Cytotechnology **41:** 37–44, 2003.

Cetyltriethylammonium bromide stimulating transcription of *Bombyx mori* nucleopolyhedrovirus *gp64* gene promoter mediated by viral factors

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Received 26 June 2002; accepted in revised form 8 January 2003

Key words: Bombyx mori nucleopolyhedrovirus, Cetyltriethylammonium bromide, *gp64* promoter, Transactivition, Transfection, Transient expression

Abstract

To characterize the effects of cetyltriethylammonium bromide (CTAB) on the transcription of *gp64* promoter from *Bombyx mori* nucleopolyhedrovirus (BmNPV), the plasmid pBmgp64Luc used in transient expression assay system was constructed by using the *luciferase* gene as a reporter under the control of BmNPV *gp64* promoter. When the *Bombyx mori* cells (Bm-N) were transfected with the pBmgp64Luc, different treatments were undertaken. We found that the transient expression activity of luciferase could not be augmented directly by CTAB treatment alone, but could be enhanced more than 2 times by BmNPV treatment alone at a multiplicity of infection (MOI) of 0.5. Through co-treatment with 0.1 μg ml^{-1} of CTAB and BmNPV at a MOI of 0.5, the enzymatic activity increased 5.21 times. We presumed that the stimulation of transcription of BmNPV *gp64* promoter by CTAB was mediated by viral factors from BmNPV. In addition, the time curves of luciferase activity in cells transfected with pBmgp64Luc and transactivated by virus were observed.

Introduction

Baculoviruses, which are large double-stranded DNA viruses with genomes of 80 to 180 kb (Ahrens et al. 1997; Ayres et al. 1994; Majima et al. 1996), replicate and are transcribed in the nuclei of infected cells. During the infection, viral genes are expressed in a coordinately regulated cascade fashion and divided into immediate early, delayed-early, late, and very late phases. The regulation of these phases appears to be determined primarily at the level of transcription (Guarino and Summers 1986). Early genes are transcribed by host RNA polymerase II prior to viral DNA replication (Fuchs et al. 1983; Grula et al. 1981; Yang et al. 1991). Late genes are transcribed by a virus-induced RNA polymerase activity that appears to be encoded mostly by viral genes (Fuchs et al. 1983; Beniya et al. 1996; Lu and Miller 1995; Todd et al. 1995).

Baculoviruses are characterized by a complex infection cycle that produces two structurally and functionally distinct virion phenotypes (Blissard and Rohrmann 1990). The virions of polyhedron-derived virus (PDV) phenotype acquire an envelope in the nucleus and are subsequently occluded in large polyhedron-shaped occlusion bodies, while the virions of the budded virus (BV) phenotype are not occluded and acquire an envelope by budding through the virus-modified plasma membrane at the cell surface. The BV phenotype serves to spread the infection from cell to cell within an infected individual. GP64, a major baculoviral envelope glycoprotein of the BV phenotype, is encoded by the virus and is required for endocytosis of the virus into host insect cells (Volkman and Goldsmith 1985). It is presented on the surface of infected cells and on virions as a homotrimer, forming typical peplomer structures (Oomens et al. 1995). During the infection cycle, GP64 is

abundantly expressed and transported to the cell surface to be incorporated into budding virions. Its synthesis peaks at 8 and 24 hr post infection (Whitford et al. 1989). Although most baculovirus structural proteins are expressed as late genes, *gp64* gene expression is regulated by a bi-phasic promoter that contains both immediate early and late promoter functions (Blissard and Rohrmann 1989). From the early promoter, GP64 is shed from infected cells early in infection before any progeny BV can be detected (Jarvis and Garcia 1994). From the late promoter during infection, the expressed GP64 is transported to the cell surface continually to compensate its pickup by assembling virus during the process of budding (Grabherr et al. 2001).

Cetyltriethylammonium bromide (CTAB), a cationic detergent, has been shown to enhance the infection of *Bombyx mori* cell line (Bm-N) infected with *Bombyx mori* nucleopolyhedrovirus (BmNPV) (Cheng and Hou 1992). This enhancement is ascribed to the neutralization of negative charge on the virion envelope by a cationic detergent, resulting in the enhancement of the attachment of virions to the negative-charged cell membrane (Yamamoto and Tanada 1978; Tuan and Hou 1988). Besides the neutralization of negative charge on the virion envelope and cell membrane, we found that CTAB appeared to affect the bi-phasic replication cycle and the pathway for producing BVs and PDVs during the infection of BmNPV *in vivo* and/or *in vitro*, resulting in the increase in BVs production and a suppression of PDVs production and expression of foreign genes controlled by the *polyhedrin* promoter. CTAB could also stimulate the transcription of the *ie-1* promoter of BmNPV in uninfected Bm-N cells about three-fold via the transient expression assay system (Zhou et al. 2002). It is uncertain whether CTAB could influence the transcriptional activity of *gp64* promoter, or whether it stimulates some viral factors, such as immediate early gene products like IE-1, a key factor in baculovirus cascade regulation and required for replication of viral DNA (Guarino and Summers 1986; Lu and Miller 1995; Guarino and Summers 1987; Kovaos et al. 1992; Kool et al. 1994), in turn, to transactivate the *gp64* promoter transcription, resulting in the increase in BVs production. In the present study, we investigated the effects of CTAB on the *gp64* promoter transcription through the mediation by viral factors from BmNPV via a transient expression assay system, from which we deduced that CTAB increased BV production when Bm-N cells are infected with BmNPV.

Materials and methods

Materials

Restriction enzymes, T4 DNA ligase, and lipofectin kit were purchased from GIBCO-BRL (USA). Geneclean kit and luciferase assay kit were obtained from Promega Corporation (USA). The remaining materials used in this work were also purchased from GIBCO-BRL (USA) or Sigma Chemical (St. Louis, MO, USA).

Reporter plasmid construction

To investigate the transient transcriptional activity of BmNPV *gp64* promoter, a reporter plasmid pBmgp64Luc was generated. Two primers, 5′-TTTCTAGATATTTAAATAAACCAAACACATG-3′ (forward) and 5′-GCGGATCCAATCTCGCTTGTGTGTTTCTTA-3′ (reverse), were devised to yield the fragment with *Xba*I and *Bam*HI sites (underlined residues) on its 5′ and 3′ termini, respectively. The product of a 437-bp fragment of the *gp64* promoter from the BmNPV-ZJ8, amplified by polymerase chain reaction (PCR), was cloned into the *Xba*I/*Bam*HI sites of the Bluescript SK(M13-). The plasmid pUL220 containing an entire *luciferase* gene (1.8-kb) with 3′ polyadenylic acid (polyA) was digested with *Bam*HI (Lei et al. 1994). The excised *luciferase* fragment was then subcloned under the control of the *gp64* promoter in the right orientation. The construction of pBmgp64Luc was devised as shown in Figure 1, using the methods described by Sambrook et al. (1989).

Control plasmid construction

To construct a control plasmid used to normalize luciferase activity from each extract, a fragment of about 3.7 kb containing the *E. coli LacZ* gene with simian virus 40 (SV40) polyadenylation signals under the control of *HSP70* promoter was excised from pAcDZ1 (López-Ferber et al. 1995) at the *Xba*I and *Bam*HI sites and then cloned into *Xba*I/*Bam*HI-digested Bluescript SK(M13-). The resulting plasmid was named pHSP70LacZ and shown in Figure 2.

Virus, cell line, and cell culture

The BmNPV-ZJ8, a wild-type BmNPV, was kindly provided by Professor Wu (Shanghai Institutes for Biological Sciences, the Chinese Academy of Sci-

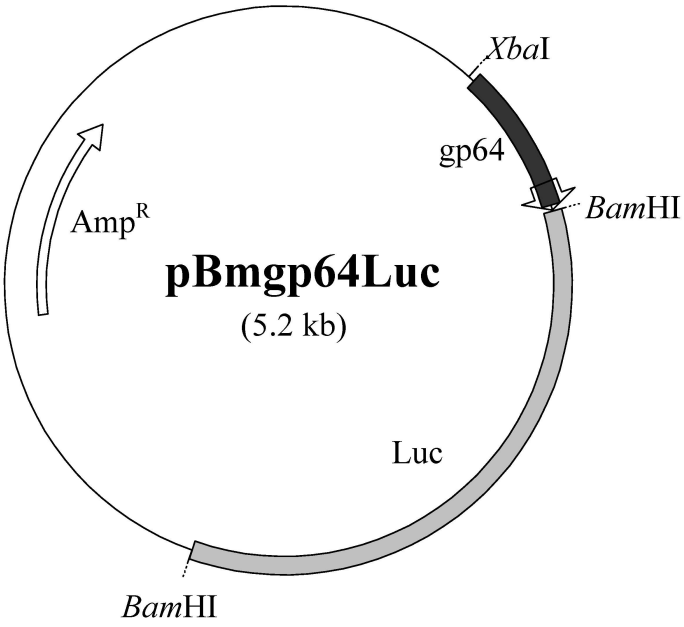

Figure 1. Diagrammatic representation of the pBmgp64Luc construct used in transient expression assay.

ences, China). The *Bombyx mori* cell line (Bm-N) was maintained in the Key Laboratory of Silkworm Biotechnology, Ministry of Agriculture, China. The cells were grown in TC-100 medium supplemented with 10% fetal bovine serum and Penicillin G (80 U ml^{-1})-streptomycin (50 μg ml^{-1}) in 15-cm^2 flasks at 27 ℃. The details for cell culture, viral propagation, viral titer, and viral maintenance were after Summers and Smith (1987).

Transfection of uninfected cells and CTAB treatment

For transfections, Bm-N cells were seeded into 15-cm^2 flasks at a density of about 5×10^5 cells ml^{-1} and cells were allowed to attach at 27 ℃ overnight. TC-100 medium was then replaced with 1 ml of serum-free medium, and 100 μl of transfection solution containing 6 μl of lipofectin and 1 μg of

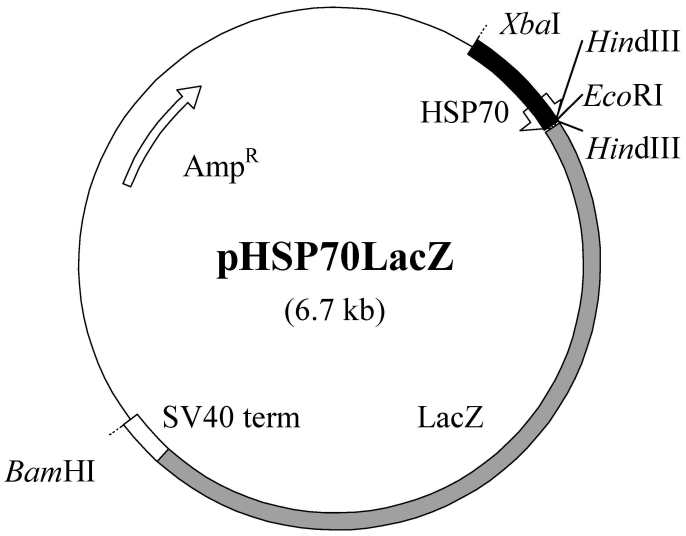

Figure 2. Diagrammatic representation of the pHSP70LacZ construct used in normalization system.

pBmgp64Luc and 1 μg of pHSP70LacZ was added to each flask. Cells were incubated at 27 °C for 5 hr then the supernatant was decanted and replaced with 3 ml of conditioned medium containing 0.1 μg ml^{-1} of CTAB. At 48 hr post-transfection (hpt), transfected cells were collected and ready for enzymatic activity assay. The treatment with no CTAB addition was made as a control. Each treatment consisted of at least three separate transfections.

Viral transactivation and CTAB treatment

To examine the transactivation of virus to *gp64* promoter, the cells, grown in each flask at 27 °C overnight, were co-transfected with 1 ml of serum-free medium supplemented with 100 μl of transfection solution containing 6 μl of lipofectin, 1 μg of pBmgp64Luc, and 1 μg of pHSP70LacZ, at 27 °C for 4 hr. Then the appropriate amount of virus was added at a multiplicity of infection (MOI) of 0.5 and the cells were incubated for another 1 hr. The serum-free medium was replaced with 3 ml of medium with or without CTAB supplement. The transfected cells, transactivated by virus, were collected every 8 hr post transfection and every 12 hr from 24 hpt for enzymatic activity assay.

Estimation of luciferase activity, specific activity of β-galactosidase, and extracted protein

The cell extracts were prepared with a luciferase assay kit (Cat. E4030). The harvested cells were washed twice by resuspension in phosphate buffered saline (PBS), then centrifuged at 5,000 *g* for 4 min at 4 °C. After washing, the cells were lysed by a single freeze-thaw cycle. The lysate was centrifuged at 4 °C to remove cell debris and supernatant stored in an ice-bath until ready for measurement. Measurements of luciferase activity (Idahl et al. 1986) on three separate transfections were taken in triplicate using a liquid scintillation spectrometer (Beckman LS6000 Series, USA). The specific activity of *E. coli* β-galactosidase was assayed by the method described by Sambrook et al. (1989). The LacZ reporter data from each extract were used to normalize the luciferase activity. The amount of protein in the lysate was measured using the Bradford method as described by Moos (1995). The data were analyzed by Statistical Analysis System (SAS, version 6.12, 1990).

Table 1. Direct effects of CTAB on the transient transcriptional activity of *gp64* promoter in uninfected Bm-N cells.Luciferase activity is indicated as cpm in 15 s. pBmgp64Luc alone represents cells transfected with pBmgp64Luc only. pBmgp64Luc+CTAB represents treatment with 0.1 μg ml^{-1} of CTAB after the cells were transfected with pBmgp64Luc. The β-gal normalizing system was introduced into each transfection. Each reaction contained 20 μg of protein extracted from the uninfected cells. The results represented the mean ± S.D. (error bars) from triplicate samples in three separate transfections at 48 hpt.

Treatments	Luc activity (cpm)
pBmgp64Luc alone	19 757.0 ± 2 435.1
pBmgp64Luc + CTAB	20 592.6 ± 4 594.3

Results and discussion

Direct effects of CTAB on the transcriptional activity of the gp64 promoter of BmNPV in uninfected Bm-N cells

To observe the transient expression level of luciferase driven by the BmNPV *gp64* promoter and the CTAB effects on it in uninfected cells via the transient expression assay system, we constructed a plasmid pBmgp64Luc by use of *luciferase* gene as a reporter gene that was under the control of the *gp64* gene promoter (Figure 1). After the Bm-N cells were transfected with this plasmid, 0.1 μg ml^{-1} of CTAB was added to the incubation medium that replaced 1-ml serum-free medium containing transfection solution to investigate the effects of CTAB on the transcription of *gp64* promoter. The transfected cells were incubated for 48 hr and then collected for luciferase activity assay. From Table 1, the luciferase activity at 48 hpt was 19 757.0 ± 2 435.1 cpm, by the transfection with the pBmgp64Luc alone, and 20 592.6 ± 4 594.3 cpm by the transfection with the pBmgp64Luc together with the CTAB supplement, from 20 μg of protein extracted from transfected cells. The data from *LacZ* were used to normalize each luciferase activity. There is no significant difference in enzymatic activity between two treatments, analyzed by SAS (F value = 2.19, $Pr > F = 0.2039 > 0.05$). It suggested that the CTAB could not affect the *gp64* promoter transcriptional activity directly.

The transactivation of gp64 gene promoter by BmNPV

Transcription from some early promoters, such as those derived from the baculovirus *39K, p26, gp64* and DNA polymerase genes, was found to be transactivated by the nuclear extracts prepared from the *Autographa californica* nucleopolyhedrovirus (AcMNPV)-infected *Spodoptera frugiperda* cells (Glocker et al. 1992). To examine the transactivation of *gp64* promoter by the viral factors from BmNPV, the BmNPV-ZJ8 was added into the transfection medium at a MOI of 0.5 for viral infection for 1 hr after Bm-N cells were transfected with the plasmid pBmgp64Luc for 4 hr. The time curves of luciferase activity in cells transfected with a pBmgp64Luc alone, and a plasmid together with the BmNPV-ZJ8, were shown in Figure 3. Each luciferase activity was normalized by the data from *LacZ*.

Before 24 hpt, the enzymatic activity with or without virus addition was too weak to analyze by SAS. At 24 hpt, the luciferase activity increased to 10 354.5 ± 3 352.1 cpm with a 1.38-fold enhancement index compared with the 7 521 ± 3 288.8 cpm of no virus treatment. Thence, the enzymatic activity was markedly increased to 121 468.0 ± 29 559.4 cpm, and the best enhancement index of 2.35 times was achieved, at 48 hpt, compared with 51 618.4 ± 13 596.2 cpm of no virus treatment, with a significant difference (F value = 134.58, Pr > F = 0.003 < 0.01). This suggested that the transcription of BmNPV *gp64* promoter could be transactivated significantly by viral factors from BmNPV-ZJ8 in Bm-N cells. Following the extension of hpt, the enzyme was accumulated inside cells and the enzymatic activity increased systematically within the whole investigation time.

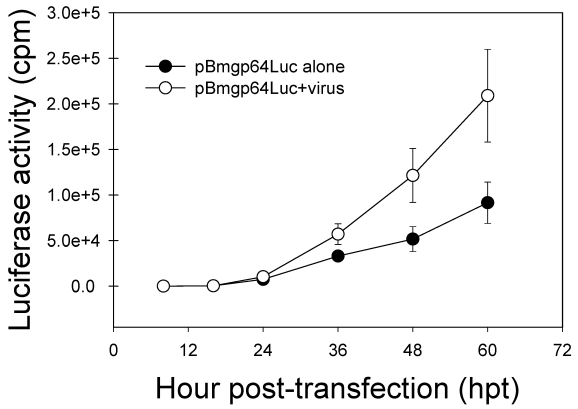

Figure 3. The time curves of luciferase activity in cells transfected with pBmgp64Luc and transactivated by BmNPV-ZJ8.The luciferase activity is indicated on the Y axis as the counts per minute (cpm) in 15 s. The investigation time is presented on the X axis as hour post-transfection (hpt). pBmgp64Luc alone represents transfection of cells with pBmgp64Luc only. pBmgp64Luc+virus represents addition of BmNPV-ZJ8 into the incubation medium at a MOI of 0.5 after the cells were transfected with the plasmid pBmgp64Luc. Before 24 hpt, the transfected cells were collected every 8 hr. Thence the cells were gathered every 12 hr. The β-gal normalizing system was introduced into each transfection. Each reaction contained 20 μg of protein extracted from Bm-N cells. The results represented the mean ± S.D. (error bars) from triplicate samples in three separate transfections.

To examine whether this constructed plasmid is functional and whether the transcription of *gp64* promoter could be transactivated by virus *in vivo*, we observed the luciferase activity in the fifth-instar silkworm larvae by injecting pBmgp64Luc into the larval bodies to transfect hemolymph cells, and injected BmNPV-ZJ8 and/or CTAB to observe effects on enzymatic activity. Unfortunately, no expected data were observed because the detected enzymatic activity was too weak to be comparable (data not shown).

Expression of the baculovirus major envelope glycoprotein gene *gp64* is regulated by transcription from both early and late promoter (Jarvis and Garcia 1994). The BmNPV *gp64* promoter construct was functional in uninfected Bm-N cells (pBmgp64Luc alone in Figure 3), indicating that transcription from the BmNPV *gp64* early promoter required no additional viral products. The addition of BmNPV-ZJ8 increased the transient expression activity of luciferase (pBmgp64Luc+virus in Figure 3), indicating that viral factors from BmNPV transactivated transcription of BmNPV *gp64* promoter.

The stimulation of gp64 gene promoter by CTAB mediated through BmNPV

The nuclear extracts, prepared from AcMNPV, or AcMNPV IE-1, a baculovirus transcriptional transactivator gene product, can transactivate the transcription of *Orgyia pseudotsugata* nucleopolyhedrovirus (OpMNPV) *gp64* promoter (Blissard and Rohrmann 1991; Glocker et al. 1992). The viral factors of BmNPV transactivated the transcription of BmNPV *gp64* promoter (Figure 3). It was previously found that CTAB could stimulate the transcription of the *ie-1* promoter of BmNPV by about 3 times in uninfected Bm-N cells (Zhou et al. 2002). To

examine whether CTAB could stimulate the transcription of BmNPV *gp64* promoter mediated by BmNPV, different treatments were undertaken and the data were shown in Figure 4.

The optimum enhancement index of transactivating the transcription of BmNPV *gp64* promoter by viral factors was found at 48 hpt (Figure 3). Therefore, the transfected cells by different treatments were gathered at 48 hpt for enzymatic activity assay. As presented in Figure 4, the luciferase activity of the pBmgp64Luc transfection alone was 50 530.7 ± 12 591.7 cpm. The pBmgp64Luc transfection together with 0.1 μg ml^{-1} of CTAB supplement was 47 365.3 ± 4 442.1 cpm with no significant difference over the no CTAB supplement (F value = 0.59, Pr > F = 0.4762 > 0.05). CTAB cannot affect *gp64* promoter transcription directly, which is similar to the result in Table 1. By transfection of cells with pBmgp64Luc together with the addition of virus to the incubation medium at a MOI of 0.5, the enzymatic activity increased to 114 813.3 ± 28 392.2 cpm with 2.27-fold enhancement index over the pBmgp64Luc transfection alone, with a significant difference (F value = 87.51, Pr > F = 0.0002 < 0.01). By the co-treatment with 0.1 μg ml^{-1} of CTAB and virus (MOI = 0.5) after the cells were transfected with the pBmgp64Luc, the enzymatic activity was significantly increased to 263 106 ± 61 551.2 cpm by 5.21-fold augmentation over the pBmgp64Luc transfection alone (F value = 44.67, Pr > F = 0.0011 < 0.01).

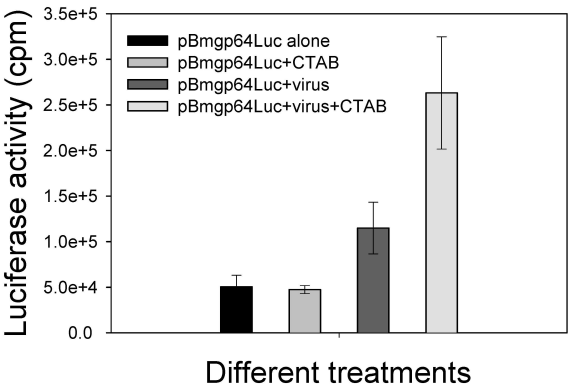

Figure 4. CTAB stimulating the transcription of BmNPV *gp64* promoter mediated by BmNPV-ZJ8.The luciferase activity is indicated on the Y axis as cpm in 15 s. Different treatments are presented on the X axis, and set as: pBmgp64Luc alone, cells transfected with plasmid only; pBmgp64Luc+CTAB, adding 0.1 μg ml^{-1} of CTAB to the medium after cells transfected with plasmid; pBmgp64Luc+virus, adding BmNPV-ZJ8 (MOI = 0.5) to transfection medium for 1 hr after cells transfected with plasmid for 4 hr; pBmgp64Luc+virus+CTAB, co-treatment with 0.1 μg ml^{-1} of CTAB and BmNPV-ZJ8 (MOI = 0.5) after cells transfected with plasmid. The β-gal normalizing system was introduced into each transfection. Each reaction contained 20 μg of protein extracted from the cells. The results represented the mean ± S.D. (error bars) from triplicate samples in three separate transfections at 48 hpt.

It has been reported that the infection of Bm-N cells with BmNPV could be enhanced by 3.2 times in the presence of CTAB (Cheng and Hou 1992). Recently, CTAB has been found to increase the BV titer by 3.7 times in BmNPV-infected Bm-N cells (Zhou et al. 2002). GP64, which is peculiar to BV, is a major virion envelope glycoprotein of baculoviruses. It is thought to play an important role in viral infection, mediating penetration of BV form into host cells through the endocytic pathway (Jarvis and Garcia 1994). Furthermore, when a foreign protein is fused at a suitable site to a copy of the entire coat protein GP64 or, alternatively, to just the membrane anchor of GP64, it is packaged into the viral coat and is present on the surface of baculovirus particles and infected insect cells. Thus, fusion to an endogenous viral glycoprotein provides the mechanism for the display of proteins on the virus and infected cell surface. The GP64 is considered suitable as the basis for the display of fusion proteins (Grabherr et al. 2001). The expression of *gp64* is regulated transcriptionally by both early and late promoter, and the *gp64* early promoter could be transactivated by IE-1 or nuclear extracts from virus-infected cells (Blissard and Rohrmann 1991; Glocker et al. 1992). CTAB appeared to stimulate *ie-1* promoter transcription (Zhou et al. 2002). In the present work, the luciferase activity significantly increased by 5.21 times by co-treatment with CTAB and BmNPV-ZJ8 in which the virus treatment alone augmented the enzymatic activity by 2.27 times. However, CTAB treatment alone did not increase the enzymatic activity significantly. Thus, we presume that CTAB stimulated some viral factors, such as IE-1, in turn to transactivate the *gp64* early promoter transcription. This transactivation of *gp64* early promoter may play one or more roles in the increase of BVs production by CTAB treatment.

Conclusion

Transient transcriptional activity of the BmNPV *gp64* promoter was effectively stimulated by CTAB me-

diated by viral factors from BmNPV-ZJ8. It implies that the stimulation of transcription of *gp64* promoter might result in the increase in BVs production when Bm-N cells are infected with BmNPV. Therefore, this work may provide some information for improvement of the production of stock virus in baculovirus expression vector system and complex eukaryotic proteins of *gp64* promoter-based constructs in baculovirus surface display system.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 30271007) and the National High Technology Research and Development Program of China (863 Program) (No.102-11-02-06).

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