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Promoter Analysis of *Bombyx mori* Nucleopolyhedrovirus *Ubiquitin* Gene

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The aim of this study was to analyze the characteristics of *Bombyx mori* nucleopolyhedrovirus (BmNPV) *ubiquitin* gene promoter and the effects of conserved motifs, such as TAAG, TATA, and CAAT, along with baculovirus enhancer homologous region 3 (*hr3*), on promoter activity. *Ubiquitin* gene of BmNPV was expressed during the late phase of virus infection. In the presence of viral factors, significant reduction of promoter activity was observed by deletion of -382 to -124 bp upstream of ATG. The fragment between -187 and -383 bp upstream of ATG, including distal TAAG, CAAT motif, and TATA box, could also drive expression of the reporter gene. The mutation of *cis*-elements TATA boxes and TAAG motifs significantly decreased the promoter's activity, while CAAT mutations enhanced promoter activity by 2- or 3-fold, as compared with the native promoter. In the presence of BmNPV, *hr3*, both located downstream of the reporter gene of the same vector, and separate vector, could significantly enhance transcription activity of *ubiquitin* promoter as compared to the control. We concluded that BmNPV *ubiquitin* gene might be regulated by dual sets of promoter elements, where TAAG and TATA box may positively regulate the expression of *ubiquitin*, while CAAT motif functions as a negative regulator. Viral factor(s) play an important role in the co-activation of *hr3* and promoter.

***Keywords*:** baculovirus, *ubiquitin* promoter, transient expression, mutation, enhancer *hr3*

Baculoviruses are double-stranded and enveloped DNA viruses, which mainly infect lepidopteran and some dipteran insects (Okano *et al*., 2006). Upon infection, they have a fixed life-cycle. The genes are expressed in a temporarily controlled and sequentially ordered fashion, which are classified as early, late, and very late phases based on their requirements for protein synthesis and viral DNA replication. Regulation of baculovirus gene expression generally takes place at transcriptional level (Lu *et al*., 1997). Most late and very late genes initiate transcription within or near the consensus (A/G/T) TAAG sequence, which is conserved in distantly related baculoviruses and appears to be a feature common to the baculoviridae family (Blissard and Rohrmann, 1990).

To date, some late and very late promoters of baculovirus have been studied in detail by mutation or functional analysis. For example, it has been reported that mutation in the TAAG site resulted in dramatic reduction in steady state levels of late transcripts (Morris and Miller, 1994; Todd *et al*., 1995). Some late genes contain more than one TAAG sequence, while others have both early and late promoter motifs. However, it is still unknown whether the activity of one promoter can influence the function and regulation of adjacent promoters (Blissard and Rohrmann, 1989; Mans and Knebel-Morsdorf, 1999; Kumar *et al*., 2001).

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Most sequenced baculoviruses encode ubiquitin proteins other than *Neodiprion lecontei* NPV and *Neodiprion sertifer* NPV (Garcia-Maruniak *et al*., 2004; Lauzon *et al*., 2004). Baculovirus ubiquitin is considered not essential to viral DNA replication, but does participate in budding of virion and virus growth in certain conditions (Reilly and Guarino, 1996). It may also be competitively combined with ubiquitin-activating (E1) enzymes, preventing the degradation of useful proteins required for virus infection (Haas *et al*., 1996). BmNPV encodes a protein of 77 amino acids with an identity of 76% to the eukaryotic ubiquitin protein and 100% to AcMNPV ubiquitin.

In this study, transcription and promoter analyses of BmNPV *ubiquitin* gene were carried out to better understand the regulation of late gene expression. A series of plasmids, containing truncated or site-directed mutated region, upstream of *ubiquitin* ORF, were constructed and sequenced, respectively, to investigate the transcriptional regulation of *ubiquitin* promoter in insect cells through transient expression assays. The investigation of virus ubiquitin will improve the recognition of viral evolution and infection, and help better understand interactions between virus and host. Furthermore, the effects of BmNPV *hr3* on the promoter activity of BmNPV *ubiquitin* were also investigated.

Materials and Methods

Materials

The *Bombyx mori* cell line (*Bm*-N) and larva JY1, *E. coli*

DH10B, and pGEM-3Z vector were maintained in the lab. The wild-type baculovirus strain, BmNPV-ZJ8, plasmid pUL220 (Lei *et al*., 1993) containing an entire ORF of *luciferase* gene, and plasmid pSK-hr3 (Zhang *et al*., 1995) containing BmNPV homologous region-3 were kindly provided by Prof. Wu Xiangfu (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences). Enzymes, reagents, and chemicals used throughout this work were obtained from Promega (USA), unless otherwise stated. All chemicals were of analytical grade. Beckman LS-600TA liquid scintillation spectrometer was used to measure luciferase activities.

Virus and cell culture

The mock- or BmNPV-infected *Bm*-N cells were propagated with TC-100 medium (GIBCO) containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Invitrogen) at 27°C as described by Summers and Smith (1987).

Construction of functional plasmid

To examine the effects of deletions or mutations on the *ubiquitin* gene promoter in transient expression assay, various reporter plasmids were generated. All plasmids were created by inserting the promoter fragment into the *Bam*HI and *Eco*RI sites of plasmid pGEM-3Z.

Polymerase chain reaction (PCR) amplification of BmNPV *ubiquitin* promoter fragments was performed with primers shown in Table 1A, using the denatured genomic DNA of BmNPV as template under normal conditions. Five clones were constructed, each containing a promoter fragment of 595 bp, 382 bp, 124 bp, 26 bp, and 197 bp, respectively. All the promoter regions contained late promoter motif TAAG. The 197 bp fragment including distal TAAG located between -187 and -383 bp upstream of ATG.

The pBm595ubiluc plasmid DNA was used as template to amplify the serial promoter fragment containing mutation of *cis*-activating elements using high fidelity *pfu* DNA

Table 1. Oligonucleotides used for plasmid construction in experiments

(A) Primers used in promoter deletion analysis. To study the effects of deletions on *ubiquitin* promoter activity of BmNPV, five plasmids were created using PCR analysis. Primers Pubi F1, Pubi F2, Pubi F3, and Pubi R were used to amplify the promoter fragment length 595, 382, and 124 bp, respectively. Primers Pubi-2nd F and Pubi-2nd R were used to amplify a 197 bp fragment containing distal TAAG, CAAT motif, and TATA box. The *Eco*RI and *Bam*HI sites in these primers were in italic and underlined. The 26 bp promoter, containing proximal TAAG motif, and TATA box, was annealed using Pcore F and Pcore R, and the cohesive end of *Eco*RI and *Bam*HI sites were in italic and underlined.

Name	Sequence (5' → 3')	Plasmid name
Pubi F1	TG*GAATTC*GATCGGATAGCGA	pBm595ubi
Pubi F2	CG*GAATTC*GTGTAACAAAGTCG	pBm382ubi
Pubi F3	TG*GAATTC*AATTGCCGTTGAAGGGAAAT	pBm124ubi
Pubi R	TTT*GGATCC*TTACACTATTACTTATAAATGACAACGG	
Pubi-2nd F	CG*GAATTC*GTGTAACAAAGTCGACAGCG	pBm197ubi
Pubi-2nd R	GAT*GGATCC*AAACGACTTAATATAACACC	
Pcore F	*AATTC*TTATAAGTAATAGTGTAAAAG	pBm26ubi
Pcore R	*GATCC*TTTTACACTATTACTTATAAG	

(B) Primers used in promoter mutation analysis. The *cis*-activating elements, TATA box, TAAG, and CAAT motifs were mutated to CGTA, TCCG, and CGGT, respectively, by PCR analysis. Primer 17 MR and 19 MR contained the proximal TAAG and TATA box mutation intermediately. The mutations of CAAT and distal TAAG, TATA were introduced in the center of every reverse complemented primer. Pubi F1 and every mutated reverse primer were used to amplify the distal fragment. Every mutated forward primer and Pubi R primer was used to amplify the proximal fragment with *Pfu* DNA polymerase. After recovery from gel, the distal and proximal fragments were allowed to anneal, after which the primers Pubi F1 and Pubi R were employed to amplify the full promoter region with desired mutation. The mutated nucleotides are in italics and underlined. All mutated sequences were confirmed by sequencing.

Name	Sequence (5' → 3')	Plasmid name
17M R	TTTGGATCCTTACACTATTACT*CG*TAAATGACAACGG	p17Mubi
195M F	GTGGCCCGGTGTTAT*CC*GTCGTTTGAAAGC	p195Mubi
195M R	GCTTTCAAACGAC*GG*ATAACACCGGGCCAC	
19M R	TTTGGATCCTTACACTATTACTTA*CG*AATGACAACGG	p19Mubi
269M F	CGTCAGCCTC*CG*TAATCTCGG	p269Mubi
269M R	CCGAGATTA*CG*GAGGCTGACG	
71M F	CGCCTG TGCAC*GG*TTCCACCATCGGTG	p71Mubi
71M R	CACCGATGGTGGAA*CC*GTGCACAGGCG	
359M F	GACAGCGAAAC*GG*TTTATCGTTTTC	p359Mubi
359M R	GAAAACGATAAA*CC*GTTTCGCTGTC	

polymerase. The mutation site was designed in the middle of two corresponding primers that were complementarily reversed. Pubi F1 and the mutated reverse primer were used to amplify the fragment containing the mutated site in the 3' end, while the mutated forward primer and Pubi R were used to amplify the fragment containing the mutated site in the 5' end. An annealed two-corresponding fragment containing the same mutated site was subsequently added to Pubi F1 and Pubi R in order to amplify the promoter fragment, which contained a site-directed mutation of a certain motif, such as TAAG, CAAT or TATA box. In this experiment, TATA boxes and TAAG motifs were mutated to CGTA and TCCG, respectively, while CAAT motifs were mutated to CGGT. Primers used in promoter mutation analysis are shown in Table 1B.

All plasmids created were confirmed by sequencing. The entire ORF of *luciferase* gene fragment was inserted into the *Bam*HI site of these recombinant plasmids, under the control of *ubiquitin* promoter in the right orientation.

To examine the effects of BmNPV *hr3* on transcription of *ubiquitin* promoter, plasmid pBm595ubiluc-hr3 was constructed by inserting *hr3*, isolated from plasmid pSK-hr3, downstream of *luciferase* gene in the pBm595ubiluc plasmid.

RNA isolation and Northern blot analysis

Cells were inoculated into 15 cm^2 flasks at a quantity of about 1×10^6 cells/ml and cultured over night. Subsequently, medium was removed and replaced with 1 ml virus inoculum at a multiplicity of infection (m.o.i.) of 10 and incubated for 1 h. The virus inoculum was replaced with 3 ml of conditioned medium. Counting of hours post-infection (h.p.i.) started the moment when virus inoculum was added. At 0, 2, 4, 8, 12, 24, 48, and 72 h.p.i., total RNA was isolated from cells in TRIzol (Invitrogen) following the manufacturer's instructions. Mock-infected cells were used as control.

Primers for PCR amplification of BmNPV *ubiquitin* gene were designed based on BmNPV genomic nucleotide sequence (GenBank accession no. NC_001962): ubi-F (forward); 5'-ATGCAAATATTCATCAAAACATTGACGGGC-3' and ubi-R (reverse); 5'-TTAATACCCTCCTCGTAATCGTA ACAC-3'. The PCR product was purified on a 1% low gelling temperature Seaplaque agarose gel and labeled with [α-^{32}P] dCTP, according to the DNA labeling system of the manufacturer (TaKaRa). The labeled products were precipitated in ethanol absolute, and resuspended in 0.1× TE buffer.

The 10 μg total RNA samples were denatured in a formaldehyde/formamide buffer and electrophoresed on a 1.2% agarose, 2.2 M formaldehyde gel (Brown and Mackey, 1997). After that, the gels were stained with ethidium bromide to confirm equivalent sample quantities and washed with RNA-free water to remove the formaldehyde. RNA was subsequently transferred to a charged nylon membrane. ULTRAhyb buffer was used for the hybridizations following the manufacturer.

Transfection and transient expression assay

For transfections, TC-100 medium was substituted with 1.5 ml of serum-free medium and cells washed 3 times. The cells ($0.5\sim1\times10^6$ cells) were transfected with 100 μl of transfection solution containing 5 μl of lipofectin, 1 μg reporter plasmid DNA, and 0.1 μg of standard plasmid (pRL-CMV, Promega) for internal control. Cells were incubated at 27°C for 3 h and infected with wild-type BmNPV (m.o.i.=1.0) for 1 h. Supernatant was decanted and 3 ml of conditioned medium was added to the cells. Mock or pUL220 transfected cells were used as blanks. Each experiment was trireplicated. Each transfection consists of at least three separate parallel experiments.

Each fifth instar silkworm larva was injected with 20 μl transfection solution into the larval haemolymph. The solution composed of 6 μl lipofectin, 1 μg reporter plasmid DNA and 0.1 μg standard plasmid.

After 3 h, the silkworm larva was infected with wild type BmNPV (5.0×10^5 PFU). Each treatment consisted of at least three separated groups (one group composing 5 larvae with approximately the same weight) and was repeated three times.

The extracts were prepared with a dual luciferase assay kit (Promega). The samples were centrifuged at 10,000 rpm for 5 min at 4°C. The harvested cells were washed three times by resuspending in phosphate buffered saline (PBS, pH 8.0), then centrifuged at 9,000 rpm for 5 min at 4°C. The cells were lysed by passive lysis buffer and a single freeze-thawing cycle. Before measurement, the lysate was centrifuged at 4°C to remove cell debris. The firefly luciferase or *Renilla* luciferase activity were measured and determined in the supernatants. The Bradford methodology was followed to measure the amount of protein in the supernatants (Bradford, 1976).

Results

Cloning and sequence analysis of BmNPV *ubiquitin* promoter

Primers were designed based on reported complete nucleotide sequence of BmNPV T3 strain, and the ATG of *ubiquitin*

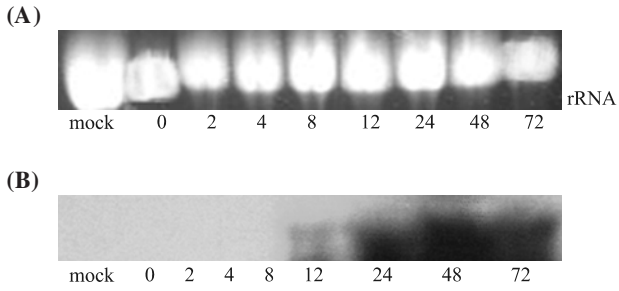

Fig. 1. Northern blot analysis was performed using total RNA extracted from BmNPV-infected or mock-infected cells. (A) Electrophoresis of 10 μg RNA from BmNPV infected *Bm*-N cells at different stages. Cells were infected at an m.o.i. of 10 and incubated at 27°C. The time of virus infection was defined as zero h.p.i.. Total RNA was isolated from wild-type BmNPV-infected *Bm*-N cells at 0, 2, 4, 8, 12, 24, 48, and 72 h.p.i.. Cells were homogenized in TRIzol and RNA was isolated from uninfected cells as control. (B) Northern blot analysis of *ubiquitin* gene from BmNPV. Transcription of BmNPV *ubiquitin* gene was first detected at about 12 h.p.i..

gene was changed to ATT by site-directed mutagenesis in the reverse primer, in order to construct non-fused reporter plasmids. Using BmNPV-ZJ8 genome DNA as template, fragments upstream ATG of *ubiquitin* ORF were amplified by PCR. These products were purified, enzyme digested, and inserted into the *Bam*HI and *Eco*RI sites of pGEM-3Z, respectively.

Sequence analysis showed that the amplified *ubiquitin* promoter region from BmNPV-ZJ8 has an identity of 98.9% and 92.8% with BmNPV T3 strain and AcMNPV C6 strain, respectively, while the conserved motifs were identical. In the BmNPV *ubiquitin* promoter region, there are two TAAG motifs at -17 and -195 bp upstream of ATG. The TATA box was located at -19 and -269 bp, while the CAAT motif was located at -71 and -359 bp upstream of ATG.

Northern blot analysis

At 27°C, *Bm*-N cells were seeded in 15 cm^2 flask at a quantity of about 1×10^6 cells/ml and cultured over night. The cells were infected with wild type BmNPV. Total cellular RNA was extracted at 0, 2, 4, 8, 12, 24, 48, and 72 h.p.i. for Northern blot analysis. Transcriptional product of *ubiquitin* gene was first detected at about 12 h.p.i. and increased thereafter. The result implied that BmNPV *ubiquitin* gene was a late gene in the virus infection cycle, as shown in Fig. 1.

Transcription of baculovirus ubiquitin promoter by trans-activation of viral factor(s)

In this study, cells were transfected with the plasmid pBm595-ubiluc containing the promoter sequence of 595 bp in length

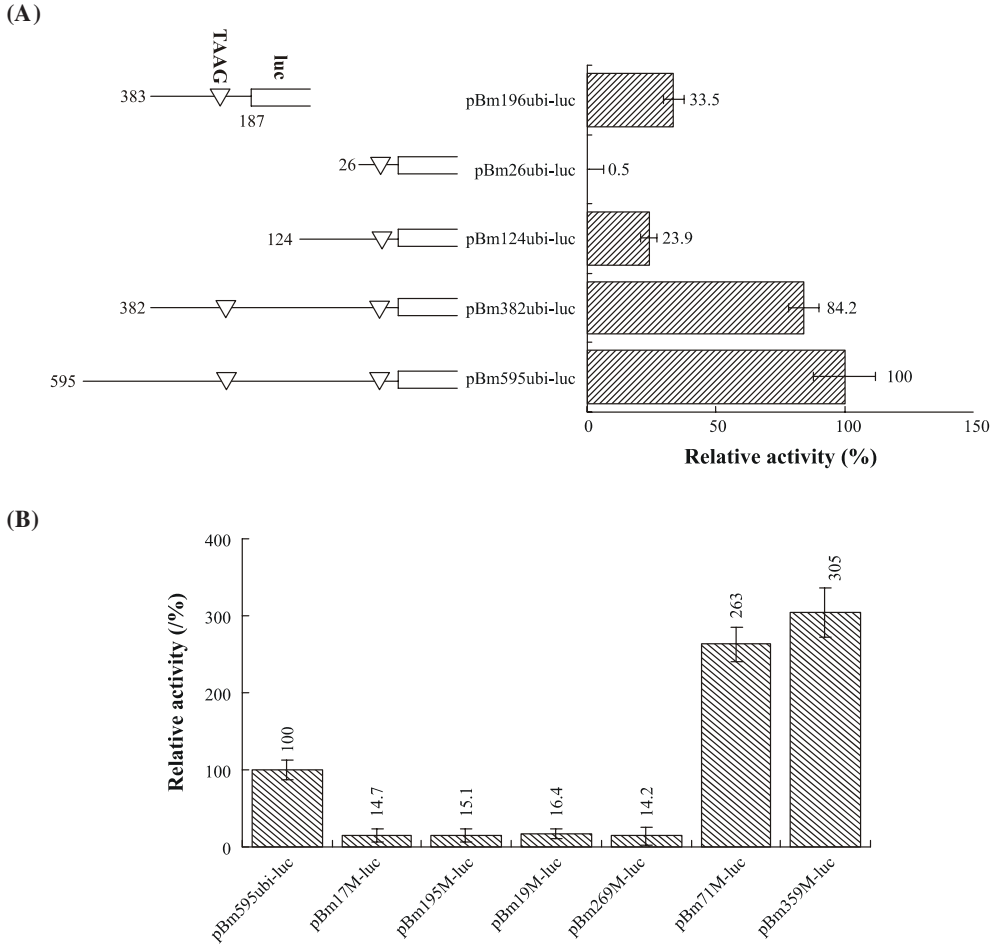

Fig. 2. Effect of deletions or mutations on promoter activity BmNPV infected *Bm*-N cells were transfected with 1 μg of each reporter plasmid DNA and 0.1 μg internal control plasmid, respectively. Cells were collected at 48 h post transfection. (A) Comparison of luciferase activities driven by deleted *ubiquitin* promoters. The BmNPV genome DNA was used as template in the PCR to amplify promoter fragment with length of 595, 382, 124, 26, and 197 bp, respectively. Five reporter plasmids were constructed, in which *luciferase* gene was subcloned under the control of the promoter. Length of promoter fragment was contained in the plasmid name. Transcription activity of each promoter was presented as stimulating index over the full-length promoter, which was arbitrarily set as 100. Each reaction contained 10 μg of protein extracted from the transfected cells. (B) Functional mutation analysis of *ubiquitin* promoter. pBm595ubiluc plasmid DNA was used as template to amplify promoter fragment, containing mutations of *cis*-activating elements. *Bm*-N cells were transfected with reporter plasmids and pRL-CMV, containing *Renilla luciferase* gene. Normalized transcription activity of each promoter was presented as stimulating index over the native promoter, which was arbitrarily set as 100. Each reaction contained 10 μg of protein extracted from the transfected cells.

and then infected with BmNPV. Analysis of transient expression assay showed that in transfected cells not infected with the virus, luciferase activity was similar to that of the mock infection assay in *Bm*-N cells. However, in *Bm*-N cells infected with the virus, luciferase activity was detected at about 13,378 cpm. These results strongly suggest that *luciferase* gene driven by *ubiquitin* promoter was not expressed without virus factor(s).

Effect of multifarious modification on the promoter activity

The modifications of the promoter region involved progressive deletions of the promoter fragment upstream of ATG and mutations of some conserved *cis*-activating elements in the promoter. The effects of these deletions on the *ubiquitin* promoter were determined by transient expression assay using the cell extracts obtained from transfected cells. As shown in Fig. 2A, in the presence of virus factors, deletion from -595 to -382 bp relative to ATG did not significantly reduce promoter activity compared to the full length 595 bp, where 84.2% promoter activity remained when the deletion occurred. However, the promoter activity was significantly reduced when deletion occurred at -382 to -124 bp upstream of ATG (only remained the 23.9% activity of full promoter). The fragment from -383 to -187 bp upstream of ATG, which contained another putative conserved motifs, could effectively drive the expression of luciferase in transient expression assay. Here, 33.5% promoter activity was observed. The 26 bp mini-fragment containing only TAAG motif and TATA box, from *ubiquitin* promoter region, had the basic promoter activity.

A series of recombinant plasmids containing a *luciferase* gene, driven by mutated *ubiquitin* promoter, were also constructed. In these plasmids, TATA box (at -19 bp and -269 bp), TAAG (at -17 bp and -195 bp) and CAAT (at -71 bp and -359 bp) motifs within the *ubiquitin* promoter region were mutated. Briefly, the TATA box, TAAG, and CAAT motifs were mutated to CGTA, TCCG, and CGGT, respectively. The mutations within TATA box and TAAG motif lead to a remarkable decrease in luciferase activity. However, mutations of CAAT to CGGT significantly enhanced luciferase activity, in comparison to its native form. The detailed results of *luciferase* driven by mutated promoter were shown in Fig. 2B.

BmNPV *hr3* enhanced transcription of ubiquitin promoter *in vivo*/*vitro*

Baculovirus homologous region (*hr*) functions as both enhancer and origin of viral DNA replication (Guarino and Summers, 1986). To examine the effects of BmNPV *hr3* on the transcriptional activity of *ubiquitin* promoter, pBm595ubiluc-hr3 was created by inserting a *hr3* fragment downstream of *luciferase* gene in the plasmid pBm595ubiluc. *Bm*-N cells were transfected with 1 μg pBm595ubiluc-hr3 plasmid, or co-transfected with 1 μg pBm595ubiluc and pSK-hr3, respectively. Each transfection contained 0.1 μg internal control plasmid, pRL-CMV. At 48 h post transfection (h.p.t.), the transfected cells were collected and the luciferase activity was assayed. Transient expression result showed that *hr3* could not increase the expression of reporter gene without virus infection.

In the presence of viral factors, both *hr3* located downstream of the reporter gene of the same vector. or in the separate plasmid, could greatly enhance promoter transcription. As shown in Table 2A, luciferase activity was 3,658 cpm in transfected *Bm*-N cells with pBm595ubiluc, while it increased to 226,444 cpm (approximately 62 fold enhancement) in cells transfected with pBm595ubiluc-hr3. A similar result was obtained when *Bm*-N cells were co-transfected with pBm595ubiluc and pSK-hr3, where luciferase activity enhanced to 49,560 cpm (14 fold increase).

Hr3 functions as enhancer in transfected silkworm larvae, while the *in vivo* transfection conditions greatly differ from that in transfected cells (Xiao *et al*., 2001). *Bombyx mori* larvae were transfected with 1 μg reporter plasmid pBm595-ubiluc-hr3 or co-transfected with pBm595ubiluc and pSK-hr3 followed by infection with BmNPV, each reaction containing 0.1 μg internal control plasmid pRL-CMV. The haemolymph was collected for the determination of luciferase activity at 48 h.p.t. (Table 2B). Upon BmNPV infection, *hr3* enhanced transcription of *ubiquitin* promoter about 422-fold in *cis*-linked, and 182-fold in *trans*-activation.

Discussion

Ubiquitin plays an important role in virus life cycles or virus-host interactions (Guarino, 1990). In this study, we described the transcriptional analyses of BmNPV *ubiquitin* promoter. Northern blot analysis revealed that transcription of BmNPV *ubiquitin* started at about 12 h.p.i., while promoter analysis showed that its transcription required the expression products of other viral gene(s), suggesting that BmNPV *ubiquitin* was a late gene.

Promoter recognization is considered as one of the key transcription processes involved in baculovirus gene expression. In a previous study (Lin *et al*., 2007) the region in AcMNPV ubiquitin promoter responding to viral factor(s) was located between -595 to -382 bp upstream of ATG. In this study, significant reduction in promoter activity was observed in mutants with deletions of -382 to -124 bp upstream of ATG as compared to the full-length promoter (595 bp) in cells infected with viruses, while the promoter activity was almost abolished with continuous deletions. This indicates that although ubiquitin from AcMNPV or BmNPV shared high identity, during evolution they formed different transcriptional regulation mechanisms to harmonize the regulation of host ubiquitin transcription signal.

In addition, a 197 bp fragment containing the distal conserved motifs, from -383 to -187 bp upstream of ATG, was found to drive the expression of *luciferase* gene. It was suggested that the two late transcript initiate sites in *ubiquitin* promoter and *cis*-acting elements responsive to viral factors were mainly located within the 5' region of promoter, consistent with the finding of two ubiquitin transcripts by Guarino (Guarino, 1990).

In the *ubiquitin* promoter region, the TAAG, CAAT motifs, and TATA box appeared twice. Transient expression assays indicated that mutation of both proximal and distal TAAG and TATA box resulted in significant reduction of *ubiquitin* activity. A main feature of all late and very late baculovirus

Table 2. Effect of *hr3* enhancer on transcriptional activity of BmNPV *ubiquitin* promoter *in vitro*/*vivo*. Luciferase activity is presented as cpm in 15 sec. Enhancing ability of BmNPV *hr3* is presented as stimulating folds over the corresponding plasmid pBm595ubiluc, which is arbitrarily set as 1.00. The *Renilla luciferase* normalizing system was introduced into each transfection. The results represent averages from three separately transfected cells at 48 h.p.t..

(A) Increased luciferase activity from BmNPV *ubiquitin* promoter by BmNPV *hr3 in vitro*. Transfection of pBm595ubiluc-hr3 or co-transfection of pBm595ubiluc and pSK-hr3 was compared with that of the pBm595ubiluc plasmid transfected *Bm*-N cells. At 3 h.p.t. cells were infected with BmNPV. Each reaction contained 5.0 μg of protein extracted from transfected *Bm*-N cells.

Plasmids	Virus infection	Luciferase activity (cpm)	Stimulating folds
pBm595ubiluc	-	20±17.89	/
pBm595ubiluc-hr3	-	48±27.72	/
pBm595ubiluc	+	3,658±241.1	1.00
pBm595ubiluc-hr3	+	226,444±19002.13	61.90
pBm595ubiluc+pSK-hr3	+	49,560±2854.47	13.55

(B) Increased luciferase activity from BmNPV *ubiquitin* promoter by BmNPV *hr3 in vivo*. Transfection of pBm595ubiluc-hr3 or co-transfection pBm595ubiluc and pSK-hr3 was compared with pBm595ubiluc in *Bombyx mori* larvae followed by BmNPV infection (5×10^5 PFU per larva). Each reaction contained 10 μg of protein extracted from transfected silkworm haemolymph

Plasmids	Luciferase activity (cpm)	Stimulating folds
pBm595ubiluc	16±16	1.00
pBm595ubiluc-hr3	6,751±328.1	421.94
pBm595ubiluc+pSK-hr3	2,904±214.3	181.50

gene transcripts is initiated within a conserved motif TAAG (Thiem and Miller, 1990). The TAAG motif may play several roles in late and very late gene expression, one serving to stabilize the RNAs or facilitate translation, while another as site for binding of a transcriptional activator or RNA polymerase (Ooi *et al*., 1989). Another motif, TATA box, was located at a relatively fixed position upstream of the transcription initiation site, assisting in directing RNA polymerase II to the transcription initiation site. Mutation of TATA box leads to the incorrect initiation of mRNA transcription. The proximal TATA box and TAAG overlapped each other in the *ubiquitin* promoter region. Thus the mutation of TAAG or TATA box may lead to incorrect reorganization of RNA polymerase. In addition, CAAT motif is a highly conserved transcriptional element in the promoter region. A previous study on the CAAT box in human delta globin gene showed that mutation of the motif could improve transcription level 2.2 fold (Yao *et al*., 2002). In this study, the mutation of two CAAT motifs was found to improve promoter activity in comparison to the native promoter, indicating that both CAAT box negatively regulated *ubiquitin* promoter activity as distal transcriptional element. According to deletion analyses, all mutation analyses results showed that the repeated motifs in *ubiquitin* promoter co-regulated the transcription of *ubiquitin* gene.

It has been confirmed that homologous region 3 (*hr3*) functions as a viral DNA replication origin and enhancer (Guarino and Summers, 1986; Zhang *et al*., 1995), which can accelerate the expression of early genes, especially the promoters containing TATA box. However, the *hr* enhancers have no effects on late and very late promoters. A previous study considered that only *cis*-linked *hr* could improve promoter transcription independent of its position and orientation (Lu and Carstens, 1993). The mechanism of *hr* enhancer is to improve the start rate of transcription, but not improve the quantity of DNA template through replication of virus DNA. In this study, no significant luciferase activity was detected in *Bm*-N cells transfected with pBm595ubiluc-hr3. It is likely that *hr3* can't independently enhance the transcription of late promoter. In the presence of virus factors, *cis*-linked and *trans*-linked BmNPV *hr3* significantly enhanced transcriptional activity of *ubiquitin* promoter, implying that in BmNPV, viral factors are considered to interact with the *ubiquitin* promoter and *hr3* enhancer. Further studies are necessary to elucidate viral functions in relation to interactions with the *ubiquitin* promoter and *hr3* enhancer and its enhancement of transcriptional activity.

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