkworms, bacteria, vectors and reagents

The silkworm strain p50, hybrid Suju×Minghu, *B. mori*-derived BmN cells, *Escherichia coli* strain DH10B, pGEM-4Z-*luc* containing a complete *luciferase* gene of firefly (Lei *et al*., 1994) and wild-type *B. mori* nucleopolyhedrovirus Zhenjiang strain (wt BmNPV-ZJ) were maintained in our laboratory. Vector pGEM-4Z and E4030 kit for the luciferase assay were from Promega Corp. (Madison, USA). The insect cell culture medium

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TC-100, fetal bovine serum (FBS) and lipofectin were from Invitrogen. Enzymes and other main reagents were from TaKaRa. Primers for PCR amplification were synthesized by Shanghai Invitrogen Biological Engineering Co., Ltd; they also did the DNA sequencing.

Cloning of CIb1 promoter fragments and construction of reporter plasmids

The genomic DNA of silkworm was prepared from about 1.0 g of the posterior part of the silkgland of 5–10 fifth instar larvae according to the method previously described (Zhao *et al.*, 2000). PCR primers were designed in accordance with the *CIb1* gene and its upstream sequence published in GenBank (AF529176). Five forward primers were located at different sites upstream the transcriptional initiation site, containing an *Eco*R I restriction site. The reverse primer was located near the translation initiation site with a *Bam*H I restriction site (underlined part): PF1, 5′-TAAGAATTCTTGTGATGAGGGTGGCTTG-3′ (−824 to −816 nt); PF2, 5′ -TATGAATTCTAAGGTCTCAAGCATAGTTACA-3′ (−672 to −649 nt); PF3, 5′ -TTCGAATTCTTGCAGTGCCTCTTCAATCCTG-3′ (−506 to −482 nt); PF4, 5′-TGGGAATTCTCTTGGGTCGTTACCCAAG-3′ (−302 to −282 nt); PF5, 5′-TCTGAATTCTAGATCGAGGTCAGATAGCGT-3′ (−72 to −51 nt); PR, 5′-CAAGGATCCGTCTCTGATTCAATATTGTATGTCT-3′ (−16 to +10 nt of complementary strand).

PCR amplification was performed with each of the five forward primers and the reverse one by using silkworm genomic DNA as template. The PCR conditions were as follows: DNA was predenatured at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 1 min and 30 s, 72 °C for 2 min, and finally extended at 72 °C for 10 min. Then PCR products were gel-isolated and purified. Thus, *CIb1* promoter fragments were generated and cloned into vector pGEM-4Z after digestion with *Eco*R I and *Bam*H I. The recombined plasmids were identified by gel electrophoresis and sequencing. By using pGEM-4Z as vector, transient expression plasmids were constructed with the *luc* gene driven by heterogeneous *CIb1* promoters.

Cell culture, transfection and transient expression

The methods for routine BmN cell culture and transfection were as previously described (Summers and Smith, 1987; Zhou *et al.*, 2002). Cells were seeded into 12 cm^2 flasks at a density of 5×10^5 cells/ml (3 ml per flask) and cultured overnight. Before transfection, the medium was removed and the cells were washed twice with serum-free TC-100 medium, then transfected with 50 μl transfection solution containing 1 μg reporter plasmid DNA and 7.5 μl lipofectin in 1 ml serum-free medium. After 4 h serum-free medium was replaced by 3 ml TC-100 medium containing 10% FBS, and this moment was set as zero time of infection. Cells transfected with pGEM-4Z-*luc* served as the blank. Three replicates were done for each experiment.

In the BmNPV infection experiment, cells were transfected with reporter plasmid for 3–4 h followed by infection of wt BmNPV-ZJ at 1.0 multiplicity of infection (MOI) for 1 h as previously described (Shen *et al.*, 2004). Cells transfected with the same reporter plasmid without infection served as positive control, and pGEM-4Z-*luc*-transfected cells as the blank.

Preparation of cell extracts and assay of luciferase activity

Cells were harvested 48 h post infection (hpi) by centrifugation at $9000 \times g$ for 5 min at 4 °C. Cell extracts were prepared with a E4030 kit (Promega Corp.) and the lysates were processed with a freeze-thaw cycle at −20 °C and room temperature followed by centrifugation at 4 °C to remove cell debris. The supernatants were used for the luciferase assay. Luciferase activities were measured by a luminometer 20/20 (Turner Biosystems Inc., Sunnyvale, CA; 2 s delay and read at 10^{th} s) as relative luminescence units (RLU) per 10 μg lysate. The total protein content of the lysates of each treatment was estimated as previously described (Zhou *et al.*, 2002) for normalization of the luciferase activity.

Results

Cloning of the CIb1 promoter from the silkworm genome

At first, a 0.8 kb *CIb1* promoter was generated from the genomic DNA of p50 by PCR with the primers PF1 and PR, and cloned into vector pGEM-4Z. The sequencing result showed that the cloned fragment was 844 bp in length and identical with that published in GenBank (AF529176) as well as in BGI-Silkworm Genome Database, *i.e.*

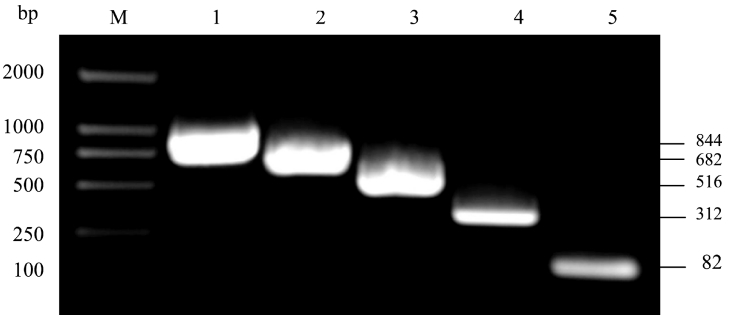

Fig. 1. *CIb1* promoter fragments amplified from genomic DNA of silkworm strain p50 by PCR. M, DNA molecular marker; lanes 1 to 5, different promoter fragments generated.

the cloned fragment was the *CIb1* promoter (Fig. 1).

Analysis of the structure of the CIb1 promoter

Within the cloned 844 bp fragment of the *CIb1* promoter there were some DNA motifs including a Bm1 (highly repetitive retroposon-like DNA element), a TATA box, a CCAAT motif (on the complementary strand), a lipopolysaccharide-response element CATTW, a transcription factor NF-$\varkappa$B binding motif GGGAACTCCT and a GATA box (He *et al*., 2003). In order to study the function of these motifs, the 844 bp *CIb1* promoter was used as the template for the generation of heterogeneous promoters by the forward primers PF2, PF3, PF4 and PF5 paired with the reverse primer PR, respectively. The sequencing results indicated that the promoter fragments produced were 682 bp, 516 bp, 312 bp and 82 bp, respectively. Compared with the 844 bp fragment, 682 bp fragment lost part of the Bm1 motif. The 516 bp one contained the same element except for the Bm1 motif, but fragment 312 bp lost the Bm1 and GGGAACTCCT motif completely, and fragment 82 bp only contained a TATA box and a CCAAT motif (Fig. 2).

Then reporter plasmids p*ZCIb1*844-*luc*, p*ZCIb1*682-*luc*, p*ZCIb1*516-*luc*, p*ZCIb1*312-*luc* and p*ZCIb1*82-*luc* were constructed with a *luc* gene under the control of heterogeneous *CIb1* promoters for the luciferase activity assay in BmN cells. The luciferase activities of cells transfected by different reporter plasmids showed significant differences (Fig. 2B). The 82 bp *CIb1* promoter (−72 nt to +10 nt upstream the transcription initiation site) containing the basic elements of an eukaryotic promoter showed a certain transcription level. The 312 bp *CIb1* promoter revealed the highest transcriptional activity, significantly higher than that of the 82 bp fragment. It suggests that there is a positive *cis*-acting element from −302 nt to −73 nt upstream the transcription initiation site. When the promoter fragment increased to 516 bp in length, its transcription activity was not increased but

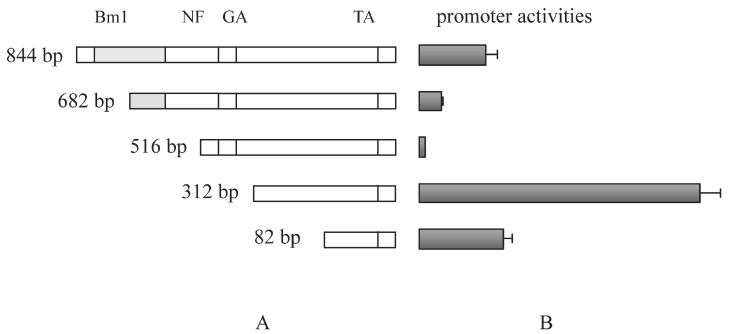

Fig. 2. *CIb1* promoter structure and its transcription activity. The gray region indicates the Bm1 element, NF means the NF-$\varkappa$B binding site, GA and TA indicate the GATA and TATA box, respectively. (A) *CIb1* promoter structure model; (B) transcription activity of heterogeneous promoters.

Table I. Transcriptional activities of heterogeneous *CIb1* promoters in the BmN cell line.

Reporter plasmid	Luciferase activity [RLU]	Relative luciferase activity (%)
p*ZCIb1*844-*luc*	1770 ± 295	100
p*ZCIb1*682-*luc*	579 ± 60	32.71
p*ZCIb1*516-*luc*	140 ± 20	7.91
p*ZCIb1*312-*luc*	7436 ± 539	420.11
p*ZCIb1*82-*luc*	2229 ± 216	125.93

BmN cells were transfected with reporter plasmid containing a luciferase gene (*luc*) driven by heterogeneous *CIb1* promoters. The data show the average of relative luciferase activity units (RLU) from three separate treatments per 10 μg protein of cell lysate after being normalized by the blank vector pGEM-4Z-*luc* (a luciferase gene inserted at the *Bam*H I site of pGEM-4Z) and total protein of cells (mean ± S.D.)

dropped significantly to 1.88% of that of 312 bp, implying that it is a negative *cis*-acting element from −506 nt to −303 nt. But when the promoter increased to 682 bp, the transcription activities raised again, perhaps a partial Bm1 functioned as a positive *cis*-acting element. The activity of the 844 bp fragment containing a complete Bm1 element increased significantly compared with that of 682 bp (Table I).

The 132 bp fragment in the CIb1 promoter significantly suppressed the transcription activity

Interestingly, when genomic DNA of Suju× Minghu instead of p50′s as the template for PCR amplification with the primers PF2 and PR was used, four fragments of different length were generated. The sequence results showed that they were 682 bp, 696 bp, 550 bp and 564 bp in length, respectively, and showed high similarity with the sequence of the *CIb1* promoter published in GenBank (AF529176) in which the 682 bp fragment is identical to that of p50 cloned above; the 550 bp one lacks a 132 bp fragment from −517 nt to −386 nt upstream the transcription initiation site. It remained unknown and needs further study whether these fragments are *CIb1* promoters or not, but they have provided a natural deletion model for the study of the *CIb1* promoter. Hence another reporter plasmid p*ZCIb1*550-*luc* with the *luc* gene under the control of 550 bp fragments was constructed to transfect BmN cells for the luciferase activity assay using p*ZCIb1*682-*luc* as control. The results showed that the 682 bp promoter revealed a lower level transcription activity. In contrast, the transcription activity of the 550 bp fragment was strikingly high reaching (3136789 ± 25853) RLU, about 5417 times of that of the 682 bp promoter, suggesting that in the 132 bp fragment a strong negative *cis*-acting element exists suppressing the transcription activity of the *CIb1* promoter.

BmNPV infection increased the transcription activity of the CIb1 promoter

BmN cells were transfected by the reporter plasmids p*ZCIb1*844-*luc*, p*ZCIb1*682-*luc*, p*ZCIb1*516-*luc* and p*ZCIb1*312-*luc*, respectively, followed by infection of BmNPV and harvested 48 hpi for the luciferase activity assay. The results showed that the luciferase activities in cells infected by BmNPV were higher than those of uninfected cells, *i.e.*, the infection of BmNPV increased the transcription activity of the *CIb1* promoter, suggesting the *CIb1* promoter response to the challenge of BmNPV. But the increases varied with the lengths of the promoter, the 682 bp and 516 bp

Table II. The effects of BmNPV infection on heterogeneous *CIb1* promoters.

Reporter plasmid	Luciferase activity [RLU]		Increase of luciferase activity (times)
	BmNPV infection	Uninfected	
p*ZCIb1*844-*luc*	59591 ± 4710	2259 ± 168	26.4
p*ZCIb1*682-*luc*	92565 ± 4989	947 ± 58	97.7
p*ZCIb1*516-*luc*	20643 ± 1103	212 ± 34	97.4
p*ZCIb1*312-*luc*	202721 ± 12724	6637 ± 214	30.5

BmN cells were transfected with reporter plasmid containing a luciferase gene (*luc*) driven by heterogeneous *CIb1* promoters for 3–4 h followed by infection of wt BmNPV-ZJ (MOI = 1.0) for 1 h. The data show the average of relative luciferase activity units (RLU) from three separate treatments per 10 μg protein of cell lysate after being normalized by the blank vector pGEM-4Z-*luc* (a luciferase gene inserted at the *Bam*H I site of pGEM-4Z) and total protein of cells (mean ± S.D.).

promoters increased much more than those of 844 bp and 312 bp (Table II).

Discussion

Most eukaryotic gene expression is tissue-specific (Liang and Pardee, 1992). *B. mori* CIb1 is mainly expressed in fat bodies and a part in other tissues including ovary (He *et al.*, 2003). Therefore, the *luc* gene driven by the CIb1 encoding gene (*CIb1*) promoter could be expressed in the *B. mori* ovary-derived BmN cell line.

Deletion assay of the *CIb1* promoter including the natural deletion models amplified from the genomic DNA of Suju×Minghu showed that the transcription activities were strikingly different among heterogeneous *CIb1* promoters. The 82 bp *CIb1* promoter containing the basic elements of an eukaryotic core promoter showed a certain level transcription. The activity of the 312 bp *CIb1* promoter was significantly higher than that of 82 bp, suggesting that from −302 nt to −73 nt upstream transcription initiation site harbours the main positive *cis*-acting elements. In contrast, the 132 bp fragment from −517 nt to −386 nt upstream transcription initiation site contains a negative regulation element strongly suppressing the promoter activity. So, when the promoter fragment increased to 516 bp which includes part of the 132 bp sequence, the transcription activity dropped rapidly. But the Bm1 element showed positive regulation function (Adams *et al.*, 1986; Wilson, *et al.*, 1988), 682 bp and 844 bp *CIb1* promoters, including partial and whole Bm1 element, respectively, showed higher transcription activities.

Injection of LPS to fifth instar silkworm larvae resulted in a measurable abundance change of the *CIb1* transcript in fat bodies (He *et al.*, 2003). But in our experiment, changes of the *CIb1* promoter activity were not detected in BmN cells challenged by LPS (data not shown), possibly because BmN cells lack some factors existing in fat bodies involved in the immunity response of the *CIb1* gene to LPS (Taniai and Tomita, 2000; Taniai *et al.*, 2006).

CIb1 promoter contains both positive and negative *cis*-acting elements. Specific transcription factors or repressors of the cells bind to them, respectively, under the control of genetic factors and environmental conditions for regulation of the *CIb1* gene transcription level to meet the demands of development and acclimation of cells or silkworms. In this experiment, the increase of *CIb1* promoter activity after infection of BmNPV was a response of cells to external challenge, suggesting further that CIb1 plays an important role in the innate immunity of silkworm.

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