Exogenous JH and ecdysteroid applications alter initiation of polydnaviral replication in an endoparasitoid wasp, *Cotesia plutellae* (Braconidae: Hymenoptera)

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Polydnaviruses are a group of double-stranded DNA viruses and are symbiotically associated with some ichneumonoid wasps. As proviruses, the replication of polydnaviruses occurs in the female reproductive organ at the pupal stage. This study analyzed the effects of two developmental hormones, juvenile hormone (JH) and ecdysteroid, on the viral replication of *Cotesia plutellae* bracovirus (CpBV). All 23 CpBV segments identified contained a conserved excision/rejoining site ('AGCTTT') from their proviral segments. Using quantitative real-time PCR based on this excision/rejoining site marker, initiation of CpBV replication was determined to have occurred on day 4 on the pupal stage. Pyriproxyfen, a JH agonist, significantly inhibited adult emergence of *C. plutellae*, whereas RH5992, an ecdysteroid agonist, had no inhibitory effect. Although RH5992 had no effect dose on adult development, it significantly accelerated viral replication. The results of immunoblotting assays against viral coat proteins support the effects of the hormone agonists on viral replication. [BMB reports 2011; 44(6): 393-398]

INTRODUCTION

Polydnaviruses (PDVs) are a group of unique double-stranded DNA viruses symbiotic with some endoparasitoid wasps (1). It comprises two genera, Ichnovirus (IV) and Bracovirus (BV), depending on the host wasp family, Ichneumonidae or Braconidae (2). These two PDV taxa are also characterized by viral morphology and serological independence or DNA hybridization analyses, suggesting their independent origin (3). IV virions are relatively uniform in size and contain biconvex nucleocapsids surrounded by two unit membranes, whereas BVs are highly variable in length and have cylindrical nucleocapsids surrounded by a single envelope (4). The outer membranes of IVs are acquired by budding off from calyx cells. In comparison, the single envelope of BV suggests its release into the oviduct lumen by cell lysis (5).

PDVs are also unique in their viral transmission mode. Their full genomes are located on their host chromosome(s) and maintain vertical transmission along with wasp generation (6). However, their replication into viral particles for horizontal transmission to their specific lepidopteran hosts occurs only in the ovarian calyx region during the host pupal stage (7, 8).

*Cotesia plutellae* (Braconidae: Hymenoptera) is a solitary endoparasitoid wasp that parasitizes the diamondback moth *Plutella xylostella* (9). *C. plutellae* bracovirus (CpBV) has been previously identified and is known to be replicated in the ovarian calyx during the late pupal stage (10). It is regarded as a major factor in reducing host cellular immune capacity in parasitized *P. xylostella* (11, 12). For example, CpBV15β effectively inhibits hemocyte-spreading behavior in *P. xylostella* (13). CpBV-lectin exhibits early expression during parasitization and has been suspected to inhibit non-self recognition in *P. xylostella* (14, 15). CpBV-PTPs impair cellular immune processes such as phagocytosis and encapsulation probably by altering the phosphorylation status of target proteins within hemocytes (16, 17). CpBV-H4, CpBV-E9K, and CpBV-ELP1 are speculated to inhibit host immune capacity (18, 19). Recently, CpBV-RNase T2 was shown to inhibit both the humoral and cellular immune responses of *P. xylostella* (20).

Despite significant roles of PDVs in the parasitism of endoparasitoids, little has been shown about endocrine signaling in PDV replication. Juvenile hormone (JH) and ecdysteroid are key endocrine signals in insect development during the immature stages (21). In *Campoletis sonorensis* IV, ecdysteroid was shown to stimulate PDV replication in an *in vitro* organ culture assay and a thoracic ligation assay (7). In *Chelonus inanitus* BV, however, ecdysteroid may be not a direct endocrine signal for the initiation of viral replication based on the titer measurements during pupal development (22).

In this study, we performed direct application of hormonal analogs during the adult development of *C. plutellae*. To determine CpBV replication, two molecular processes were monitored. One monitored a specific stage of CpBV genome...
amplification by quantitative polymerase chain reaction (qPCR), whereas the other used PCR to analyze a specific stage of CpBV genome excision/rejoining at the viral excision/rejoining DNA site, where the proviral CpBV was replicated into its episomal viral form. Using these molecular monitoring techniques, this study analyzed the effects of JH and ecdysteroid on initiation of CpBV replication within the host *C. plutellae*.

**RESULTS**

**Excision/rejoining sites of different CpBV segments are conserved**

Molecular processes of PDV replication include excision of amplified PDV segments from wasp chromosome(s) (8). The excision sites appear to be conserved among different segments and even different PDV species (23). The conserved sequence ('AGCTTT') was searched in the CpBV segments (Fig. 1). All 23 known CpBV segments contained this putative excision/rejoining site sequence as well as consensus sequences around the excision sites.

To test the putative excision/rejoining sites, CpBV-S3 was chosen and used to design two segment-specific primer sets: rFP1/rRP1 for the non-excision site and rFP2/rRP2 for the excision site (Fig. 2A). PCR using non-excision site primers resulted in a consistent predicted product during the different developmental stages of *C. plutellae*. However, PCR using excision site primers showed the predicted PCR products only in the late pupal periods (P4 and P5) and adult stage, during which viral replication occurred in the ovary of the abdomen. This suggests that CpBV replication began 4 days after pupal ecdysis. TEM study showed that the pupal female ovary on day 5 was actively producing virus, as detected from virogenic stroma in the nucleus (Fig. 2B).

**CpBV replication is accelerated by ecdysteroid, but delayed by JH**

To analyze the effects of endocrine signaling on CpBV replication, we began by testing the effects on adult development of *C. plutellae*. We topically applied both JH and ecdysteroid agonists to wasp pupae. Pyriproxyfen, a JH agonist, clearly inhibited adult development, especially during the young pupae stage. However, RH5992, an ecdysteroid agonist, did not show any effect at any pupal stages on the adult development of *C. plutellae* (data not shown). When the hormone agonists were treated on day 1 of the pupae stage, pyriproxyfen had a significant inhibitory effect at concentrations above 0.01 μg. On the other hand, RH5992 did not have any effect on the adult development at concentrations in the 0.0001-1 μg range (data not shown). In response to 0.1 μg hormonal treatments, the pupal developmental rate was completely inhibited by the JH analog but appeared to be slightly accelerated by the ecdysteroid agonist, especially 5 days after treatment (data not shown).

The effects of both hormones on CpBV replication were analyzed in terms of CpBV segment amplification, excision, and viral capsid formation (Fig. 3). Using rFP1/rRP1 primers with qPCR, CpBV segment amplification was initiated in 4-day-old control pupae (left panel in Fig. 3A). However, JH agonist
viral DNA amplification during replication has not been observed for PDVs. A study on CiBV previously proposed two hypothetical models of viral DNA amplification: amplification of the clustered viral genome area followed by excision of each segment, and alternatively, a rolling circle model after excision using rFP2 and rRP2 primers by quantitative real-time PCR. Each treatment was replicated three times. The PCR products were visualized on agarose gel by staining with ethidium bromide. (B) Immunoblotting of CpBV coat proteins (left lane, Coomassie staining) using CpBV polyclonal antibody. Two coat proteins (right lane, immunoblotting) were detected, as indicated by thick, stained bands at approximately 30 and 35 kDa. (C) Effects of two hormones on synthesis of viral coat proteins.

**Fig. 3.** Effects of two hormone agonists on CpBV replication during the pupal period (day 1-day 5: D1-D5) of *Cotesia plutellae*. Pyriproxyfen (PYR, a JH agonist) and RH5992 (an ecdysteroid agonist) were topically applied on 1-day-old pupae at 0.1 μg. (A) Measurement of CpBV-S3 amplification using rFP1 and rRP1 primers or excision using rFP2 and rRP2 primers by quantitative real-time PCR. Each treatment was replicated three times. The PCR products were visualized on agarose gel by staining with ethidium bromide. (B) Immunoblotting of CpBV coat proteins (left lane, Coomassie staining) using CpBV polyclonal antibody. Two coat proteins (right lane, immunoblotting) were detected, as indicated by thick, stained bands at approximately 30 and 35 kDa. (C) Effects of two hormones on synthesis of viral coat proteins.

**Discussion**

The genomes of PDVs, as proviruses, are present in host wasp chromosome(s) (24). In BVs, viral integration of an ancestral rugulivirus type was estimated to have occurred 100 MYA ago (25). The integrated viral genome segments are amplified, excised, and then encapsulated during replication without re-entry back into wasp host chromosomes (26). Thus, viral replication occurs for the horizontal transfer of viral particles to the parasitized lepidopteran host, whereas the integrated form of the viral genome is vertically transmitted along with wasp generation (1). This study showed a virogenic stroma in the nuclei of pupal ovarian follicles of *C. plutellae* around the calyx area. The virogenic stroma, which possesses viral particles undergoing viral assembly and release processes, looks similar to those of other PDVs (27).

Viral production during the pupal stage of *C. plutellae* was supported by the excision period of CpBV segments. All 23 CpBV segments shared a putative excision site (‘AGCTTT’), which has been reported in other PDVs (23). In CpBV, PCR analysis around the excision site clearly showed the products on P4 or later, indicating initiation of viral segment replication. Our qPCR and immunoblotting data also show that segment amplification of CpBV as well as viral coat protein synthesis began on P4. All previously analyzed PDVs begin their replication during the pupal stage (8, 28-31). For PDV replication, including CpBV, a viral DNA polymerase or any associated factor(s) that use host DNA polymerase are necessary. However, no PDV possessing DNA polymerase has been identified since its gene is not likely to be encapsulated in viral particles (32). Current approaches for identifying PDV genomes using ovarian EST or analysis of BAC clones containing PDV segments clearly suggest that viral coat protein genes, which are not encapsulated, are actively expressed during viral replication (33, 34).

Viral DNA amplification during replication has not been observed for PDVs. A study on CiBV previously proposed two hypothetical models of viral DNA amplification: amplification of the clustered viral genome area followed by excision of each segment, and alternatively, a rolling circle model after excision of the clustered area (22). Partial amplification of the viral genome area in the wasp chromosome may be understood by the molecular process of chorion gene amplification during chorionogenesis of *Drosophila melanogaster* (35). Upon amplification, DNA replication occurs at multiple origins, followed by repetitive amplification (36). Our qPCR data indicate that amplification and excision began on the same day (day 4 pupal stage, ‘P4’). Moreover, immunoblot assay against the viral particles indicated that P4 pupae contained viral coat proteins. Considering that viral coat protein genes are not encapsulated in other PDVs, including CpBV, the viral coat protein genes must have been actively expressed when the CpBV was amplified and excised.

RH5992 accelerated CpBV replication while pyriproxyfen delayed it. RH5992, an ecdysteroid analog, did not affect the adult emergence rate, but it did significantly accelerate CpBV production, including amplification, excision, and viral coat protein formation. By measuring ecdysteroid titers during the pupal stages of *C. inanitus*, ecdysteroid peaks in the early pupal stages were found to occur during intensive cell proliferation and differentiation of the ovarian calyx (22). Thus, the effect of ecdysteroid on PDV replication, including CpBV replication, in this study may be understood in terms of early...
ovarian morphogenesis for preparation of subsequent massive DNA replication. Alternatively, the ecdysteroid peak may be directly associated with viral replication only in the ovarian calyx, as other tissues in wasp also possess the CpBV genome. In choriogenesis of D. melanogaster, only the follicle cells in the terminal oocytes undergoing DNA amplification of the chorion gene express Broad-Complex by the EcR-USP complex in response to ecdysteroid signaling (37). The Broad-Complex induces expression of endoreplication genes, which in turn recognize the replication origin and induce gene amplification (38). The inhibitory action of the JH agonist against viral replication may be explained based on its interference of Broad (38). The inhibitory action of the JH agonist against viral replication may be explained based on its interference of Broad gene expression as well demonstrated in tissue remodeling during metamorphosis (39). Ecdysteroid signaling during cellular processes may occur in the ovarian calyx cells of C. plutellae to amplify the CpBV genome, followed by excision and viral particle formation. This hypothesis needs to be explored in a future study.

MATERIALS AND METHODS

Insect rearing
P. xylostella larvae were reared on cabbage leaves at 25 ± 1°C under a photoperiod of 16 : 8 (L : D) h. Adults were fed 10% sucrose solution. Late second instar P. xylostella larvae (4 days after oviposition at 25 ± 1°C) were parasitized by C. plutellae at a 1 : 2 (wasp: P. xylostella) ratio for 24 h. Adults emerged from their cocoons (11 days after parasitization at 25 ± 1°C), were collected, and allowed to mate for 24 h before parasitization.

Hormone treatment
Pyriproxyfen (95% technical, Dongbang-Agro, Seoul, Korea) and RH5992 (96% technical, Kyungnong, Seoul, Korea) were used as agonists of JH and ecdysteroid, respectively. Different concentrations of both analogs were dissolved in acetone and topically applied to pupae of C. plutellae in a 5 μl volume.

Genomic DNA (gDNA) extraction
From the different developmental stages of C. plutellae, gDNAs were extracted using extraction buffer (10 mM Tris, 0.1 M EDTA, 20 μg/ml RNase, 0.5% SDS, pH 8.0), in which each developmental sample consisted of 100 larvae, 100 pupae, or 30 adults. Larvae were 7-8 days old after parasitization. Pupae of different ages (from 1 to 5 days) were chosen after pupation. One-day-old adults after emergence were separated into male and females. After proteinase K treatment, gDNAs were purified by phenol extraction and ethanol precipitation.

Test PCR for excision/rejoining site and quantitative real-time PCR (qPCR)
For analysis of excision process of CpBV DNA replication, qPCR was designed using primers (rFP2: 5'-TCC GTC CAA ATA CGG TG A-3' and rRP2: 5'-GGA GAG AGA AGCATA TGC AGA G-3') specific to CpBV DNA segment #3 (CpBV-S3) (see Fig. 2A). Using gDNAs isolated from the different developmental stages of C. plutellae, multiple PCR reactions were performed consisting of 35 cycles of 30 s at 94°C, 30 s at 53°C, and 1 min at 72°C.

To monitor amplification and excision rates of CpBV-S3 within the ovary, qPCR reactions were performed with primers specific to the non-excision site (rFP1: 5'-GAC GTC TTA GTG TGA AGC AT-3' and rRP: 15'-CAT TCC TTC CAG CCG TG-3') or with rFP2 and rRP2 primers specific to the excision site (see Fig. 2A) using a Real-Time PCR ABI Prism 7500 (Prism 7500, Applied Biosystems, Foster City, CA, USA) along with SYBR green chemistry of Accupower Greenstar ™ PCR premix (Bioneer) and real-time fluorescence measurements. Each 20 μl reaction mixture consisted of 1 x Greenstar ™ PCR Master Mix, 10 mM MgCl₂, 0.5 mM of primers, and 90 ng of DNA. Initial incubation at 95°C for 15 min was carried out to activate Hotstar Taq DNA polymerase. qPCRs consisted of 40 cycles of 30 s at 94°C, 30 s at 53°C (amplification site)/53°C (excision site), and 1 min at 72°C. B-Actin gene was used as a control with primers: 5'-TGG CAC ACC ACC TTC TAC-3' and 5'-CAT GAT CTG GGT CAT CTT CT-3'. Each cycle was scanned to quantify the PCR products of each treatment with three replicates. Amplification plots in real-time were constructed using ABI PRISM ™ 7500. Quantitative analysis of DNA amplification or excision frequencies was carried out using the comparative Ct (ΔΔCt) method (40).

Ultrastructure of ovarian calyx cells using transmission electron microscope (TEM)
Ovaries were isolated from 5-day-old pupae and fixed in 2.5% glutaraldehyde in 0.01 M phosphate buffer (pH 7.2) for 2 h at 4°C. After several washes in phosphate buffered saline (PBS, 100 mM phosphate, 0.7% NaCl, pH 7.4), the ovaries were post-fixed with 1% osmium tetroxide for 30 min at 4°C and then washed again with PBS. The ovary samples were then dehydrated through a graded ethanol series, substituted with propylene oxide, and then embedded in Epon 812 for 48 h at 60°C. Sections (< 90 nm) were cut on a Leica Ultracut UCT ultramicrotome using a diamond knife (Ultracut UCT, Leica Microsystems, Wetzlar, Germany). Ultrathin sections were transferred onto 200 mesh copper grids and stained with uranyl acetate for 20 min, and then with lead citrate for another 10 min. The sections were examined by TEM (H-7650, Hitachi, Tokyo, Japan) at 80 kV.

Immunoblotting analysis of CpBV coat proteins
Proteins were extracted from pupae and adults of different ages using PBS, in which each sample consisted of 100 pupae or 100 adults. The extracted protein samples were diluted with PBS and mixed with the same volume of denaturing buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8). After boiling for 5 min at 95°C, the samples (50 μg per lane) were separated by 10% SDS-PAGE. Electrophoresis was performed under denaturing conditions (41) until
tracking dyes migrated to the end of the gel. The separated proteins on the gel were transferred onto nitrocellulose paper by the method of Towbin et al. (1979) (42). Non-specific sites were blocked by 5% skim milk for 1 h at room temperature. After three washes with PBS, the membrane was incubated for 2 h at room temperature with polyclonal antibody recognizing coat proteins of CpBV (43). The polyclonal antibody was raised in rabbit using an antigen sample of CpBV viral particles isolated from the ovarian calyx of *C. plutellae*. After three washes with PBS, the membrane was incubated for 1 h at room temperature with secondary antibody (1/2,000 dilution) conjugated with alkaline phosphatase substrate solution containing nitro blue tetrazolium/5-mono-4-chloro-3-indolyl phosphate (NBT/BCIP, Sigma-Aldrich Korea, Seoul, Korea) in 10 mM phosphate buffer (pH 9.5).

**Sequence analysis of CpBV segment excision site**

The conserved PDV excision site sequence obtained from previous related PDVs (8, 44) was used to locate sites in different CpBV segments (NCBI accession number): S2 (DQ075354), S3 (DQ075355), S4 (DQ075356), S5 (DQ075357), S8 (DQ075358), S9 (DQ075359), S10 (EF067319), S11 (DQ075360), S14 (EF067320), S16 (EF067321), S21 (EF067322), S22 (EF067323), S27 (DQ067324), S28 (AY651829), S30 (AY651828), S33 (AY651830), S35 (EF067325), S36 (EF067326), S37 (EF067327), S38 (EF067328), S41 (EF067329), S50 (EF067330), S51 (EF067331), and S52 (EF067332). Multiple alignment of these terminal CpBV repeats was performed using the DNAsStar program (Version 5.02, DNAsStar Inc., Madison, WI, USA).

**Statistical analysis**

The means were compared by a least squared difference (LSD) test of one way ANOVA using PROC GLM of SAS program (45) and discriminated at Type I error = 0.05.

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**REFERENCES**


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Bokri Park and Yonggyun Kim


