Expression and Localization of *Bombyx mori* V-ATPase 16 kDa Subunit c

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V-ATPase plays a central role in lepidopteran midgut ion transport physiology, and lepidopteran midgut turned out to be a model tissue for the study of V-ATPase. In the present study, the 5'-RACE method is used to obtain the 5'-UTR of V-ATPase c subunit gene from *Bombyx mori*. Sequence analysis of the promoter region and 3'-UTR of V-ATPase c subunit gene revealed that the transcription of the V-ATPase c subunit gene may be regulated by multi-ways. RT-PCR analysis showed that *B. mori* V-ATPase c subunit mRNA expresses in the whole developmental stages of *B. mori*. We also constructed a transient vector to determine the subcellular localization of the *B. mori* V-ATPase c subunit, and the result demonstrated that it is located in the membrane and some specific regions of BmN cells. Real-time PCR analysis further indicated that the c subunit mRNA expression was up-regulated significantly at 24 and 72 h in the midguts of resistant *B. mori* larvae after being inoculated with *B. mori* nucleopolyhedrovirus, suggesting that it may be related to the immune response against virus infection.

Key words: V-ATPase Subunit c, *Bombyx mori*, BmNPV

Introduction

The vacuolar-type H+-ATP synthetase (V-ATPase) is ubiquitously expressed in eukaryotic cells (Harvey and Nelson, 1992), not only in plasma membrane but also in vacuolar membranes, including endosomes, lysosomes, clathrin-coated vesicles and chromaffin granules (Nelson, 1989; Takase *et al.*, 1994). It is a multi-subunit enzyme that consists of a peripheral V1 domain responsible for ATP hydrolysis and an integral V0 domain that transports protons across membranes (Forgac, 1999; Nishi and Forgac, 2002; Stevens and Forgac, 1997). V-ATPases play an important role in pH regulation of the intracellular compartments, the extracellular space and the cytoplasm (Beyenbach and Wieczorek, 2006; Forgac, 2007; Wagner *et al.*, 2004). The subunit c of V-ATPase belongs to the integral V0 domain, forming a hexameric complex with subunits c' and c'' as a core of the V0 domain (Wilkens *et al.*, 2004). It is known that the subunit c is one of the most conserved membrane proteins. It is vital for V-ATPase assembly and forms the principal pathway for proton translocation (Finbow and Harrison, 1997).

V-ATPase is turned out to be localized in the lepidopteran larval midgut (Wieczorek *et al.*, 2003) and serves the following special physiological functions: regulating ionic composition and energizing fluid secretion, amino acid absorption and sensory transduction (Harvey and Wieczorek, 1997). It has already been known that baculovirus budded viruses (BVs) enter the cell via clathrin-mediated endocytosis (Long *et al.*, 2006). V-ATPase may play a role in regulating the pH value to promote the membrane fusion between virus and endosome (Kingsley *et al.*, 1999). This is a necessary step for the virus release into the cytosol.

An early work in our laboratory demonstrated that V-ATPase is located in goblet cell apical membranes of the silkworm (*Bombyx mori*) larval midgut epithelium (unpublished). We have previously reported the cloning of the subunit c gene from *B. mori* larval midgut (Lü *et al.*, 2007), which is highly homologous to c subunits from

other 15 species. The V-ATPase c subunit from *B. mori* contains four membrane-spanning helices and is strongly expressed in Malpighian tubules, not in fat body. In the present study, we analyzed the 5' promoter region and 3'-UTR (untranslated region) of the c subunit gene, the sublocalization of the c subunit in BmN cells and the expression patterns in different developmental stages. Besides, the transcription expression levels of the V-ATPase c subunit gene in *Bombyx mori* nucleopolyhedrovirus (BmNPV)-infected strains NB (resistant to BmNPV) and 306 (susceptible to BmNPV) were determined by real-time PCR.

Experimental

Insect, cell line, and virus

The *B. mori* strain C108 (standard strain of silkworm), highly susceptible silkworm strain 306, highly resistant silkworm strain NB, BmN cell line, and BmNPV T3 isolate were maintained in our laboratory.

Insect rearing, RNA isolation, and cDNA synthesis

All larvae from three silkworm strains (C108, 306, and NB) were reared with fresh mulberry leaves at 27 °C under a 12 h light/12 h dark photoperiod. The egg, 1st to 5th instar larvae, pupa and imago from *B. mori* C108 were collected and the midguts from C108 larvae at day 5 of the 5th instar were dissected. The larvae of 306 and NB were reared up to the 5th instar. Each newly molted 5th instar larva was orally inoculated 10^6 viruses (Obs, 5 μl, enough to get 100% infection in the susceptible 306 strain) using a pipette. The central regions of midguts from these larvae were dissected 0, 6, 12, 24, 48, 72 and 96 h post inoculation (hpi).

All collected larvae and dissected midguts were immediately frozen in liquid nitrogen. Total RNA was extracted from frozen samples with RNeasy mini kit (QIAGEN, Valencia, CA, USA) according to the user manual and treated for 20 min at 37 °C with RNase-free DNase I (TaKaRa, Dalian, China). Finally, the total RNA was inspected with Genespec III (Naka Instruments Co., Ltd., Tokyo, Japan). The cDNAs were generated from these RNA using M-MLV RTase (Promega, Madison, USA) and an oligo-DT primer following the manufacturer's instructions.

5'-Rapid amplification of cDNA ends (5'-RACE)

5'-RACE was performed using SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Gene-specific primers of EVc-R, 5'-ggatccATGGCTGAAAATAATC-3', and QVc-R, 5'-CACGCACGCCTGCATCTC-3', were designed based on the *B. mori* V-ATPase c subunit gene (GenBank accession no. EU082222). Briefly, the primers, UPM and primer EVc-R or QVc-R, respectively, were used for 5'-RACE under the following conditions: denauaturation at 94 °C for 3 min, 35 cycles at 94 °C for 30 s, at 68 °C for 30 s, at 72 °C for 1 min, and elongation at 72 °C for 7 min. The PCR products were examined by electrophoresis in 1% agarose gel following ethidium bromide staining and purified using a gel extraction kit (AxyGen, Taizhou, China), then ligated into pMD18-T vectors (TaKaRa) and transformed into *E. coli* TG1 strain. The plasmid was purified, and sequencing was performed using an automatic sequencer CEQ8000 (Beckman, Fullerton, USA).

Nucleotide sequences analysis

The 5'-RACE sequence was assembled with *B. mori* genomic sequence using the DNAstar software. Potential transcriptional factor binding sites in the 5' flanking region of the *B. mori* V-ATPase c subunit gene were analyzed by SIGNAL SCAN (http://thr.cit.nih.gov/molbio/signal), TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html), and AliBata 2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). The first 250 nucleotides of 3'-UTR were analyzed using the RNADraw software to predict the secondary structure.

Expression of B. mori V-ATPase c subunit mRNA in different developmental stages

PCR was performed on cDNAs from the egg, 1st to 5th instar larvae, pupa and imago from C108 using the following primers for the *B. mori* V-ATPase c subunit gene: QVc-F, 5'-CGGCGTCTGCTATCATCTTCA-3', and QVc-R, 5'-CACGCACGCCTGCATCTC-3'. As an internal control for adjustment of template RNA quantity, a 284-bp fragment of *Bm-actin A3* (GenBank accession no. X04507) was amplified in parallel to each sample using the following primers: Bm-actin-F,
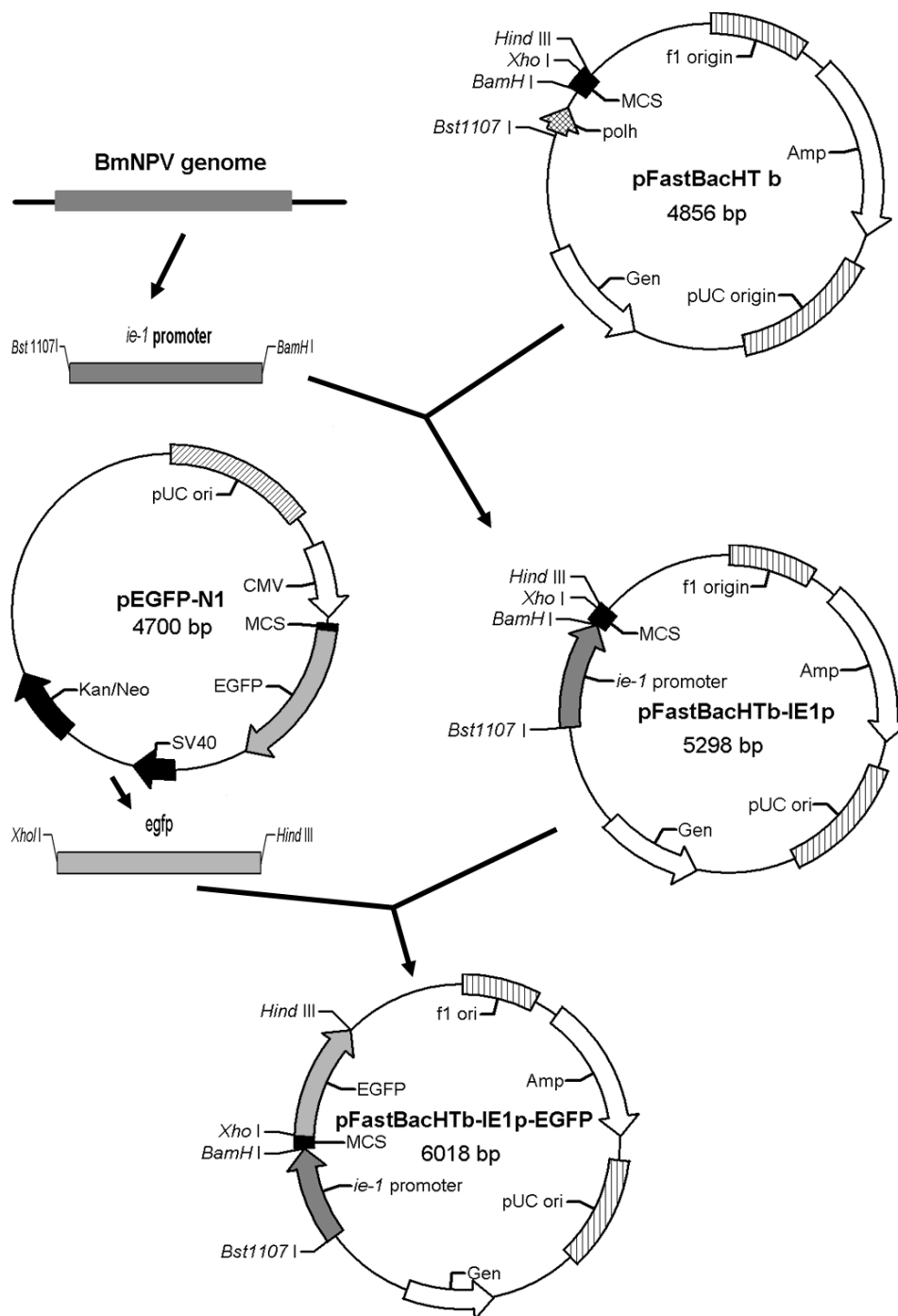
Fig. 1. Construction of the transient expression vector pFastBacHTb-IE1p-EGFP.

5'-GCGCGGCTACTCGTTCACTACC-3', and Bm-actin-R, 5'-GGATGTCCACGTCGCACT-TCA-3'. The PCR reactions were carried out for 40 amplification cycles (94 ºC/60 s, 58 ºC/45 s, and 72 ºC/45 s) in a GeneAmp 2400 System thermocycler (Applied Biosystems, Foster City, CA, USA). The PCR products were electrophoresed on 1% agarose gel stained with ethidium bromide.

Construction of transient expression vectors of V-ATPase c subunit

The *polh* promoter of pFastBacHT b (Gibco BRL, Carlsbad, USA) was replaced by the *ie-1* promoter, and amplified from BmNPV T3 genomic DNA using the primers IE1-F and IE1-R. The *egfp* was amplified from plasmid of pEGFP-N1 with the primers EGFP-F and EGFP-R, and was inserted in *Xho* I/*Hind* III sites of the above vector to generate pFastBacHTb-IE1p-EGFP (Fig. 1). The transient vector pFastBacHTb-IE1p-EGFP was identified by digestion with *Bst1107* I and *Bam*H I, *XhoI* I, and *Hind* III, respectively. We also designed a pair of primers, HTb-F and HTb-R, to identify the pFastBacHTb-IE1p-EGFP vector according to the sequence of the pFast-BacHT b plasmid. The full length V-ATPase c subunit cDNA was amplified from cDNA of *B. mori* midgut with the primers EVc-F and EVc-R, and was subcloned to the *Bam*H I/*Xho* I sites of pFastBacHTb-IE1p-EGFP vector without stop codon to construct pFastBacHTb-IE1p-Vc-EGFP. All primer sequences are listed in Table I.

Subcellular localization of V-ATPase c subunit in BmN cells

BmN cells were transformed with pFastBacHTb-IE1p-Vc-EGFP DNA using a lipofectin-mediated transformation method (Invitrogen). The vector of pFastBacHTb-IE1p-EGFP was used for mock transformation. Cells were examined under a fluorescence microscope (Leica, Wetzlar, Germany).

Real-time PCR analysis of V-ATPase c subunit mRNA in BmNPV-infected 306 and NB larvae

The cDNAs from larvae midguts of the BmNPV-infected 5th instar strains 306 and NB were used as templates. A 305-bp region in the *B. mori* V-ATPase c subunit gene was used to design the forward primer QVc-F, 5'-CGGCGTCTGCTAT-CATCTTCA-3', and the reverse primer QVc-R, 5'-CACGCACGCCTGCATCTC-3'. The gene of the *B. mori* translation initiation factor 3 subunit 4 (GenBank accession no. DQ443289) (TIF-3) was used as an internal control using the primers TIF-3-F, 5'-AGATGACGGGGAGCTTGATGGT-3', and TIF-3-R, 5'-GAGGGCGGAATGTACTT-GTTGC-3'. The real-time PCR was conducted on an Mx3000P instrument (Stratagene, San Diego, CA, USA) using SYBR *Premix Ex Taq* kit (TaKaRa) following the protocol in triplicate. Each amplification was performed using 25 μl of reaction mixture under the following conditions: initial denuaturation at 95 ºC for 2 min, followed by 40 cycles at 95 ºC for 30 s, 58 ºC for 30 s, 72 ºC for 20 s, and finally 72 ºC for 5 min. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The expression levels were normalized using the threshold cycle (C_t) value obtained for the *TIF-3* amplifications run in the same template, and the relative expression level was analyzed with the $2^{-\Delta\Delta Ct}$ method (Wong and Medrano, 2005).

Table I. Primers used in the construction of transient expression vectors.

Target gene	Accession no.	Primers (forward, reverse)[a]
BmNPV T3 *ie-1* promoter	L33180	IE1-F: 5'-gtatacGATTTGCAGTTCGGGAC-3'
		IE1-R: 5'-ggatccAGTCGTTTGGTTGTTCA-3'
egfp	U55762	EGFP-F: 5'-ctcgagATGGTGAGCAAGGGCG-3'
		EGFP-R: 5'-aagcttTTACTTGTACAGCTCGTC-3'
B. mori V-ATPase c subunit	EU082222	EVc-F: 5'-ggatccATGGCTGAAAATAATC-3'
		EVc-R: 5'-ctcgagTTTTGTGTACAGGTAGA-3'
pFastBacHTb		HTb-F: 5'-CGGATGAAGTGGTTCGCATC-3'
(sequence primers)		HTb-R: 5'-TCAGGTTCAGGGGGAGGTGT-3'

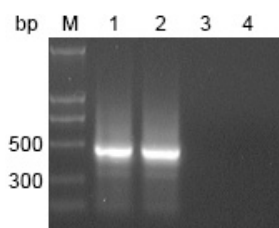[a] The restriction sites are underlined.

Fig. 2. The second PCR of 5'-RACE of the *B. mori* V-ATPase c subunit gene. M, DNA ladder; lanes 1 and 2, PCR product; lane 3, amplification only with UPM; lane 4, amplification only with primer QVc-R.

Fig. 3. Predicted secondary structure of the first 250 nt of the 3'-UTR of the *B. mori* V-ATPase c subunit gene.

Results

Sequence analysis of B. mori V-ATPase c subunit gene

We previously obtained a gene through the *in silico* cloning method encoding the V-ATPase c subunit from *B. mori* (GenBank accession no. EU082222). To determine the transcription initiation site, 5'-RACE was performed. A PCR fragment of about 500 bp in length was obtained (Fig. 2). We cloned this fragment to the pMD18-T vector and sequenced it. All the sequence was assembled with the *B. mori* genomic sequence, a 751-bp promoter sequence in the 5'-flanking region of the *B. mori* V-ATPase c subunit gene (readers may obtain the sequence information by contacting the senior author directly), was finally identified, which contains 166 bp 5'-UTR. The transcription initiation site was determined by 5'-RACE, it was 166 bp upstream of the start codon. The promoter region contained the putative binding sites for several important transcription factors, including the GATA-1 box (-51 to 59, -89 to -98), zeste binding motif (-61 to -67, -513 to -517, -669 to -674), activation protein-1 (AP-1, -463 to -471), TBP (TATA box protein) binding motif (-337 to -343, -361 to -367, -632 to -639). Moreover, a relatively long 1279-bp 3'-UTR of the *B. mori* V-ATPase c subunit gene was also determined. The analysis result of the first 250 nucleotides of 3'-UTR is shown in Fig. 3, which represents a loop-stem structure.

Expression of B. mori V-ATPase c subunit mRNA in different developmental stages

RT-PCR was carried out on cDNAs from developmental stages of *B. mori* C108. The result revealed (Fig. 4) that the *B. mori* V-ATPase c subu-
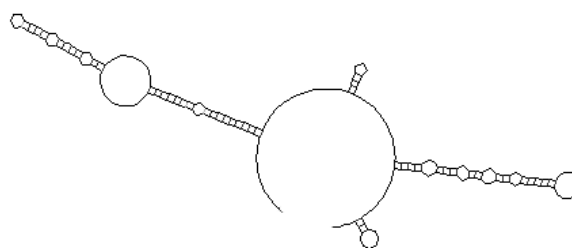
nit gene expressed in the whole developmental stages of *B. mori*.

Subcellular localization of B. mori V-ATPase c subunit in BmN cells

The sequencing results (data not shown) revealed that the transient vector pFastBacHTb-IE1p-EGFP was successfully constructed. The intracellular localization of the *B. mori* V-ATPase c subunit was performed by the transient expression vector of pFastBacHTb-IE1p-Vc-EGFP, which is able to express the V-ATPase c subunit and EGFP fusion protein in BmN cells after transformation. Green fluorescence was determined under a fluorescence microscope. The result (Fig. 5) revealed that the *B. mori* V-ATPase c subunit localized primarily in the cell membrane and in some specific regions of cytoplasm in BmN cells, which can not be recognized.

Real-time PCR analysis of V-ATPase c subunit mRNA in BmNPV-infected 306 and NB larvae

The amplification specificities for the V-ATPase c subunit and TIF-3 genes were determined by analyzing the melting curves. Only one peak was present in the melting curves for two genes above, indicating that the amplifications were spe-
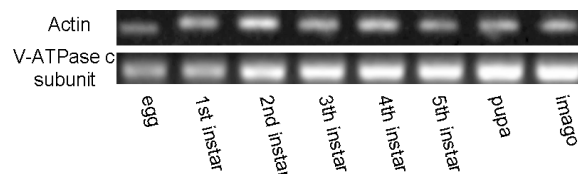

Fig. 4. Expression of the *B. mori* V-ATPase c subunit mRNA in different developmental stages.

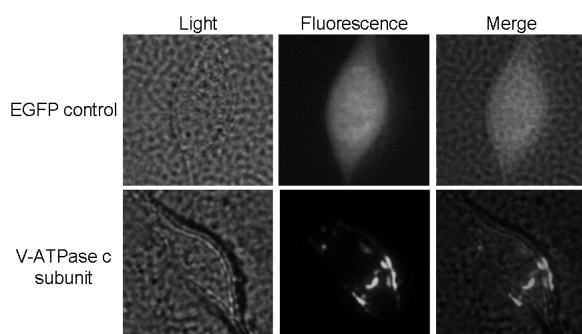| Light	Fluorescence	Merge

EGFP control

V-ATPase c subunit

Fig. 5. Subcellular localization of the *B. mori* V-ATPase c subunit in BmN cells. From left to right: bright field, fluorescence (green), and merged images.

cific (data not shown). The transcript expression of the V-ATPase c subunit in midguts of the *B. mori* strains 306 and NB at different times after inoculation with BmNPV is shown in Fig. 6. The transcript expression level was slightly lower in the BmNPV-infected susceptible strain 306 larvae than in the BmNPV-infected resistant strain NB larvae just after virus inoculation (0 hpi). Following BmNPV infection, the expression significantly increased in resistant larvae at 24 hpi compared to the expression at 0 hpi. It also increased in susceptible larvae, but this was not significant. Then the expression deceased gradually in both susceptible and resistant larvae. The transcription expression significantly decreased at 72 to 96 hpi in susceptible larvae, which may be due to starvation after BmNPV infection. However, it

increased at 72 hpi in resistant larvae and maintained a constant level. The increase of V-ATPase c subunit mRNA in midguts of resistant larvae at 24 and 72 hpi indicated that V-ATPase may be related to the immune response against BmNPV infection.

Discussion

Silkworm is an economically important insect and is regarded as a model insect of Lepidoptera. Studies on the certain related genes of *B. mori* have attracted more attention. Previously we identified a gene encoding the *B. mori* V-ATPase c subunit using the *in silico* cloning method. The ORF and tissue distribution of the V-ATPase c subunit gene was analyzed. It is highly homologous with other species and expressed strongly in Malpighian tubules, not in fat body (Lü *et al.*, 2007).

In the present study, we found that the *B. mori* V-ATPase c subunit gene contained a 751-bp promoter sequence in the 5' flanking region. Several putative binding sites for GATA-1 box (2 sites), zeste (3 sites), TBP (3 sites), and AP-1 were present within the promoter region (Fig. 2), suggesting that the transcription of the *B. mori* V-ATPase c subunit gene may be regulated by multiple transcription factors. Zeste is in fact a DNA-binding protein that could interact simultaneously with two chromosomes suggesting that the interaction affects the regulatory properties of the target gene (Pirrotta *et al.*, 1988). The zeste
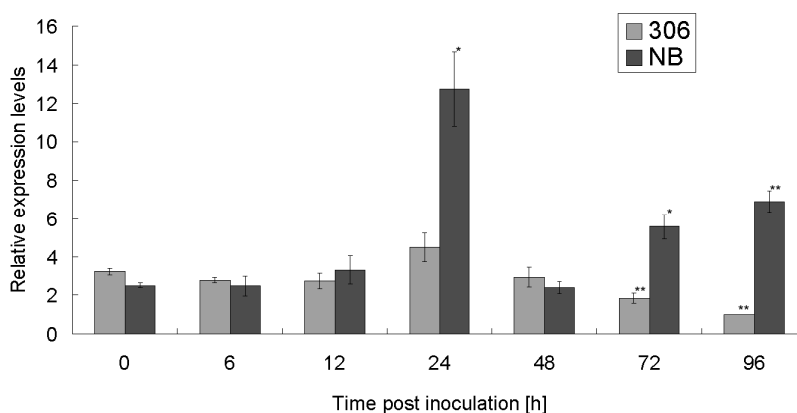
Fig. 6. Expression of V-ATPase c subunit mRNA in BmNPV-infected *B. mori* midguts at different times. TIF-3 was used as internal control. Data are expressed as the mean ± SD in three experiments. The significant differences to the expression levels at 0 hpi ($p < 0.05$ and < 0.01) are denoted with * and **, respectively.

binding sites are found in the immediate vicinity of the promoter as well as in the vicinity of distant regulatory elements and can form an intragenic loop by zeste-zeste interactions to facilitate the action of distant enhancers on the promoter (Benson and Pirrotta, 1988). The TATA box serves as the binding site for an RNA polymerase II-associated transcription factor known as TFIID factor (Nakajima *et al.*, 1988; Parker and Topol, 1984) which functions at an early step during the assembly of transcription initiation complexes at RNA polymerase II promoters (Buratowski *et al.*, 1989; Van Dyke *et al.*, 1988). Some studies demonstrated that transposase could bind to the TBP binding motifs which are near the TATA box to repress the transcription by interfering with the TFIID-TATA box interaction, thereby blocking the assembly of an RNA polymerase II transcription complex (Kaufman and Rio, 1991).

Translational control of protein synthesis by specific sequences of the corresponding mRNAs in the 3'-UTRs is a feature of many transcript-specific regulatory mechanisms (Mazumder *et al.*, 2003). The RNA secondary structure prediction of the first 250 bases in *B. mori* V-ATPase c subunit mRNA 3'-UTR (Fig. 3) revealed a loop-stem structure, which might be a potential protein-binding domain required for translational repression. So it may be a regulatory manner for transcription of the *B. mori* V-ATPase c subunit gene, although its function, if any, is unknown.

The RT-PCR result (Fig. 4) revealed that the V-ATPase c subunit gene expresses in the whole *B. mori* developmental stages and their expression levels are generally higher than the expression levels of the *B. mori* actin A3 gene, which is in accordance with the conclusion that V-ATPase genes are considered housekeeping genes (Finbow and Harrison, 1997).

Identifying the subcellular localization of protein is particularly helpful in the functional annotation of gene products. In our earlier work, the *B. mori* V-ATPase c subunit gene was found to be expressed in the ovary (Lü *et al.*, 2007). We used the transient vector to examine the subcellular localization of the *B. mori* V-ATPase c subunit in BmN cells. The result (Fig. 6) showed that the V-ATPase c subunit is localized not only in the cell membrane but also in some regions in the cytoplasm, but we can not recognize its accurate location. It seems to be located in endomembranes

of BmN cells as reported previously (Harvey and Nelson, 1992).

BmNPV is considered to be a major silkworm pathogen causing significant economic loss in sericulture. NB is one of the silkworm stains that is highly resistant to BmNPV and was first identified in our laboratory (Chen *et al.*, 1991), while the silkworm strain 306 is highly susceptible. Some results from our laboratory revealed that BmNPV invades the midguts of both *B. mori* resistant and susceptible strains, but viral proliferation is inhibited in the resistant strain (unpublished). It has been clearly that BmNPV exclusively infects columnar cells of *B. mori* larval midgut epithelial cells and replicates in columnar cells within 24 hpi; then virus infection advances rapidly (Rahman and Gopinathan, 2004). Therefore, larval midgut cells might be a key place that is closely linked to the resistance to BmNPV. The real-time PCR results (Fig. 6) showed that the transcript expression of the V-ATPase c subunit in the midguts of the silkworm resistant strain NB would be up-regulated significantly at 24 and 72 hpi, indicating that V-ATPase may be involved in the immune response against BmNPV infection. V-ATPase is the most important transporter to regulate the pH value within relatively narrow limits in eukaryotes. Some processes in organisms have been identified to require an acidic environment generated by V-ATPase (Forgac, 2007). The degradation of viral macromolecules might be recognized and sorted to lysosomes by antiviral immune responses of the resistant strain after BmNPV infection in the resistant silkworm strain, because acidification of late endosomes by V-ATPase is important in intracellular trafficking of newly synthesized lysosomal enzymes from the Golgi apparatus to lysosomes (Forgac, 2007), and the processing of lysosomal enzymes through hydrolyses also requires optimal acidic pH (Hasilik, 1992; Pisoni and Thoene, 1991). The transcription regulation of the V-ATPase c subunit gene should be dependent on the potential transcriptional binding sites in the promoter region and the loop-stem structure in 3'-UTR analyzed above. Nevertheless, the detailed regulatory mechanism of V-ATPase c subunit mRNA expression in response to the treatment with BmNPV in resistant silkworm larvae, and the question if V-ATPase is involved in the *B. mori* immune response to BmNPV infection or not require further studies.

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