Foreign Insect Hormones Stimulating the Transcription of the ie-1 Promoter of Bombyx mori Nuclear Polyhedrosis Virus in Vivo and in Vitro

Yajing Zhou,1,2 Qingli Xiao,1 Zhifang Zhang,1,4 Jialu He,1 and Yuanxing Zhang2

1Key Laboratory of Silkworm Biotechnology, Ministry of Agriculture, Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, 212018, Jiangsu, China
2State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China

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Via a transient expression assay system, an experimental study was undertaken to characterize the effects of insect ecdysone and juvenile hormone analogue on the transient expression of the luciferase gene under the control of the immediate-early gene (ie-1) promoter of Bombyx mori nuclear polyhedrosis virus. The results demonstrated that the transcriptional activity of the ie-1 promoter was increased to a certain extent by different insect hormone treatments in uninfected insect cells or fifth instar silkworm larvae transfected with a plasmid containing a luciferase gene driven by the ie-1 promoter. By ecdysone treatment alone, an increase of 5–7-fold was reached in Bm-N, or Bm-5 cells, or in the early developmental stage of fifth instar larvae. By treatment with juvenile hormone analogue alone, about 2-fold, in Bm-N, Bm-5, and Sc-21 cells, or about 5-fold increase in the middle developmental stage of larvae was given, respectively. By co-treatment with ecdysone and juvenile hormone analogue, the increase was given between that of ecdysone and juvenile hormone analogue treatment alone. In addition, the synergistic effects of foreign/endogenous hormones on the activity of ie-1 promoter are discussed.

Key words: Bm-N cell line; ecdysone; ie-1 promoter; juvenile hormone analogue; silkworms

Baculoviruses replicate in arthropods, specifically in lepidopteran insects. In the replication cycle, eubaculovirinae have two types of morphology, the budded virus (BV) phenotype and occluded virus (OV) particles which are enveloped nucleocapsids embedded within occlusion bodies.1–2) The BV causes cell to cell infection in vivo and in vitro and the OV causes host to host infection.3–4) During infection, viral genes are expressed in a coordinately regulated cascade fashion and divided into four general classes based on their kinetics of expression5–6): immediate-early, early-delayed, 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.7–9) Early genes are transcribed by host RNA polymerase II; therefore no viral gene products are necessary for the expression of early genes.8,10

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.11–13) It also appears to be required for viral DNA replication.14–15) The BmIE1, a protein product of the immediate early-1 gene of Bombyx mori nuclear polyhedrosis virus (BmNPV), can stimulate the promoter of the 39K gene of Autographa californica nuclear polyhedrosis virus (AcMNPV).16) As a co-activator of the cytoplasmic actin gene promoter of the silkworm Bombyx mori in transfected cells, the BmIE1 increased the level of transcription from this promoter by two orders of magnitude.17)

Conventional baculovirus expression vectors are recombinant viruses that can express a foreign gene in insect cells under the control of the polyhedrin promoter, which provides high-level transcription during the very late phase of infection. Unlike the polyhedrin promoter, the ie-1 promoter is active in uninfected cells, in the absence of any other viral factors.11,12) Therefore, ie-1 promoter based-constructs can be used to isolate genetically transformed insect cells. This nonlytic insect cell expression system provides a high-level expression of recombinant proteins under ie-1 control without viral infection, with the additional advantage of continuous production in a cellular environment in contrast to that generated by
a baculovirus infection. Insect hormones, especially ecdysone and juvenile hormone, mainly function as regulators of developmental events during the insect life cycle, to control the ecdysis and metamorphosis of the insect, and influence the synthesis of DNA, RNA, and related proteins in larvae. Co-treatment with ecdysteroid and juvenile hormone analogue (JHA) of silkworm larvae at fifth instar increased the silk production up to 6%. 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 increased by co-treatment with ecdysone and JHA.

It has been previously reported that JHA treatment of fifth instar silkworm larvae or pupae can augment the foreign gene expression level or polyhedra numbers by 16% or 35% respectively. Ecdysone could also stimulate the foreign gene expression and viral replication efficiently in the Bombyx mori baculovirus expression vector system (Bm-BEVS). It is uncertain whether applying insect hormones onto the host influences the transcriptional activity of the ie-1 promoter, then sets off a chain reaction of other genes depending on the presence of the ie-1 product. In this study, we investigated the effects of ecdysone and JHA on the expression of the luciferase gene under the control of the BmNPV ie-1 promoter through using a transient expression assay system, from which we deduced the effects of foreign insect hormones on the transcription of the ie-1 promoter.

**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 otherwise stated.

**Reporter plasmid.** The reporter plasmid containing a luciferase gene under the control of the BmNPV ie-1 promoter, pBmIE1-Luc, was previously constructed in our laboratory. Purified BmNPV DNA and two primers, 5’-TAG AAT TCA TCC CAA CGG CGC AGT GTA-3’ (forward) and 5’-ATG GAT CCA ATA GTC GTA TGG TCC ACG-3’ (reverse), were used for amplification of the ie-1 promoter region. These primers included EcoRI and BamHI restriction enzyme sites at their 5’ ends. The product of a 360-bp fragment of the ie-1 promoter amplified by polymerase chain reaction (PCR) was cloned into the EcoRI/BamHI sites of the pGEM3Z (+). The plasmid pUL220 containing an entire luciferase gene (1.8-kb) was digested with BamHI. The digested luciferase fragment was then placed under the control of the ie-1 promoter in the right orientation.

**Cell line and cell culture.** The Bombyx mori cell line (Bm-N, Bm-5) and the Spodoptera frugiperda cell line (Sf-21), maintained in the Key Laboratory of Silkworm Biotechnology, Ministry of Agriculture, Peop. Rep. China, were cultured with TC-100 medium with 10% fetal bovine serum. The cells were incubated at 27°C and subcultured every 3–5 days using a split ratio of 1: 2–3. The details of cell culture were referred to Summers and Smith's. 

Ecdysone, JHA, and silkworm variety. Used throughout this work, the insect ecdysone was 20β-hydroxyecdysone prepared by the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Peop. Rep. China. The juvenile hormone analogue ZR512 was kindly provided by Professor Weizheng Cui (Shandong Agricultural University, Peop. Rep. China). JY1, the overexpression variety of silkworm, maintained in our laboratory, was routinely grown at 25–26°C.

**Transient expression in uninfected cells or silkworm larvae and hormone treatments.** The cells were seeded into 15-cm² flasks at a density of about 5 × 10⁶ cells/ml (3 ml per flask) and cultured for 24 h then transfected with 60 µl of transfection solution containing 5-µl lipofectin and 1-µg plasmid DNA in 1 ml of serum-free medium for 4–6 h. To generate a cell line transiently expressing luciferase, cells were incubated for another 48 h at 27°C by replacing serum-free medium with 3 ml of conditioned medium containing 3 µg/ml of ecdysone, or JHA, or a mixture of 3 µg/ml of ecdysone and 3 µg/ml of JHA. The treatment without hormone was made as a control. Each treatment was repeated 3 times.

Similarly, each larva at fifth instar was injected with 10-µl transfectional solution into the larval hemolymph containing 5-µl lipofectin and 1-µg plasmid DNA at different stages after molting through different treatments with ecdysone or JHA. In silkworm larvae rearing and silk production, the traditional application protocol of insect hormones is through feeding ecdysone by spreading it onto the mulberry leaves, or spreading JHA onto the surface of the larval bodies, at an appropriate concentration, to increase silk production. We previously investigated the effects of insect hormones on foreign gene expression in the fifth instar silkworm larvae infected with a recombinant baculovirus. Spreading JHA onto the surface of the larval bodies and injection or feeding ecdysone onto the larvae effectively augmented foreign gene expression. In this work, therefore, the protocols of applying hormones were decided with reference to these mentioned as above, and we made some changes. Each larva was treated with hormones through directly injecting it with different doses of ecdysone, or feeding ecdysone by spreading it onto the mulberry leaves 12 h before
transfection, or spreading 100 ppm of JHA onto the surface of the larval bodies at 12 h before transfection, or injecting with ecdysone together with JHA treatment by spreading 100 ppm of JHA onto the larval bodies at 12 h before transfection. A treatment with sterile water was done a control. Each treatment consisted of at least 3 separated groups (selecting 5 os with about the same weight as one group) and was repeated three times. The Bm-N, Bm-5, Sf-21, and hemolymph cells in larvae at 48 h post-transfection (hpt) were harvested by centrifugation at 10,000 g for 5 min at 4°C and were ready for the luciferase activity assay.

Measurement of the luciferase activity. The cell extracts were prepared with a kit from Promega (Cat. E4030). The harvested cells were washed twice by being resuspended in phosphate buffered saline (PBS), then centrifuged at 6,000 g for 5 min at 4°C. After washing, the cells were lysed by a single freeze-thaw cycle at −70°C with the kit. The lysate, melted in water-bath at 37°C, was centrifuged at 4°C to remove cell debris and supernatant in an ice-bath was used for the luciferase assay. The measurements on three separate experiments were taken in triplicate using a liquid scintillation spectrometer (Beckman LS6000 Series, USA). The amount of protein in the lysate was measured using the Bradford method as described. The data were analyzed by Statistical Analysis System (SAS).

Results

Effects of insect hormones on the transcriptional activity of the ie-1 promoter in uninfected cells

To examine whether the foreign hormones can increase the transcriptional activity of the BmNPV ie-1 promoter in uninfected cells, we investigated the effects of different insect hormone treatments on the expression level of luciferase under the BmNPV ie-1 promoter control, in three cell lines (Bm-N, Bm-5, and Sf-21) by using a transient expression assay system. We had found that adding 3 μg/ml of ecdysone to medium gave an optimum increase of about 3 times of the titer of budded virus (BV) in Bm-N cells infected with a wild type BmNPV. In this work, the applied quantity of hormones was defined as 3 μg/ml of ecdysone and 3 μg/ml of JHA by adding them to the medium together or separately.

The luciferase assays, from 3 μg of protein extracted from Bm-N or Bm-5 cells, or 2 μg of protein extracted from Sf-21 cells, are shown in Fig. 1. The analysis of variance procedure by SAS showed (procedure not shown) that, by addition of 3 μg/ml of ecdysone alone to the medium, the luciferase activity at 48 hpt was significantly increased by about 5-fold in Bm-N or Bm-5 cells, or about 3-fold in Sf-21 cells, compared with the control. By addition of 3 μg/ml of JHA alone to the medium, about a 2-fold increase appeared, with significant differences in the three cell lines (F value = 13.17, Pr>F = 0.0001 < 0.01). When a mixture of 3 μg/ml of ecdysone and 3 μg/ml of JHA was added to the medium, the index of increase in Bm-N cells was near that by JHA treatment alone, while in Bm-5 or Sf-21 cells, the index of increase was near that by ecdysone treatment alone, with no significant differences. However, the trend of the increase was all between that of ecdysone and JHA treatment alone in these cells. These results indicated that the foreign insect hormone treatments, especially ecdysone treatment alone, efficiently increased the transcription of ie-1 promoter in uninfected cells.

Effects of insect hormones on the transcriptional activity of ie-1 promoter in silkworms during the stage of fifth instar larvae

Effects of ecdysone

We had investigated the effects of insect hormones on the foreign gene expression in the fifth instar silkworm larvae infected with a recombinant baculovirus, and found that 4-μg ecdysone per os or 100 ppm JHA treatment optimally augmented phytase gene expression. In this study, each larva was injected with or fed on 4 μg of ecdysone at 36 h (an early developmental stage), or 72 h (a middle developmental stage), or 108 h (a late developmental stage) after molting of fifth instar larvae, then transfected with 10 μl of transfection solution 12 h later.
Figure 2 showed that, through the analysis of variance procedure by SAS, the activity of luciferase at 48 hpt was significantly increased by about 5.1, 3.2, or 2.6-fold, respectively, by injecting 4 µg of ecdysone per os at different developmental stages. Meanwhile, the activity of luciferase was significantly increased by about 4.3, 2.5, or 1.9-fold, respectively, by feeding 4 µg of ecdysone per os (F value = 29.97, Pr > F = 0.0001 < 0.01). The best time for applying ecdysone was at an early developmental stage (36 h after molting) compared with the other developmental stages, and the effect caused by ecdysone injection was better than that by feeding. These data indicated that the ecdysone treatment could also stimulate the transcription of ie-1 promoter in uninfected silkworm larvae efficiently.

Because the cellular environment in the transient expression system differed greatly from that generated by a baculovirus infection, we investigated whether the ecdysone dose-dependent effect in transient expression systems differs from that in baculovirus expression system. Each larva was injected in with 0, 0.5, 1, 2, 3, 4, 5, or 6 µg of ecdysone, or was fed with 0, 2, 4, or 6 µg of ecdysone, at 36 h after molting of the fifth instar, then transfected with 10 µl of transfection solution 12 h later.

Figure 3 presents the transient expression activity of luciferase at 48 hpt, which markedly rose following the increase of injected ecdysone dose per os from 1 to 4 µg, then decreased slowly from 4 to 6 µg. And the greatest increase, of about 7-fold, was reached at 4 µg of ecdysone per os. No significant differences were observed from 0 to 1 µg (F value = 0.38, Pr > F = 0.7243). From Fig. 4, the greatest increase was also reached at 4 µg by feeding with ecdysone per os. However, this increase, about 4.3-fold, was lower than that by direct injection. These results were in agreement with that in the baculovirus expression system.

Effects of JHA

To examine the effects of JHA on the activity of the ie-1 promoter, the larvae were spread with 100 ppm of JHA onto their bodies at 36 h (an early developmental stage), 72 h (a middle developmental stage), and 108 h (a late developmental stage) after molting of fifth instar larvae, then transfected with 10 µl of transfection solution 12 h later. Figure 5 shows that the largest increase over the control, about 5.1-fold at 48 hpt, with JHA treatment at 72 h after molting, i.e., a middle developmental stage during the fifth instar, was obtained, compared with the treatment at 36 h (1.7-fold) or 108 h (2-fold) after molting, with significant differences (F value = 16.5, Pr > F = 0.0016 < 0.01).

Effects of co-treatment with ecdysone and JHA

Similar to the trend in uninfected cells, the co-
Fig. 4. Dose-dependent Effects of Ecdysone Given by Feeding on the Transcriptional Activity of BmNPV ie-1 Promoter in Larvae.

Stimulating folds of luciferase activity are indicated on the Y axis over the control. Ecdysone dose is indicated on the X axis as \( \mu \text{g/os} \). The control with sterile water treatment was arbitrarily set at 1.0. Each larva was fed with a different dose of ecdysone at 36 h after molting of fifth instar larvae and then injected with 10 \( \mu \text{l} \) of transfection solution 12 h later. Each reaction contained 3 \( \mu \text{g} \) of protein extracted from larval hemolymph cells. The results represented averages from three separate treatments.

Fig. 5. Effects of Different JHA Treatments on the Transcriptional Activity of BmNPV ie-1 Promoter in Larvae.

The increase of luciferase activity is indicated on the Y axis as stimulating folds over the control with sterile water treatment, which was arbitrarily set at 1.0. Different JHA treatments are indicated on the X axis. Each reaction contained 3 \( \mu \text{g} \) of protein extracted from larval hemolymph cells. The results represented averages from three separate treatments. JHA indicates the treatment with JHA; JHA/36 h, JHA/72 h, and JHA/108 h represent that 100 ppm of JHA was spread onto the larval bodies at 36, 72, and 108 h after molting, then transfection solution was injected 12 h later, respectively.

treatment by injection with ecdysone together with JHA treatment by spreading 100 ppm of JHA onto the larval bodies, then transfection with 10 \( \mu \text{l} \) of transfection solution 12 h later, showed that the amount of stimulation was between that of ecdysone treatment and JHA treatment alone at different developmental stages during the fifth instar. From Fig. 6, at the early, middle, or late developmental stage of the fifth instar, the co-treatment with ecdysone and JHA treatment by spreading 100 ppm of JHA onto the bodies of fifth instar larvae at 36 h, 72 h, and 108 h after molting, then transfection solution was injected 12 h later, respectively.

Fig. 6. Effects of Co-treatment with Ecdysone and JHA on the Transcriptional Activity of BmNPV ie-1 Promoter in Larvae.

The increase of luciferase activity is indicated on the Y axis as stimulating folds over the control with sterile water treatment which was arbitrarily set at 1.0. Different co-treatments with ecdysone and JHA are indicated on the X axis. Each reaction contained 3 \( \mu \text{g} \) of protein extracted from larval hemolymph cells. The results represented averages from three separate treatments. E+JHA represents the co-treatment with ecdysone and JHA; E+JHA/36 h, E+JHA/72 h, and E+JHA/108 h represent that each larva was injected with 4 \( \mu \text{g} \) of ecdysone together with JHA treatment by spreading 100 ppm of JHA onto the bodies of fifth instar larvae at 36 h, 72 h, and 108 h after molting, then transfection solution was injected 12 h later, respectively.

Discussion

The developmental events of silkworm larvae and synthesis of fibroin are mainly regulated by the balance of ecdysone and juvenile hormone inside the larval hemolymph. During the early developmental stage of larvae at the fifth instar, the juvenile hormone titer appears at a higher level while ecdysone is lower, relatively. On the contrary, ecdysone appears in a higher level while juvenile hormone appears in a lower one during the late developmental stage. Ecdysone mainly functions by stimulating the synthesis of large biomolecules and accelerating the mature course of organelles associated with fibroin synthesis and metamorphosis during the late developmental stage. However, juvenile hormone inhibits larvae from metamorphosis and postpones the dis-
integration of cells and organs, keeping the structure and function of cells and organs in order and extending the period of time for protein synthesis.\textsuperscript{22,36}

Therefore, during the early developmental stage of fifth instar larvae, applying ecdysone by injection significantly stimulated the transient expression of the luciferase gene (Ecdysone/36 h of Injection in Fig. 2, Fig. 3) because a lower level of inner ecdysone was improved by foreign ecdysone, which benefits the synthesis of DNA, RNA, and relative proteins. By treatment with foreign ecdysone during the middle or late developmental stage, this ability was weakened systematically (Ecdysone/72 h and Ecdysone/108 h of Injection in Fig. 2) following the increase of inner ecdysone titer. The treatment by ecdysone feeding gave a similar response with the same trend (Feeding in Fig. 2).

On the contrary, during the early developmental stage, only a 1.7-fold increase of luciferase activity was produced by JHA treatment (JHA/36 h in Fig. 5) due to the inner juvenile hormone titer with a higher level. Following the inner juvenile hormone level reduction during the middle developmental stage, the treatment with JHA partly compensating for the relative lower titer of inner juvenile hormone led to a great increase of 5.1-fold of luciferase activity (JHA/72 h in Fig. 5) through maintaining the function of protein synthesis system of larvae in order. During the late developmental stage, this effect was only about 2-fold (JHA/108 h in Fig. 5). It might be attributed to the higher level of inner ecdysone and the delayed action of JHA treatment which can not entirely relieve the decline of cells and organs from ecdysone.

Interestingly, an expected accumulative increase did not appear by co-treatment with ec dysone and JHA. These two opposite effects seemed to counterbalance one another partially. Therefore, the increase by co-treatment with ec dysone and JHA was not as good as that of ec dysone (in an early and late developmental stage) or JHA treatment (in a middle developmental stage) alone and between that of two treatments during the different developmental stages of fifth instar larvae (Fig. 6).

Meanwhile, a similar tendency was seen in uninfected cells. The ec dysone treatment increased luciferase activity more than that of JHA treatment alone. And the increase by co-treatment with ec dysone and JHA was between that of the two treatments (Fig. 1).

In a coordinately regulated cascade of gene transcription initiated by a hormone, it 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 is initiated.\textsuperscript{35,37} Therefore, it is of great immediate significance to find whether there exists a HRE in the ie-1 gene, or insect hormones improve the basic efficiency of protein synthesis in the host.

Because of the feedback effect on the endocrine glands resulting from foreign hormones entering the hemolymph and the disturbance caused by endogenous hormones, the real physiological effect of foreign hormones on the transfected host and the relationship between foreign/endogenous hormones and the foreign gene expression need to be further questioned.

From all the above, the transcriptional activity of the ie-1 promoter was effectively stimulated by treating the host with insect hormones. This implies that potential application with insect hormones for foreign gene expression driven by the ie-1 promoter in stably transformed cell lines and in transgenic silkworm, Bombyx mori, is highlighted.

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