Biochemical and Biophysical Research Communications 461 (2015) 102–108

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification and characterization of three novel hemocyte-specific promoters in silkworm *Bombyx mori*

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ARTICLE INFO

Article history:
Received 18 March 2015
Available online 7 April 2015

Keywords:
Bombyx mori
Hemocytes
Specific promoter
Recombinant AcMNPV (rAcNPV)

ABSTRACT

Insect hemocytes play essential roles in the metabolism, metamorphosis and immunity, which are closely related events of growth and development. Here, four novel hemocyte-specific genes were obtained and conformed in our study, namely, Bmintβ2, Bmintβ3, BmCatO, and BmSw04862, respectively. Subsequently, their promoter sequences were cloned, and their activity in hemocytes, fat body, and silk gland were analyzed using recombinant AcNPV vector system in vivo. Our results showed that Bmintβ2, Bmintβ3, and BmCatO were hemocyte-specific promoters in the silkworm, *Bombyx mori*. Interestingly, Bmintβ2, and Bmintβ3 promoter regions were both located in their first intron. Further analysis of a series of BmCatO promoter truncations showed that a 254 bp region could function as a promoter element in the tissue-specificity expression. In summary, the results of this study revealed that we have identified three hemocyte-specific promoters in silkworm that will not only great significance for better understanding of hemocyte-specific gene, but also has potential applications in insect hematopoiesis and innate immunity research.

1. Introduction

Hemocytes play vital roles in insect growth and development [1–4]. Insect are continually exposed to a wide range of pathogens, such as viruses, bacteria, fungi, and protozoa. Thus, insects have evolved an effective immune system to protect themselves from pathogens [5]. Insects lack an acquired immune system but have a well-developed innate system, which including humoral and cellular defenses. Insects hemocytes not only act as mainly executors of cellular immunity, such as phagocytosis, encapsulation, and nodules, also involve in humoral immunity, such as melanization [6–10].

Drosophila hemocytes have been well-understood during the past few decades [11–15], and researches focus on *Drosophila* have enhanced our understanding of hematopoiesis, hemocytes function, and regulator mechanisms [16–21]. Hemocytes and their function are also investigated widely in certain insect order, such as Mosquito [22,23], *Bombyx mori* [8,24], *Manduca sexta* [25,26] *Pseudoplusia includens* [27,28], *Spodoptera frugiperda* [28] and Honey Bee [29]. But due to the current lack of molecular marker and efficient genetic operation, our knowledge are still limited of the further exploitation in those insects.

The silkworm *B. mori* is not only an important insect model for silk production, but also an excellent fundamental research model. Here, in order to create a tool for hemocytes research and application of *B. mori* hemocyte-specific genes. Microarray data were analyzed and four hemocyte-specific genes were obtained in our study. Subsequently, promotes were cloned and their activity were analyzed using recombinant AcNPV vector system.

2. Materials and methods

2.1. Biological materials

B. mori strain p50 was used in this experiment. The larvae reared with mulberry leaves as described previously [30]. The cultured silkworm embryo-derived BmE cell lines used in this study were cultured in Grace medium (Invitrogen) supplemented with 10% (V/V) FBS (Invitrogen) [31].

2.2. Bioinformatic screening of hemocyte-specific genes in silkworm

Silkworm hemocyte-specific genes were analyzed using microarray data from SilkDB (http://www.silkdb.org/microarray/).

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http://dx.doi.org/10.1016/j.bbrc.2015.03.176
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Subsequently, BLAST searches were conducted using GeneBank (http://www.ncbi.nlm.nih.gov/), SilkwormDB (http://www.silkdb.org/silkdb/), and KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/). Primers were designed and the fragments of the candidate genes were acquired by polymerase chain reaction (PCR).

2.3. Verification of the hemocyte-specific genes and 5′ full RACE

To demonstrate whether the genes obtained were hemocyte-specific, we further investigated their expression profiles in different tissues with PCR and qRT-PCR. Subsequently, 5′ RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) were performed using GeneRacer™ kit (Invitrogen). All primers used in our study were shown in supplementary data table S. Each putative protein was further validated by domain prediction using Pfam (http://pfam.sanger.ac.uk/) and SMART (http://smart.embl-heidelberg.de/).

2.4. Cloning the promoter sequence

Total DNA was extracted from silkworm larvae using the Takara MiniBEST Universal Genomic DNA Extraction Kit (Takara, Japan). The predict promoter region were downloaded from the SilkDB. The primers for all promoters are listed in supplementary data table S.

2.5. Transfection and construction of recombinant AcMNPV

The reconstructed pFastBac™ Dual vector were transfected into *E. Coli DH10Bac* cells (Invitrogen) to make recombinant bacmids [32]. Recombinant baculovirus bacmids were verified by PCR analysis using the M13 reverse primer and the forward primer of the candidate genes' promoter, and construct into BmSWU3 cells in 24-well plates with Lipofectamine® 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol.

2.6. Injection of recombinant AcMNPV into silkworm larvae

The cell culture medium contain recombinant viruses were collected by centrifugation at 800×g for 5 min, and stored at −80°C until to use. Virus titer was determined as described previously [33]. 20μL of the recombinant viral solution (10^6 PFU/larva) was injected into the hemocoel of the silkworm larvae on the 1st day of 5th instar. Five days post-injection, hemocytes, fat body, and silk gland were collected or dissected out, after fixed in 4% paraformaldehyde (PFA) and washed in ice-cold PBS, the samples were observed and photographed using a fluorescence microscope (Nikon 80i) and/or confocal microscope (Olympus FV1000).

2.7. Flow cytometric analysis

The larval silkworm hemolymph was collected and analyzed as described previously with little modified [34]. Briefly, the collected hemocyte was centrifuged at 1000×g for 5 min at 4 °C. The hemocytes was washed twice and resuspended in 1×PBS. Samples were analyzed on an Accuri C6 (BD, USA). GFP and DsRed fluorescence were detected by using a 488 and 575/26 bandpass filter, respectively.

2.8. Quantitative real-time PCR (qRT-PCR)

For qRT-PCR analysis, a StepOne Plus™ Real-Time PCR system (Applied Biosystems, USA) and the SYBR Master Mix (Promega, USA) were used under the following conditions: 95°C for 10min, followed by 40 cycles of 5s at 95°C and 30 s at 60°C. The primers used in this experiment were showed in Supplementary data table S. Bm*GAPDH* was used as an internal control.

3. Results

3.1. Screening of hemocyte-specific genes in silkworm

The microarray data provide an effective tool to screening the tissue-specific gene in silkworm, *B. mori* [35]. In the present study, we analyzed transcriptional levels in ten silkworm tissues (See materials and methods) and BmN-SWU1 cell line which derived from the ovarian tissues. In all, twenty-four hemocyte-specific high expression probes were screened out (Fig. 1A). The probe sequences were used as queries to BLAST against the SilkDB (http://www.silkdb.org/silkdb/), and KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/). Relative EST sequences, predicted CDS sequences, and/or full-length cDNA sequences if any were downloaded and analyzed. We cloned all candidate genes in our study, and analyzed their expression profiles in different tissues, including hemocytes, head, testis, ovary, midgut, malpighian tube, epidermis, silk gland, and fat body with PCR (data not shown). Finally, four candidate genes were screening out four genes, namely, Bmintβ2 (probe: sw18286), Bmintβ3 (sw08491), BmCatO (sw05834, and sw17255), and BmSw04862 (sw04862, a novel gene in silkworm). The qRT-PCR results showed that those four gene specifically high expressed in hemocytes (Fig. 1B).

Bmintβ2 and Bmintβ3 have been reported in our previous study [36], and both consist of three domains, a large extracellular portion, a single transmembrane segment (Tm), and a short cytoplasmic domain (Fig. 1C). BmCatO belong to Cathepsin family, which was a group of protease predominantly located in lysosomes [37,38]. BmSw04862 was a novel gene, there was no homologous gene in SilkDB and GeneBank (data not shown). The deduced amino acid sequences contain a signal peptide and a single transmembrane segment (Fig. 1C).

3.2. Gene structure analysis and identification of promoters region

5′RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was used to analyze hemocyte-specific promoters. The gene structures were shown in Fig. 2A. Bmintβ2 and Bmintβ3 both closely clustered on nscaf2847 which was located on chromosome 4 in silkworm genome with different transcriptional orientations, and only 841bp distance with each other in the genome (Fig. 2A).

At the beginning, three truncated promoter fragments from Bmintβ2 promoter were cloned, and named pBmIntβ2-1 (1998bp, −1877/+121bp), pBmIntβ2-2 (3070bp, −2949/+121bp), and pBmIntβ2-3 (5347bp, −5226/+121bp) (Fig. 2A). The truncated promoters were subcloned into modified pFastBac™ Dual vector (Fig. 2B). The recombinant plasmid (pHS), together with positive control (pPH) and negative control (pΔP) (Fig. 2B), were first transfected into *E. Coli DH10Bac* cells, then constructed into BmSWU3 cells, and finally the recombinant viruses were used to infection silkworm larvae as described in materials and methods. Just as expected, the negative control group only detected EGFP signal, and the positive control group detected both EGFP and DsRed signal in hemocytes, fat body, and silk gland (Fig. 3A). Those data suggested that the system we designed was reliable. But disappointingly, the experiment group did not capture any DsRed signal by fluorescence microscopy and qRT-PCR analysis (data not shown). Subsequently, we try to select the sequences which contain the first intron, and named pBmIntβ2-4 (3591bp, −2293/+1298bp), pBmIntβ2-5 (1180bp, +118/+1298bp), and pBmIntβ2-6 (959bp, +196/+1154bp), respectively (Fig. 2A). Surprisingly, the DsRed signal was observed in hemocytes (Fig. 3A). Interestingly,

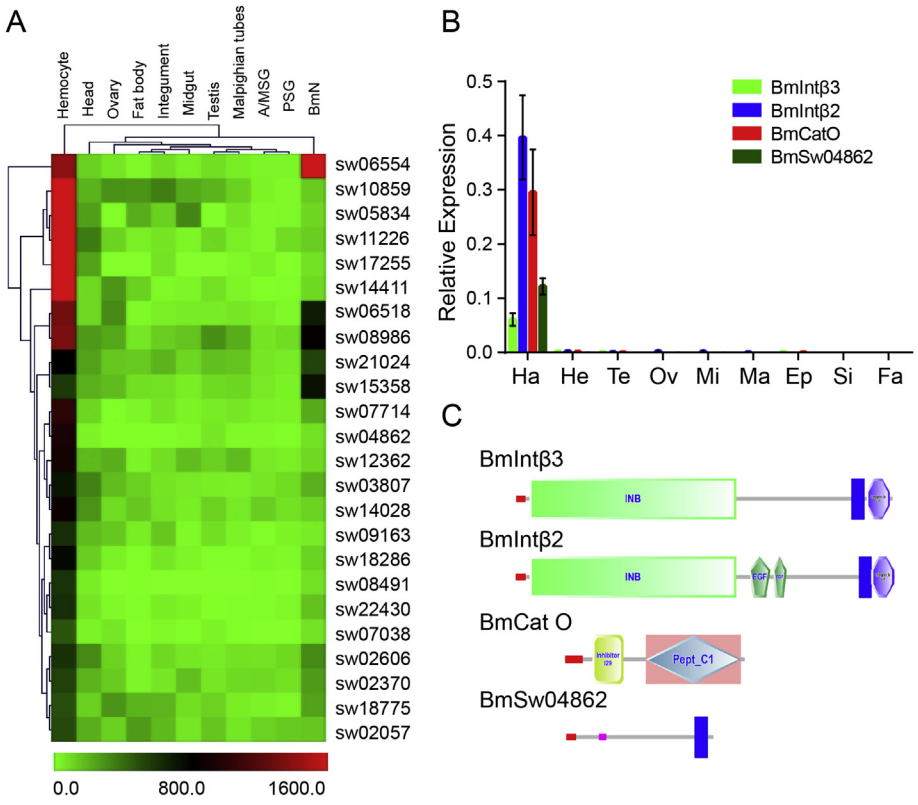

Fig. 1. Screening and analysis of hemocyte-specific gene in silkworm. A, a heat-map of the hemocyte-specific probes on day 3 of the fifth instar larvae of silkworm, *Bombyx mori*. Gene expression levels are represented by green (lower expression) and red (Higher expression) boxes. The columns represent eleven different tissues or cell line, namely, hemocytes, head, ovary, fat body, integument, midgut, testis, malpighian tubes, anterior/median silk gland (A/MSG), posterior silk gland (PSG), and BmN cell line (BmN). B, tissue expression analysis of different hemocyte-specific candidate genes by qRT-PCR. Abbreviation: Ha, hemocytes; He, head; Te, testis; Ov, ovary; Mi, midgut; Ma, Malpighian tube; Ep, epidermis; Si, silk gland; Fa, fat body. *BmGAPDH* was used as an internal control. The results are expressed as the means ± SD of three independent biological replicates. C, conserved domains of the hemocyte-specific genes in silkworm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

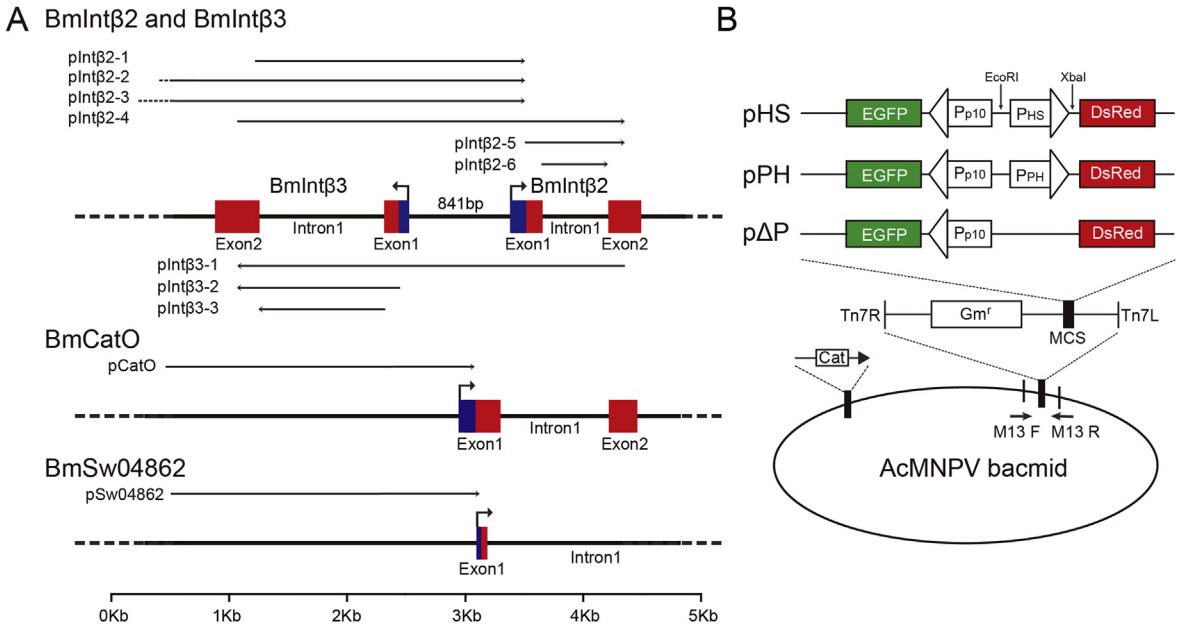

Fig. 2. Promoter cloning strategies of the candidate genes and vector construction. A, schematic representation of the candidate hemocyte-specific promoters clone. Exons and introns are represented by color box and black solid lines, respectively. 5′UTRs are represented by blue box. Short black arrows represent transcriptional start sites. B, schematic representation of the recombinant bacmid procedure. Promoter sequences were inserted into the plasmid with EcoRI and XbaI, pPH and pΔP were used as positive and negative control, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

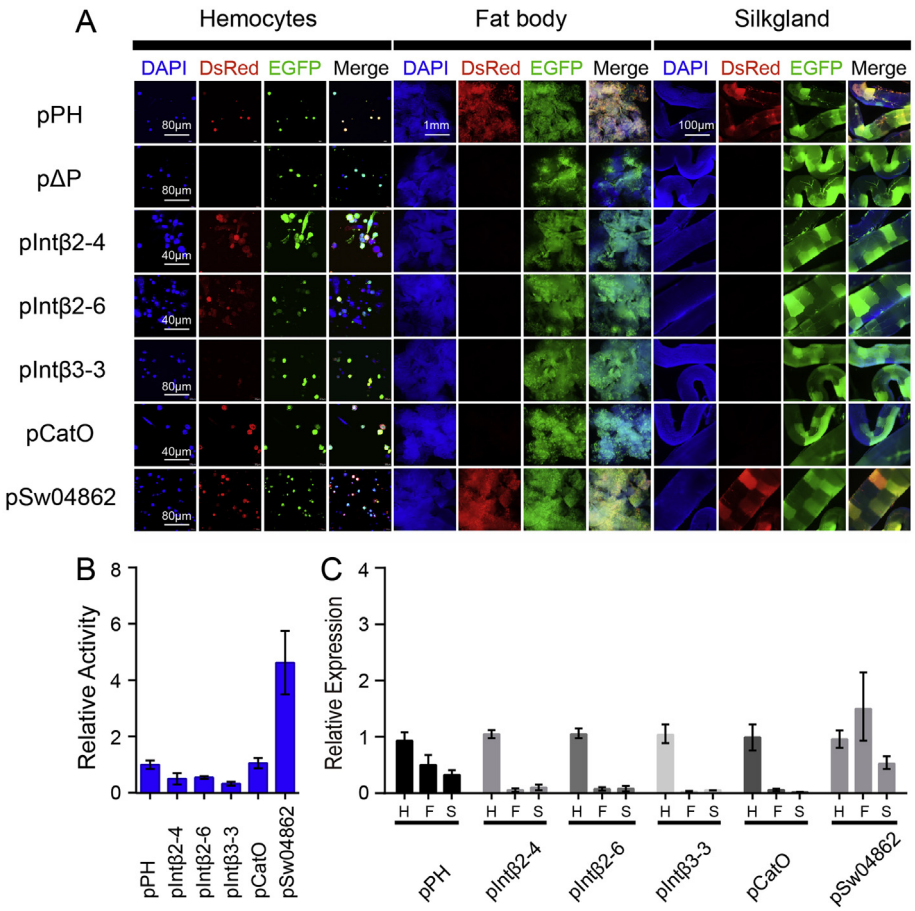

Fig. 3. Activity analysis of promoters in different tissues. A, Fluorescent observation of the larvae hemocytes, fat body, and silk gland. B, the relative activity of different promoters in hemocytes using flow cytometry. C, Expression analysis of DsRed in larvae hemocytes (H) fat body (F), and silk gland (S) at 72 h post-injected different recombinant viruses with qRT-PCR. The housekeeping gene *BmGAPDH* was used as an internal control.

there were no signal in fat body and silk gland(Fig. 3A). Those results suggested that the promoter region located in the first intron. A similar approach was used to study the promoter of Bmintβ3, and a similar result was obtained (Fig. 3A).

A 1912bp promoter fragment from BmCatO promoter, and a 2194bp promoter fragment from BmSw04862 were cloned, named pBmCatO (1912bp, −1836/+76bp), and pBmSw04862 (2194bp, −2168/+26bp), respectively (Fig. 2A). As shown in Fig. 3A, those two promoters were active in hemocytes, and consistent with BmIntβ2 and Bmintβ3, DsRed signal was not observed in fat body and silk gland. However, pBmSw04862 was shown broad activity in the survey tissues (Fig. 3A).

To demonstrate the relative activity of those promoters, we further detected the fluorescence intensity using a flow cytometry. The result showed that all of the promoters were active in hemocytes in vivo (Fig. 3B). We have also investigated the DsRed transcriptional levels in different tissues with qRT-PCR (Fig. 3C), consistent with the results above. BmInt1, Bmintβ3 , and BmCatO were specific high expressed in hemocyte, but BmSw04862 widely expressed in detected tissues (Fig. 3C).

3.3. *Truncation analysis of BmCatO promotor tissue-specificity related region in vivo*

We have previously shown that the promoter of BmCatO was active in hemocytes specifically, and showed higher activity than other hemocyte-specific promoters investigated in our study (Fig. 3). The sequence was analyzed using the Gene Quest Module of DNASTAR software, as shown in Supplementary data Fig. S, TATA box-was located at −49 ahead of the translation start site (TSS), and the putative transcriptional binding sites were also identified and labeled in Fig. S.

To demonstrate the hemocyte-specific region and minimal promoter region, a series of truncation analysis were conducted in the following research. As shown in Fig. 4A, five recombinant AcNPVs were constructed and named pBmCatO (1912bp, −1836/+76bp, complete region), pBmCatO1 (1530bp, −1454/+76bp), pBmCatO2 (938bp, −862/+76bp), pBmCatO3 (409bp, −333/+76bp), and pBmCatO4 (156bp, −80/+76bp). Deletion of the region located −1836bp to −333bp did not has a significant effect on promoter activity in hemocytes (Fig. 4A). Fluorescence observation results showed that the first four fragments showed no activity, but BmCatO4 fragment display a strong activity in silk gland (Fig. 4B) and fat body (data not shown), qRT-PCR also present same results (Fig. 4C).

4. Discussion

Tissue-specific promoter is a valuable tool for organization development and gene function study. Benefited from the finding of a wide range of specific genes and molecular markers, great progress has been made in Drosophila hematopoiesis [39–42]. Develop up to now, the research of *Drosophila* hematopoiesis is becomes more and more prevalent and convenient. With the

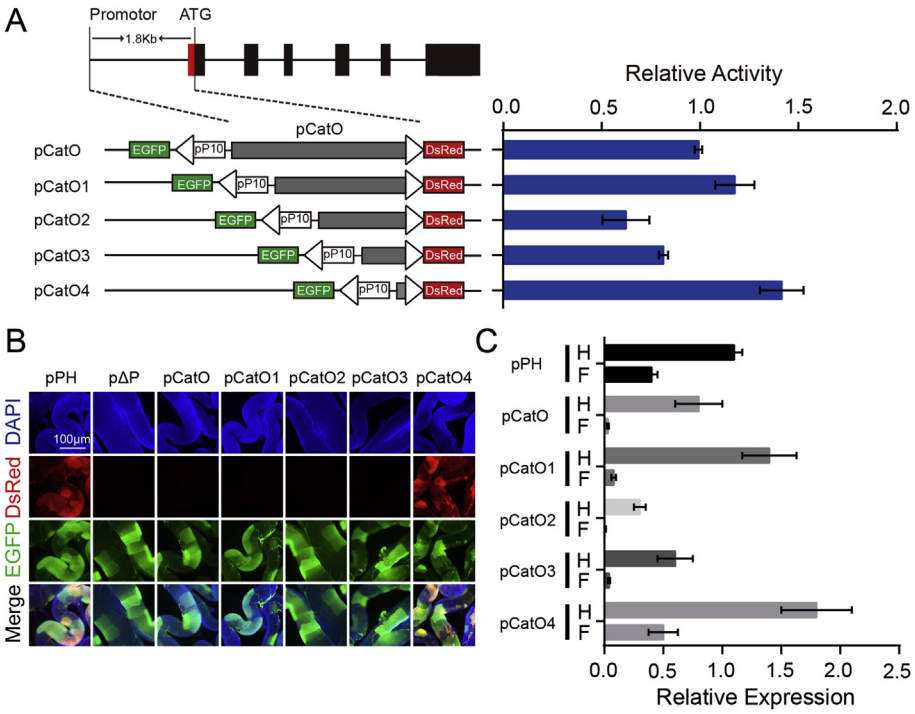

Fig. 4. 5′ truncation analysis of BmCat O promoter in vivo. A, different 5′ truncation region and their activity of the *BmCat* O promoter in hemocytes analysis by flow cytometry. B, Fluorescent observation of silk gland injected with different recombinant viruses. C, Expression analysis of DsRed in larvae hemocytes (H) and silk gland (S) injected with different recombinant viruses with qRT-PCR.

increasing application of transgenic technology [43], more and more tissue-specific promoters have been reported in silkworm. Ser1 promoter can drive EGFP specifically expressed in silk gland [44], further study showed that a 25 bp fragment is the tissue-specific region of ser1 promoter [33]. Zhao et al[45] showed that Fib H promoter is a anterior silk gland specific promoter. Besides, related studies have been reported in midgut and fat body in silkworm [46].

In an attempt to create a tool for hemocytes research. Four genes, namely, BmIntβ2, Bmintβ3, BmCatO, and BmSw04862, with high transcriptional levels only in hemocytes were selected after analysis of microarray data [35]. BmIntβ2 and Bmintβ3 belong to integrin superfamily, which is a group of cell surface glycoprotein, it mediate cell to extracellular matrix (ECM) and cell-to-cell interactions as well as transduce the bidirectional transmembrane signal [47]. Many integrin members have been identified in insects, and its expression profiles show obvious tissue-specific, especially in hemocyte-specific in Lepidoptera [26,48–50]. In our previous study [36], we showed that BmIntβ2, and Bmintβ3 were specific expressed in silkworm hemocytes, What's more, MsIntβ1, a homologs gene of BmIntβ2, and Bmintβ3 is specific exprssed in plasmatocytes in *Manduca sexta* [26]. These results suggested that BmIntβ2, and Bmintβ3 promoters are hemocyte-, even some hemocyte lineage specific in silkworm probably.

BmCatO belong to cathpsin family, which are widely exist in almost all organisms, function in intracellular protein degradation/turnover via catalyzation of protein hydrolysis [51–53], and play important roles in various physiological process [54]. Previous studies revealed that certain cathpsin members highly and/or specifically expressed in hemocytes and play important roles in wing disc differentiation [55], larval molting and metamorphosis by participating in the functioning of hemocytes in insects [56]. BmSw04862 is a novel gene in silkworm, no homologous genes in GeneBank. The deduced amino acid sequences contain a signal peptide and a single transmembrane segment (Fig. 1C), and the BmSw04862-EGFP fusion protein located in nuclear membrane and cytoplasm (data not shown).

Silkworm is susceptibility to wild-type AcNPV [57,58], studies demonstrated that the actin3 promotor can control EGFP expressed in hemocytes, silk gland, and fat body with recombinant AcMNPV system [32]. Some silkworm promoter also have been studied by using this system, such as Ser1 [33,44], Cathepsin B [59] and D [60,61], E74B [62], and USP [63]. In the present study, to increase the credibility, multiple control were designed (Fig. 2B). First, p10 promoter control the expression of EGFP, which could act as a marker of virus infection cells. Second, positive and negative control also considered, in fact, positive control (pPH promoter control DsRed expression) shown extensive activities in detected tissues, and negative control (no promoter control DsRed expression) not detected any red signal in our study (Fig. 3A). These facts indicate that recombinant AcNPV could be a efficient and reliable tool to study the promoter in certain silkworm tissues.

In the present study, to obtain the hemocyte-specific region, the upstream region of BmCatO has been well-studied by the region truncation (Fig. 4). The result suggested that the core region promoter located in the region −80bp to +76bp, was crucial for basal transcriptional of BmCatO. A 254bp region (from −333bp to −80bp) is the dominant region for hemocyte-specific activity.

In summary, we have identified three hemocyte-specific promoters in silkworm. The results not only can provide a good tool for silkworm hemocytes research, but also provide fundamental knowledge for similar study in other insects, especially in Lepidoptera.

Conflict of interest

The authors declare no potential conflicts of interest.

Acknowledgments

This work was supported in part by the National Basic Research Program of China (2012CB114603), the Research Fund for the Doctoral Program of Higher Education of China (20130182110003), the Natural Science Foundation of Chongqing (CSTC2013jcyjys0007), and the Fundamental Research Funds for the Central Universities (XDJK2013B020, SWU112033).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.176.

Transparency document

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