# Construction and Application of a Baculovirus Genomic Library

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A random genomic library of the baculovirus *Bombyx mori* nucleopolyhedrovirus (BmNPV) was constructed and viral factors were identified by screening the regulator(s) for *helicase* gene expression. DNAs of 238 library plasmids were used to co-transfect with the reporter plasmid, pHp510-luc, in which the *luciferase* (*luc*) gene was driven by the baculovirus *helicase* promoter. Results showed that eight plasmids of the library strengthened the luciferase activity more than 1000-fold. Sequence analyses revealed that all of the eight plasmids contained an intact *ie-1* coding region. To confirm the reliability of the screening library, pHp510-luc was co-transfected with the cloned early gene which revealed that the BmNPV IE-1 was the only early factor that could stimulate the *helicase* promoter. The function analyses suggested that genome-wide screening factors through the library are powerful means to investigate the transcriptional regulation of dsDNA viruses.

*Key words:* Baculovirus, Genomic DNA Library, Helicase Promoter

## Introduction

The *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a typical baculovirus, containing approx. 128 kb of double-stranded circular DNA. The BmNPV genome contains open-reading frames, which potentially encode more than 100 proteins (Gomi *et al*., 1999).

Gene expression of the BmNPV is temporally regulated. Viral immediate early and delayed early genes are recognized by host RNA polymerase II and expressed before viral DNA replication. On the contrary, the expression of late and very late genes is dependent on viral DNA replication and the presence of early viral gene products (Blissard and Rohrmann, 1990). The delayed early *helicase* gene, which was found in all baculovirus genomes sequenced to date, is one of six essential genes in baculovirus DNA replication in transient assays (Kool *et al*., 1994; Okano *et al*., 2006). According to the transient expression assays using luciferase as reporter, the *helicase* promoter can initiate detectable transcription without any viral product in Bm cells, and the transcription level is much higher when the Bm cells were infected with the BmNPV (Xiao *et al*., 2001).

A genomic library of the baculovirus was thought to be of great value for studying the regulation pattern of virus gene expression. Using an AcMNPV genomic library consisting of 12 overlapping fragments constructed in phage DNA, a series of late expression factors (LEFs) were identified based on subtraction of clones of the library (Passarelli and Miller, 1993; Todd *et al*., 1995). However, it was difficult to identify the viral factors transactivating early gene expression when the subtraction strategy was used. Meanwhile, only the viral factors of substantial transactivation result rather than subtle modulating effects could be identified using this method. Moreover, it was inconvenient to further identify viral factors supporting the late and very late gene expression from the relatively large fragment of the library.

In the present report, a random BmNPV genomic library was constructed by combining partially digestion with a 4-bp site recognition enzyme and the "partial filling-in" method. Since the BmNPV infection could stimulate the expression of the *helicase* gene, genome-wide scanning was conducted for screening the transactivator via transient assays.

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## Experimental

### *Materials*

T4 DNA ligase, platinum *pfx* DNA polymerase, lipofectin, TC-100 medium and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Restriction endonucleases, pGEM-T easy vector, DNA purification kit, luciferase assay kit and luciferase reporter vector, pRL-TK, phRL-SV40 and pRL-CMV using *Renilla luciferase* as reporter gene, were obtained from Promega Corporation (Madison, WI, USA). *Taq* DNA polymerase was purchased from TaKaRa (Dalian, China). *E. coli* strain DH10B and plasmid pUC19 were kept in our laboratory. The plasmid pHp510-luc, containing the 510-bp *helicase* promoter from the BmNPV and the *luc* gene, was from a previous work in our laboratory (Xiao *et al.*, 2002).

### *Virus, cell lines, and silkworm larvae*

The BmNPV ZJ-8 strain, a local isolate of the wild-type BmNPV, was maintained in our laboratory. Bm-N cells were propagated at 27 °C in TC-100 insect medium supplemented with 10% heat-inactivated (56 °C, 30 min) FBS. Details for cell culture were referred to Summers and Smith (1987). Silkworms of JY1 variety were maintained in our laboratory. Silkworm larvae were fed on mulberry leaves and routinely reared throughout this experiment (Lü, 1990).

### *Construction of the BmNPV genomic library*

After infected about 4–5 d by the wild-type BmNPV ZJ-8 strain, hemolymph of silkworm larvae was collected on ice and centrifuged for 10 min at 4 °C. The precipitate was washed several times with distilled water and resuspended in 0.1% SDS for 30 min at room temperature. After centrifugation, the clean polyhedra were re-suspended with 200 $\mu$l TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and mixed with an equal volume of 0.1 M $Na_2CO_3$ and 0.15 M NaCl buffer (pH 10.5) at 37 °C for 30 min. SDS was added for a final content of 0.5%, and the mixture was placed on ice for 10 min. After extraction with phenol (pH 7.0) and chloroform, respectively, two volumes of cold absolute ethanol were added to the supernatant. The virus genomic DNA was precipitated by centrifugation at 8000 × *g* for 10 min, washed with 70% ethanol and dissolved in TE buffer.

To construct a random genomic library, about 10 $\mu$g of the BmNPV DNA were partially digested by 100-fold diluted *Sau*3A I, and fragments ranging from 3 to 5 kb were recovered from agarose gel. The cohesive ends were filled in partially by incubating with dATP and dGTP in the presence of Klenow fragment of DNA polymerase I. The plasmid vector pUC19 was fully digested by *Sal* I, and subsequently filled in by adding dTTP, dCTP and Klenow fragment. The vector and genomic fragments were mixed and ligated. The chemically transformed DH10B cells were cultured on IPTG/X-Gal plates. White colonies were selected and cultured; one-step screening of recombinant plasmids was conducted by assessing the size of the recombinant plasmid (Beuken *et al.*, 1998). Plasmid DNA of 238 colonies that contained appropriate insertion was extracted for further transient assays.

### *Cloning of primary early genes of the BmNPV*

According to the sequence of the BmNPV T3 strain (GenBank accession No. L33180), primers were designed and used to amplify early genes of the BmNPV. The sequences of primers are presented in Table I.

Using BmNPV ZJ-8 DNA as template, the early gene fragment was amplified by *pfx* DNA polymerase and A-tailed using *Taq* DNA polymerase and dATP. The target fragments were recovered and cloned into the pGEM-T easy vector. The sequences were confirmed by sequencing with universal T7 (5'-TAATACGACTCACTAT AGGG-3') and SP6 (5'-ATTTAGGTG ACAC TATAG-3') primers.

### *Transfection in insect cells*

For transfections, Bm-N cells were inoculated in a 24-well plate at a density of about $5 \cdot 10^5$ cells/ml and allowed to attach at 27 °C overnight. The complete medium was then replaced with 1 ml of serum-free TC-100 medium. In 50 $\mu$l co-transfection solution, there were 2 $\mu$l lipofectin, 0.3 $\mu$g reporter plasmid DNA, 0.1 $\mu$g internal control plasmid DNA, and, if necessary, 0.3 $\mu$g of each plasmid DNA from the random library. pBlueScript DNA was introduced in certain reactions to maintain a constant quantity of DNA. The mixture was allowed to react for 15 min at room temperature, and then added to monolayer cells. The cells were incubated at 27 °C for 4–6 h;

Table I. Primers used for amplifying BmNPV primary early genes.

| Primer | Sequence of primer | Amplified fragments |
|---|---|---|
| ie1-F | GCACAGACAAAATGTGCCACACTTG | *Ie-1* coding region and 429-bp 5'UTR |
| ie1-R | CCAACTCCCATTGTTATTATGCAAC | |
| ie2-F | GTGCTTACGAGTTGACAAGTGTCTG | *Ie-2* coding region and 453-bp 5'UTR |
| ie2-R | ATCAGATTATGGATGTACTGCTAACC | |
| p30-F | CAGTAGCGCCCGAGTACTTGCAAATC | *P30* coding region and 424-bp 5'UTR |
| p30-R | GGCATCACGATATTAAAGAGGGTGTG | |
| p35-F | GACTCGTTGAAACGTGTTCGTC | *P35* coding region and 404-bp 5'UTR |
| p35-R | CTCAAATCTTGCGTTACGAGTAG | |
| pe38-F | CATATGCCGTTACATTATCAATGTGAC | *Pe-38* coding region and 448-bp 5'UTR |
| pe38-R | GTCACATTGATAATGTAACGGCATATG | |
| me53-F | GATTTGTATGTCGGCGGTGTACATGTG | *Me-53* coding region and 444-bp 5'UTR |
| me53-R | CCAAATCAGGCCTGATTCAATAACG | |

Primers were designed according to the sequence of the BmNPV T3 strain (GenBank accession No. L33180). F, forward; R, reverse.

then the supernatant was replaced with 500 $\mu$l of complete medium, and the cells were continued to incubate for another 48 h. The co-transfections with strong stimulation effects screened from the library were verified more than 2 times. The positive clones were sequenced by Sunbiotech Company, Ltd. (Beijing, China).

*Luciferase activity assay*

For the luciferase assay, at 48 h post transfection (hpt), the transfected Bm-N cells were collected and centrifugated at 10,000 × *g* for 5 min at 4 ºC. Cell extracts were prepared with a luciferase assay kit (Promega). The harvested cell pellets were washed twice by suspending them in cold phosphate buffered saline (PBS), and then centrifuged at 8,000 × *g* for 4 min at 4 ºC. The washed cells were lysed by adding PLB buffer. After a freeze-thawing cycle, the lysate was centrifuged at 4 ºC to remove cell debris, and the supernatant was stored in an ice-bath during the enzymatic activity assay. The amount of protein in the lysate was measured using the Bradford method (Smith, 1995). Measurements of dual-luciferase activities were conducted using a liquid scintillation spectrometer (Beckman LS6000 Series, USA) (Idahl *et al*., 1986). The luciferase activity was indicated as counts per min (CPM) in 15 s.

## Results

*Construction of the BmNPV genomic library*

Since the largest coding region of the BmNPV is 3669 bp (*helicase* gene), 3- to 5-kb genomic fragments were desired to ensure the presence of the largest coding region in an individual clone of the library. To determine the optimal digestion of the BmNPV, serial diluted *Sau*3A I was used to digest the genome DNA at certain reaction time. Through electrophoresis, we found that 100-fold diluted *Sau*3A I could produce ideal 3- to 5-kb DNA fragments. The reaction was scaled up when the optimal conditions were determined. The 3- to 5-kb fragments were recovered from the gel and partially filled in. *Sal* I-digested pUC-19 DNA was also partially filled in and then ligated with the 3- to 5-kb fragments. Sufficient colonies were obtained by chemical transformation. By use of blue/white screening (Sambrook *et al*., 1989), white colonies were selected and subjected to one-step confirmation of the insert fragment size. About 85% insertions larger than 2.5 kb were determined. Plasmid DNA of 238 colonies containing appropriate insertion was prepared for further transient assays.

The equation $N = 1n(1 - P)/1n[1 - (I/G)]$ can be used to evaluate the quality of a constructed library (Seed *et al*., 1982). $N$ stands for the number of required independent clones, $P$ stands for the probability of containing a particular sequence, $I$ is the average size of the cloned fragments in kb, and $G$ is the size of the target genome in kb. Since the BmNPV was about 128 kb in size, the cloned fragments were about 3.5 kb in average. 167 colonies were required to obtain a representation of 99%. So the 238-plasmid library was sufficient for analysis, and the random genomic library of the BmNPV was successfully constructed.

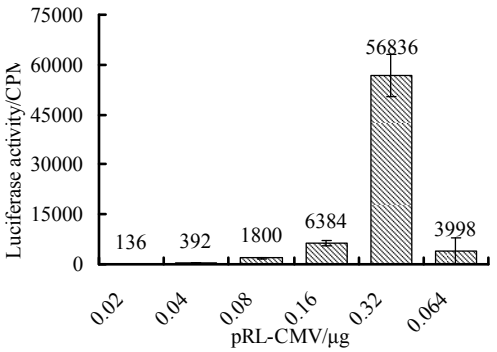


Fig. 1. Expression effects of transfection of various amounts of pRL-CMV. Different amounts of pRL-CMV plasmid DNA were used in the transfection assays as indicated. The luciferase activity is indicated as CPM in 15 s. Each reaction contained 10 $\mu$g protein extracted from the transfected cells. The results represent averages from three separate transfections at 48 hpt.

Table II. Dose-dependent manner of IE-1 for transactivation of the *helicase* promoter.

| T-ie1 | Luciferase activity [CPM/15 s] | Fold-stimulation[a] |
|---|---|---|
| non | 8 ± 3.5 | 1 |
| 0.003 $\mu$g | 20 ± 6.7 | 2.5 |
| 0.03 $\mu$g | 6444 ± 355 | 805.5 |
| 0.3 $\mu$g | 299628.1 ± 5983 | 37453.5 |

The results represent averages from three separate transfections at 48 hours post transfection. The plasmid pRL-CMV was introduced as internal control.

[a] The transactivation ability of IE-1 is presented as stimulating folds of pKS-hel510-luc plasmid (0.3 $\mu$g) transfection alone that was arbitrarily set as 1.0. Each reaction contained 10 $\mu$g protein extracted from the transfected cells. The transfected amount of the *ie-1* gene is indicated.

*Setting up of the internal control systems*

The $\beta$-glacotosidase system was used as internal control for the normalization of transfection efficiencies in our previous studies (Chen *et al*., 2004; Zhang *et al*., 2004). This detection system is very stable but less sensitive. In addition, the background of cell proteins also interferes the measurement. Since neither a sensitive nor a simple internal control system was introduced for insect cells transfection, we attempted to use *Renilla luciferase* with a mammalian virus-derived promoter as the normalizing approach in insect cells.

Three plasmid DNAs, pRL-TK, phRL-SV40 and pRL-CMV, were used as internal controls in mammalian cells with promoters from three mammalian viruses. When transfected to Bm-N cells, pRL-TK and phRL-SV40 gave no detectable results of *Renilla* luciferase activity, but the transcription efficiency of the *CMV* promoter was very high in Bm cell lines. The details are shown in Fig. 1. Further analysis demonstrated that pRL-CMV could also function in Sf-21 cells and in silkworm larvae or pupae (data not shown).

*Genome-wide screening of transactivator(s) for the helicase promoter*

The *helicase* promoter can initiate a slight expression of luciferase in Bm cells without any viral product. Since the transcription level was much higher when the Bm cells were infected with the BmNPV, there should be certain transactivator(s) for the augmentation of the *helicase* promoter activity. The constructed genomic library provided high-throughput, genome-wide screening of the viral transactivation factors for the BmNPV *helicase* promoter. Eight plasmids with more than 1000-fold stimulation of luciferase activity were obtained from all 238 plasmids via co-transfection of library DNA with the reporter plasmid. The sequence results of the 8 plasmids indicated that all these plasmids contain an intact *ie-1* gene (Fig. 2). Further cloning of the *ie-1* coding region and its 429-bp 5'UTR confirmed that the transactivation effect was obtained from IE-1 but not any other products of flanking genes. A dose-dependent manner of IE-1 for the transactivation of *helicase* promoter was also observed through adding various amounts of the *ie-1* gene to the transient expression assay system (Table II).

*Transactivation effect of the early gene on the helicase promoter in the BmNPV*

A previous study by Lu and Carstens (1993) revealed that a greater transactivation ability of the *helicase* promoter was observed with PE-38 than with IE-1 in AcMNPV. But in our experiment, pe-38 didn't increase the activation of the *helicase* promoter from the BmNPV. To validate the accuracy of library screening, the *pe-38* gene including its 448-bp 5'UTR was amplified and cloned into the pGEM-T easy vector. The sequence of *pe-38* was verified by direct sequencing. The tran-

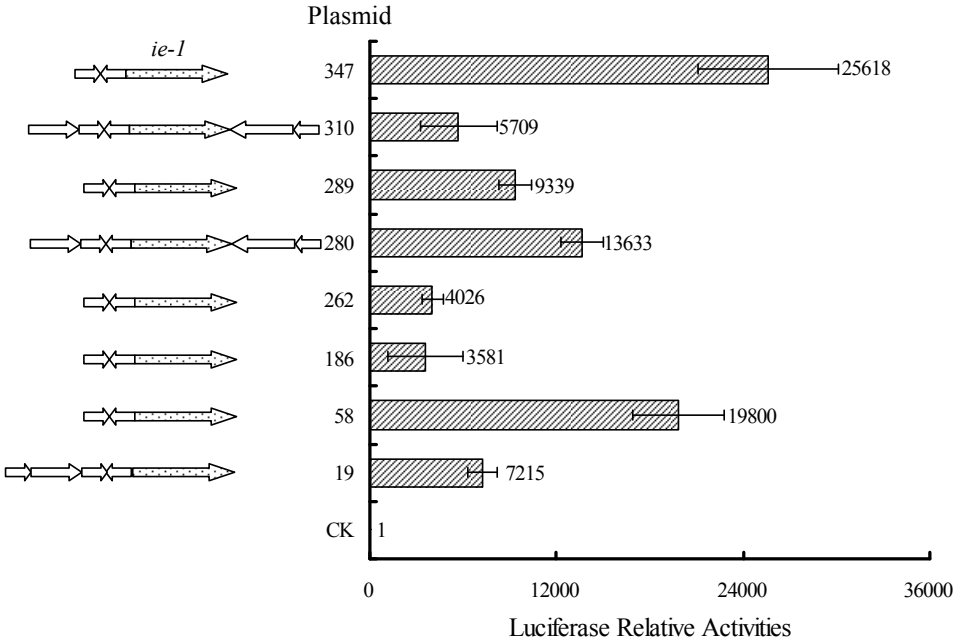


Fig. 2. Plasmids involved in transactivation of the *helicase* promoter screened by the BmNPV random library. Bm-N cells in a 24-well plate were transfected with reporter plasmid and each genomic library DNA. Eight plasmids from the library could stimulate the transcription of the *helicase* promoter and the plasmid number is indicated. The arrows represent the intact ORFs which were contained in the indicated plasmid. The luciferase activity was indicated as CPM in 15 s. Each reaction contained 10 $\mu$g protein extracted from the transfected cells. The results represent transfections prepared at 48 hpt. The average CPM value obtained from the control transfections of the reporter plasmid was 28.

sient assays showed that pe-38 did not enhance the transcription activity of the *helicase* promoter from the BmNPV. Another 4 primary early genes of the BmNPV (Table I) were also cloned but no transactivation affection was observed in transient assays. So, we concluded that no other early factor, but IE-1, could stimulate the transcription of the *helicase* promoter in the BmNPV.

## Discussion

A genomic DNA library is widely used in molecular biology, such as for gene isolation and analysis of gene expression or regulation. The enzymes *Sau*3A I or *Mbo* I, recognizing 4-bp site GATC and generating fragments compatible with several convenient phage $\lambda$ and cosmid vectors, have been proved to be useful to construct partial digestion libraries of genomic DNA. The 4-bp recognition site is expected to cut every 256 base pairs in the genome, and sufficient random cleavage of target DNA can generally be obtained by using partial digestions with these enzymes. By the partially filling-in method, neither the cohesive ends of vectors nor genomic fragments could be self-ligated since the ends were incompatible, which was helpful to reduce the background (Zabarovsky and Allikmets, 1986). It was crucial that high-molecular weight BmNPV genomic DNA should be incubated with limited amounts of *Sau*3A I restriction enzyme for variable times. To obtain the desired enrichment of 3- to 5-kb fragments, a series of pre-experiments were performed before scale-up for the final digestion.

Researches on the molecular biology of the baculovirus mainly focused on the AcMNPV and BmNPV, the representative virus strains of the baculovirus. It was presumed that highly conserved BmNPV and AcMNPV were derived from the same ancestors (Gomi *et al.*, 1999). In the present study, we constructed a BmNPV genomic library and identified its function in screening viral factors, which participate in the transcription of the target promoter. Based on the results of genome-

wide screening and further analysis, *ie-1* was the only viral gene whose product could function as transactivator on the *helicase* promoter from the BmNPV. The result was inconsistent with previous research that *ie-1* and another early gene, *pe-38*, improve the transcription of the *helicase* promoter, because the BmNPV genome *ie-1* ORF does not contain any *Sau*3A I sites, but the *ie-2*/*pe-38* locus contains 8 *Sau*3A I sites. To confirm if there are some shortcomings of the library, we cloned the *pe-38* gene and used it for transfection. The result showed that *pe-38* had no affection on the transcription of the *helicase* promoter from the BmNPV. In our studies about the *ubiquitin* promoter from the AcMNPV and BmNPV, we also found that, although they are very conserved, the transcription model was different (Lin *et al.*, 2008a, b). This suggests that the viral genes of the AcMNPV and BmNPV may have different approaches in transcription and regulation of some viral genes. All other 230 plasmids with fragments from the BmNPV failed to gave high stimulation effects on the transcription of the *helicase* promoter. This result was also confirmed by co-transfections of the reporter plasmid DNA with 6 cloned major early genes, including *pe-38* and *ie-1*. It also suggested that the regulation profile acquired by high-throughput screening with the BmNPV genomic library was reliable. From this point of view, the regulation of gene expression in the BmNPV was distinct from that in the AcMNPV. This finding gave another evidence for the diversity of baculoviruses even the BmNPV was evolved from a common ancestral virus similar to the AcMNPV.

At present, the baculovirus is broadly used in bio-insecticides and expression of foreign genes. More and more baculoviruses were studied and utilized; the genomic library of the baculovirus is a useful means in the transcription and regulation study of the baculovirus.

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Beuken E., Vink C., and Bruggeman C.-A. (1998), One-step procedure for screening recombinant plasmids by size. Biotechniques **24**, 748–750.

Blissard G.-W. and Rohrmann G.-F. (1990), Baculovirus diversity and molecular biology. Annu. Rev. Entomol. **35**, 127–155.

Chen Y., Yao B., Zhu Z., Yi Y., Lin X., Zhang Z., and Shen G. (2004), A constitutive super-enhancer: homologous region 3 of *Bombyx mori* nucleopolyhedrovirus. Biochem. Biophys. Res. Commun. **318**, 1039–1044.

Gomi S., Majima K., and Maeda S. (1999), Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. J. Gen. Virol. **80**, 1323–1337.

Idahl L.-A., Sandstrom P.-E., and Sehlin J. (1986), Measurements of serum glucose using the luciferin/luciferase system and a liquid scintillation spectrometer. Anal. Biochem. **155**, 177–181.

Kool M., Ahrens C.-H., Goldbach R.-W., Rohrmann G.-F., and Vlak J.-M. (1994), Identification of genes involved in DNA replication of the *Autographa californica* baculovirus. Proc. Natl. Acad. Sci. USA **91**, 11212–11216.

Lin X., Chen Y., Yi Y., Yan J., and Zhang Z.-F. (2008a), Promoter analysis of *Bombyx mori* nucleopolyhedrovirus *ubiquitin* gene. J. Microbiol. **46**, 429–435.

Lin X.-A., Chen Y., Xu W.-H., Yi Y.-Z., and Zhang Z.-F. (2008b), Characterization of the *Autographa californica* nucleopolyhedrovirus *ubiquitin* gene promoter. Z. Naturforsch. **63c**, 277–283.

Lu A. and Carstens E.-B. (1993), Immediate-early baculovirus genes transactivate the *p143* gene promoter of *Autographa californica* nuclear polyhedrosis virus. Virology **195**, 710–718.

Lü H.-S. (1990), The Sericultural Science in China. Shanghai Scientific and Technical Publishers, Shanghai.

Okano K., Vanarsdall A.-L., Mikhailov V.-S., and Rohrmann G.-F. (2006), Conserved molecular systems of the Baculoviridae. Virology **344**, 77–87.

Passarelli A.-L. and Miller L.-K. (1993), Three baculovirus genes involved in late and very late gene expression: *ie-1*, *ie-n*, and *lef-2*. J. Virol. **67**, 2149–2158.

Sambrook J., Fritsch E.-F., and Maniatis T. (1989), Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA.

Seed B., Parker R.-C., and Davidson N. (1982), Representation of DNA sequences in recombinant DNA libraries prepared by restriction enzyme partial digestion. Gene **19**, 201–209.

Smith J.-A. (1995), Analysis of proteins. In: Short Protocols in Molecular Biology, 3rd ed. (Ausubel F.-M., Brent R., Kingston R.-E., Moore D.-D., Seidman J.-G., Smith J.-A., and Struhl K., eds.). John Wiley & Sons Inc., New York, pp. 332–333.

Summers M. D. and Smith G. E. (1987), A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures. Texas Agricultural Experiment Station Bulletin, No. 1555, College Station, TX, USA.

Todd J.-W., Passarelli A.-L., and Miller L.-K. (1995), Eighteen baculovirus genes, including *lef-11*, *p35*, *39k*, and *p47*, support late gene expression. J. Virol. **69**, 968–974.

Xiao Q.-L., Zhang Z.-F., Yi Y.-Z., He J.-L., and Wu X.-F. (2001), Functional analysis of *helicase* gene promoter and homologous region 3 enhancer in *Bombyx mori* nuclear polyhedrosis virus. Acta Biochim. Biophys. Sin. **33**, 525–530.

Xiao Q.-L., Zhang Z.-F., Yi Y.-Z., He J.-L., and Wu X.-F. (2002), Identification of functional region of *helicase* gene promoter in *Bombyx mori* nuclear polyhedrosis virus. Acta Biochim. Biophys. Sin. **34**, 560–564.

Zabarovsky E.-R. and Allikmets R.-L. (1986), An improved technique for the efficient construction of gene libraries by partial filling-in of cohesive ends. Gene **42**, 119–123.

Zhang T.-Y., Kang L., Zhang Z.-F., and Xu W.-H. (2004), Identification of a POU factor involved in regulating the neuron-specific expression of the gene encoding diapause hormone and pheromone biosynthesis-activating neuropeptide in *Bombyx mori*. Biochem. J. **380**, 255–263.