Int. J. Indust. Entomol. Vol. 4, No. 2, 2002, pp. 137~141

International Journal of
Industrial Entomology

Effects of Insect Hormones on the Replication of Nucleopolyhedrovirus

Ya Jing Zhou[1,2], Yong Zhu Yi[1], Qing Li Xiao[1], Zhi Fang Zhang[1,*], Jia Lu He[1] and Yuan Xing Zhang[2]

[1] *Key Laboratory of Silkworm Biotechnology, Ministry of Agriculture, Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, Jiangsu, China*

[2] *State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, P. R. China.*

(Received 11 March 2002; Accepted 26 April 2002)

An experimental study was undertaken to quantify the effects of insect hormones on the replication of nucleopolyhedrovirus (NPV). The results demonstrated that $TCID_{50}$ at 72 h post-infection (hpi) rose systematically from 0.55×10^8/ml, for untreated cells, up to 1.67×10^8/ml at 3 μg/ml, then dropped down to 1.45×10^8/ml at 4 μg/ ml, by adding ecdysone to the culture medium for Bm-N cells infected with a wild-type *Bombyx mori* nucleopolyhedrovirus (BmNPV). The optimum enhancement of about 3 times on budded virus (BV) titer at 72 hpi was given at 3 μg/ml of ecdysone. While the polyhedra number had no obvious variation within the range of concentrations from 0 to 4 μg/ml. By addition of juvenile hormone analogue (JHA) into the media with this concentration range, the BmNPV $TCID_{50}$ and polyhedra number at 72 hpi did not show significant changes. Also, the addition of either 3 μg/ml of ecdysone or 3 μg/ml of JHA to the culture media did not appear to affect the $TCID_{50}$ and polyhedra number significantly in infected Sf-21 cells with the *Autographa californica* nucleopolyhedrovirus (AcMNPV).

Key words : Baculovirus, Insect cells, Ecdysone, Juvenile hormone analogue, Replication

Introduction

Both *Bombyx mori* nucleopolyhedrovirus (BmNPV) and *Autographa californica* nucleopolyhedrovirus (AcMNPV) contain an *egt* gene encoding ecdysteroid UDP-glycosyltransferase (EGT). EGT with a molecular weight of 60 kD, consisting of 506 amino acid residues, can catalyze the glycosyltransfer reaction between UDP-glucose and ecdysone to form 22-O-β-D-pyranoglucoside (O'Reilly and Miller, 1991; O'Reilly *et al.*, 1991). This resulted in the inactivation of ecdysone and the interruption of hormone equilibrium between ecdysone and juvenile hormone (JH) inside insect larvae (O'Reilly and Miller, 1989; Wang *et al.*, 2000).

Insect hormones, especially ecdysone secreted from prothoracic gland (ptg) and JH secreted from corpus allatum (ca), mainly function as regulation of growth and development in insect life cycle. They control the ecdysis and metamophosis following the variations of ecdysone and JH inside larvae cyclically. The silkworm pupae infected with AcMNPV appear in a state of artificially induced "diapause pupa" and can not be into eclosion because of this glycosyltransfer reaction (Zhang *et al.*, 1993). By treatment with ecdysone, the physiological balance of these pupae can be recovered and then be into eclosion again.

In addition, insect hormones appear to influence the synthesis of DNA, RNA, and proteins in larvae. Treatment with ecdysteroid and juvenile hormone analogue (JHA) onto the silkworm larvae at fifth-instar increased the silk production up to 6% (Cui *et al.*, 1993). For the posterior silk gland cells cultured *in vitro*, the activity of their absorption of the labeled precursors of nuclear acid and protein could be efficiently enhanced by treatment with ecdysone and JHA (Cui *et al.*, 1991).

It has been previously reported that applying JHA onto the fifth-instar silkworm larvae or pupae augmented the foreign gene expression level or polyhedra number by 16% or 35%, respectively (Wang *et al.*, 2001). Ecdysone could also stimulate the foreign gene expression and viral

*To whom correspondence should be addressed.
Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, 212018, Jiangsu, China.
Tel: 86-511-5616659; Fax: 86-511-5615044;
E-mail: zjsbsri@public.zj.js.cn

replication efficiently in the silkworm baculovirus expression vector system (Silkworm -BEVS) through affecting the physiological balance of endogenous hormones in the host (Zhang *et al.*, 2001). It is uncertain whether applying insect hormones onto the host cells cultured *in vitro* influences the nucleopolyhedrovirus (NPV) replication too. In present study, we investigated the effects of insect hormones on the NPV replication by adding ecdysone or JHA into the culture media of infected host cells with BmNPV or AcMNPV, respectively.

Materials and Methods

Reagents and chemicals

The reagents and chemicals used throughout this study were purchased from Life Technologies (Gaithersburg, MD, USA) and Sigma Chemical (St. Louis, MO, USA), unless stated otherwise.

Cell line and cell culture

The *Bombyx mori* cell line Bm-N and the *Spodoptera frugiperda* cell line Sf-21, maintained in the Key Laboratory of Silkworm Biotechnology, Ministry of Agriculture, P. R. China, were cultured with TC-100 medium supplemented with 10% fetal bovine serum. The cells were incubated at 27°C and subcultured every 3 - 5 days using an appropriate split ratio. The details for cell culture were referred to Summers and Smith (1987).

Virus stock and insect hormones

The wild-type *Bombyx mori* nucleopolyhedrovirus Bm NPV-ZJ8 and AcMNPV were kindly provided by Professor Xiangfu Wu (Shanghai Institute of Biochemistry, Chinese Academy of Sciences, P. R. China). The methods for viral propagation and maintenance were undertaken as Summers and Smith (1987). The insect ecdysone used throughout this work was 20-β-hydroxyecdysone prepared by Sericultural Research Institute, Chinese Academy of Agricultural Sciences, P. R. China. The JHA ZR 512 was kindly provided by Professor Weizheng Cui (Shandong Agricultural University, P. R. China).

Virus infection and hormone treatment

To examine the effect of hormones on the virus infection, a series of 15 cm^2 flasks were seeded at a density of 1×10^6 cells per flask and the cells were allowed to attach for 12 hrs. After the cells were attached, the medium was removed and 1 ml serum-free medium containing appropriate amount of virus was added (MOI = 0.5) for infection. The cells were incubated at 27°C for 1 hr. Then the inoculum was removed and the cells were washed once with 1 ml medium and then 3 ml conditioned medium containing different concentrations of ecdysone or JHA was added. The cells were incubated at 27°C for 3 days. The treatment without hormones was made as the control. The supernatant was collected for determination of the $TCID_{50}$ value and the infected cells were used for measurement of polyhedra.

Virus titer and polyhedra measurement

The virus titer was measured using an end-point dilution assay (Summers and Smith, 1987). The polyhedra inside cell nuclei were measured microscopically using a hemocytometer (BOECO, Germany) after sonication by a supersonic oscillator (SONIPREP 150, England) in an ice-bath for 10 min.

Results

Effects of insect hormones on the BmNPV replication in infected Bm-N cells

Effects of ecdysone

On $TCID_{50}$: To investigate the effects of ecdysone on the budded virus (BV) titer, different dose of ecdysone was added in the culture medium for Bm-N cells infected with the BmNPV-ZJ8, respectively. The variation of $TCID_{50}$ at 72 hpi was shown in Fig. 1. $TCID_{50}$ increased linearly from 0.55×10^8/ml, for untreated cells, up to 1.67×10^8/ml in the presence of 3 μg/ml of ecdysone, then decreased to 1.45×10^8/ml at 4 μg /ml of ecdysone. This suggested that the treatment with 3 μg/ml of ecdysone gave an optimum enhancement on BV propagation.

To observe the variation of $TCID_{50}$ to time, 3 μg/ml of ecdysone was added to the medium. Once the infection initiated, 100 μl of supernatant was collected for $TCID_{50}$

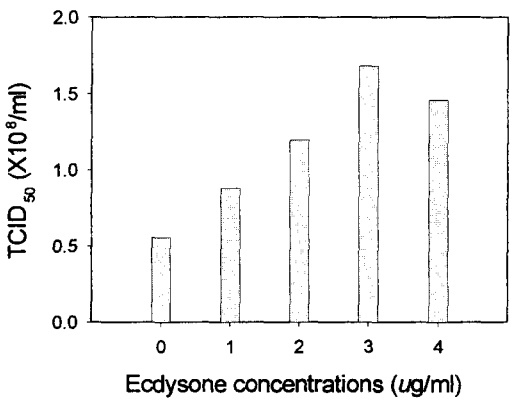

Fig. 1. Dose dependent effects of ecdysone on the $TCID_{50}$ in Bm-N cells after infection with the BmNPV-ZJ8.

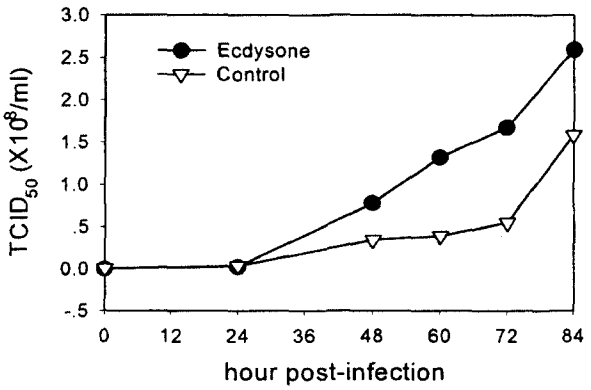

Fig. 2. The curves of $TCID_{50}$ to time in Bm-N cells after infection with BmNPV-ZJ8.

Table 1. Effects of different dose of ecdysone on the polyhedra inside nucleus of Bm-N cells with BmNPV-ZJ8 at 72 hpi

Ecdysone concentrations (μg/ml)	0.00	1.00	2.00	3.00	4.00
Polyhedra number ($\times 10^6$/ml)	3.75	3.73	3.45	3.35	3.90

measurement every 24 hrs. Then the sample was made every 12 hrs from 48 hpi. Fig. 2 showed that $TCID_{50}$ had no changes at 24 hpi. After 24 hpi, it markedly increased and the best enhancement of about 3 times, compared with the control, was achieved at 72 hpi. Ecdysone appeared to enhance the BV production of BmNPV efficiently.

On polyhedra number : Whether does the ecdysone influence the polyhedra propagation of BmNPV inside nucleus of infected cells, too? Different dose of ecdysone ranging from 0 to 4 μg/ml was added to the cultured medium. The polyhedra number at 72 hpi was shown in Table 1. Within the investigating range of concentrations, the polyhedra number fluctuated between 3.35×10^6/ml and 3.90×10^6/ml irregularly. It suggested that ecdysone had no significant effects on the polyhedra propagation in infected Bm-N cell nucleus with the BmNPV-ZJ8.

Effects of JHA

It was previously reported that treating fifth-instar larvae with JHA augmented the foreign gene expression and polyhedra propagation inside infected host silkworms with a recombinant or wild-type BmNPV (Wang *et al.*, 2001). Whether does the JHA enhance the BmNPV replication in host cells, too? Different dose of JHA was added in the Bm-N cell medium after infection with the BmNPV-ZJ8. Table 2 revealed that $TCID_{50}$ undulated between 1.64×10^8/ml and 2.10×10^8/ml within the ranging of concentrations of JHA from 0 to 4 μg/ml. JHA did not appeared to affect the $TCID_{50}$ markedly, compared with the ecdysone. The polyhedra number undulated between 3.00×10^6/ml and 3.80×10^6/ml irregularly within 0-3 μg/ml of JHA concentrations. At 4 μg/ml of JHA, it appeared a slight suppression on polyhedra production.

Table 2. Effects of different dose of JHA on the $TCID_{50}$ and polyhedra inside nucleus of Bm-N cells with BmNPV-ZJ8 at 72 hpi·

JHA concentrations (μg/ml)	0.00	1.00	2.00	3.00	4.00
$TCID_{50}$ ($\times 10^8$/ml)	1.90	1.85	1.64	2.10	1.90
Polyhedra ($\times 10^6$/ml)	3.48	3.80	3.00	3.45	2.88

Table 3. Effects of ecdysone and JHA on the $TCID_{50}$ and polyhedra inside nucleus of Sf-21 cells with AcMNPV at 72 hpi

	Control	Ecdysone (3 μg/ml)	JHA (3 μg/ml)
$TCID_{50}$ ($\times 10^8$/ml)	0.76	1.03	0.76
Polyhedra ($\times 10^6$/ml)	5.29	3.85	4.72

Effects of insect hormones on the AcMNPV replication in infected Sf-21 cells

To investigate the effects of ecdysone and JHA on AcMNPV replication in the host Sf-21 cells, 3 μg/ml of ecdysone or JHA was added to the medium respectively after infection with the AcMNPV. The variation of $TCID_{50}$ and polyhedra number were shown in Table 3.

By addition of 3 μg/ml of ecdysone, over the control the $TCID_{50}$ increased from 0.76×10^8/ml up to 1.03×10^8/ml while polyhedra decreased from 5.29×10^6/ml down to 3.85×10^6 /ml with a slight difference which was compared with that in Bm-N cells with BmNPV-ZJ8. By addition of 3 μg/ml of JHA, either $TCID_{50}$ or polyhedra number had no significant changes.

Discussion

Faulkner (1981) and Kelly (1982) reported that baculoviruses had a unique, bi-phasic replication cycle. After the infection of insect cells, BVs are produced and released into the medium by budding through the cell membrane, causing cell to cell secondary infection. Later in the infection stage, this process switches to the occlusion of the OV particles in newly synthesized polyhedra in the cell nucleus. Finally, the infected cells disintegrate, releasing the polyhedra. In the present study, ecdysone showed an enhancement on BV propagation with no significant

effects on polyhedra production in infected Bm-N cells with BmNPV (Fig. 1, Fig. 2, and Table 1).

In a coordinately regulated cascade of gene transcription initiated by a hormone, it specially combines with cytoplasmic acceptor protein to form a complex. After this complex is bound to the specific DNA-binding motif (hormone response element, HRE), gene transcription initiates (Yao *et al.*, 1993; Lü, 1998). Therefore, only special hormone can activate gene transcription in special cell lines and this gene product is needed for cells and there exists this acceptor in cells indeed. This specialness might be one of the reasons why ecdysone enhanced the BV propagation only in Bm-N cells infected with BmNPV-ZJ8 while not in Sf-21 cells with AcMNPV (Table 3).

During the virus infection, viral genes are expressed in a coordinately regulated cascade fashion and divided into four general classes based on their kinetics of expression (Friesen and Miller, 1986; Blissard and Rohrmann, 1990): immediate-early, delayed-early, late, and very late. The early and late phases of gene expression are separated by viral DNA replication. Late gene transcription begins at or near the onset of viral DNA replication (Grula *et al.*, 1981; Fuchs *et al.*, 1983; Hur and Weaver, 1990). Early genes are transcribed by host RNA polymerase II: therefore no viral gene products are necessary for the expression of early genes (Fuchs *et al.*, 1983; Hoopes and Rohrmann, 1991). IE-1, the best-characterized transcriptional activator, encoded by an immediate-early gene of baculovirus, has been shown to function as transcriptional regulator to activate the expression of some early viral genes, such as *39K* and *p35*, as well as that of itself (Guarino and Summers, 1986, 1987; Kovaos *et al.*, 1992). It also appears to be required for viral DNA replication (Kool *et al.*, 1994; Lu and Miller, 1995). The BmIE1, a protein product of immediate early-1 gene of BmNPV, can stimulate the promoter of the *39K* gene of AcMNPV (Huybrechts *et al.*, 1992). As a co-activator of the cytoplasmic actin gene promoter of the silkmoth *Bombyx mori* in transfected cells, the BmIE1 increased the level of transcription from this promoter by two orders of magnitude (Lu *et al.*, 1996). We found that via a transient expression assay system, the treatment with 3 μg/ml of ecdysone on uninfected Bm-N or Bm-5 cells, transfected with a plasmid containing *luciferase* reporter gene that is under the control of the *ie-1* promoter, could stimulate the *ie-1* promoter transcriptional activity by about 5-fold. By the treatment with 3 μg/ml of JHA, the *ie-1* promoter transcriptional activity could be enhanced about 2-fold (the manuscript has been accepted by Bioscience, Biotechnology, and Biochemistry). In this work, ecdysone treatment increasing the BmNPV titer (Fig. 1 and Fig. 2) was possibly due to some products of immediate-early genes, such as IE-1, that interrupted the equilibrium of cascade fashion regulated by the early genes, resulting in the enhancement of related genes associated with the BV production. Although the JHA treatment could also enhance the *ie-1* promoter transcriptional activity, at the same time, it might stimulate some negative factors that are unfavorable for BV production. The counterbalance of these two opposite effects resulted in no marked effects of JHA on the BmNPV titer apparently (Table 2).

GP64, which is peculiar to BV, is a major virion envelope glycoprotein of some baculoviruses. It plays an important role in viral infection, mediating penetration of BV form into host cells through the endocytic pathway (Jarvis and Garcia, 1994). *gp64* early promoter could also be transactivated by IE-1 (Blissard and Rohrmann, 1991). In the present work, it is uncertain whether ecdysone affected the transient transcriptional activity of *ie-1* promoter from BmNPV, in turn to transactivate the *gp64* early promoter, resulting in a rise in BV titer in Bm-N cells infected with BmNPV. The real mechanism for insect hormones to affect the replication of NPV in host cells needs addressing further.

Acknowledgement

This work was supported by the National Natural Science Foundation (Project No: 39970571) and "863" High Technology Project of China.

References

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

Blissard, G. W. and G. F. Rohrmann (1991) Baculovirus gp64 gene expression: analysis of sequences modulating early transcription and transactivation by IE-1. *J. Virol.* **65**, 5820-5827.

Cui, W. Z., Z. D. Wu and J. L. Xu (1991) Effects of juvenile hormone and ecdysone on absorption of the marked precursors of nuclear acid and protein in the silkworm posterior silk gland *in vitro*. *Acta Sericologica Sinica* **17**, 39-44.

Cui, W. Z., Y. Y. Zhang, F. Y. Liu and J. L. Xu (1993) Effects of using juvenile hormone mixed with molting hormone on silkworm development and silk production. *Acta Sericologica Sinica* **19**, 236-238.

Faulkner, P. (1981) Baculovirus; in *Pathogenesis of invertebrate microbial diseases*. Davidson, E. W. (ed.), pp. 3-37, Alanheld, Osmun & Co., Totowa, NJ.

Fuchs, L. Y., M. S. Woods and R. F. Weaver (1983) Viral transcription during *Autographa californica* nuclear polyhedro-

sis virus infection: A novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. *J. Virol.* **48**, 641-646.

Friesen, P. D. and L. K. Miller (1986) The regulation of baculovirus gene expression; in *Current topics in microbiology and immunology: the molecular biology of baculovirus.* Doerfler, W. and P. Boehm (eds.), 131. pp. 31-49, Springer-Verlag, Berlin.

Grula, M. A., P. L. Buller and R. F. Weaver (1981) Alpha amanitin-resistant viral RNA synthesis in nuclei isolated from nuclear polyhedrosis virus-infected *Heliothis zea* larvae and *Spodoptera frugiperda* cells. *J. Virol.* **38**, 916-921.

Guarino, L. A. and M. D. Summers (1986) Functional mapping of a trans-activating gene required for expression of a baculovirus delayed-early gene. *J. Virol.* **57**, 563-571.

Guarino, L. A. and M. D. Summers (1987) Nucleotide sequence and temporal expression of a baculovirus regulatory gene. *J. Virol.* **61**, 2091-2099.

Hur, N. E. and R. F. Weaver (1990) Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol.* **71**, 195-202.

Hoopes, R. R. and G. F. Rohrmann (1991) *In vitro* transcription of baculovirus immediate early genes: Accurate mRNA initiation by nuclear extracts from both insect and human cells. *Proc. Natl. Acad. Sci.* U. S. A. **88**, 4513-4517.

Huybrechts, R., L. Guarino, M. V. Brussel and V. Vulsteke (1992) Nucleotide sequence of a transactivating *Bombyx mori* nuclear polyhedrosis virus immediate early gene. *Biochim. Biophys. Acta* **1129**, 328-330.

Jarvis, D. L. and J. R. A. Garcia (1994) Biosynthesis and processing of the *Autographa californica* nuclear polyhedrosis virus gp64 protein. *Virology* **205**, 300-313

Kelly, D. C. (1982) Baculovirus replication. *J. Gen. Virol.* **63**, 1-13.

Kovaos, G. R., J. Choi, L. A. Guarino and M. D. Summers (1992) Functional dissection of the *Autographa californica* nuclear polyhedrosis virus immediate-early 1 transcriptional regulatory protein. *J. Virol.* **66**, 7429-7437.

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

Lu, A. and L. K. Miller (1995) The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication. *J. Virol.* **69**, 975-982.

Lu, M., R. Johnson and K. Iatrou (1996) Trans-activation of a cell housekeeping gene promoter by the IE1 gene product of baculoviruses. *J. Virol.* **218**, 103-113.

Lü, H. S. (1998) Molecular Biology of Insect Viruses. China Agricultural Scientech Press, Beijing, China.

OReilly, D. R. and L. K. Miller (1989) A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. *Science* **245**, 1110-1112.

OReilly, D. R. and L. K. Miller (1991) Improvement of a baculovirus pesticide by deletion of the EGT gene. *Biotechno.* **9**, 1086-1089.

OReilly, D. R., O. W. Howarth, H. H. Rees and L. K. Miller (1991) Structure of ecdysone glucoside formed by a baculovirus ecdysteroid UDP-glucosyltransferase. *Insect Biochem.* **21**, 795-802.

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

Wang, H. W., Z. F. Zhang, W. G. Li and J. L. He (2000) Regulation effect of baculovirus *egt* gene on the expression of insect hormone. *Acta Sericologica Sinica* (supplement) **26**, 102-106.

Wang, H. W., Z. F. Zhang, Q. L. Xiao, W. G. Li and J. L. He (2001) Insect juvenile hormone enhancing gene expression in silkworm baculovirus vector system. *Chinese J. Biotechnol.* **17**, 590-593.

Yao, T. P., B. M. Forman, Z. Jiang, L. Cherbas, J. D. Chen, M. McKeown, P. Cherbas and R. M. Evans (1993) Functional ecdysone receptor is the product of ECR and ultraspiracle genes. *Nature* **366**, 476-479.

Zhang, Z. F., J. L. He, N. M. Zhou, G. Hua and X. F. Wu (1993) Replication of *Autographa californica* nuclear polyhedrosis virus and expression of recombinant baculovirus foreign gene in silkworm pupae. *Acta Sericologica Sinica* **19**, 203-207.

Zhang, Z. F., J. L. He, B. Yao, Y. J. Zhou, Y. Chen and Y. Z. Yi (2001) China Patent Application No: 01127289.9.