Stimulation of polydnavirus replication by 20-hydroxyecdysone

B. A. Webb and M. D. Summers

Department of Entomology, Texas A & M University, College Station (Texas 77843, USA)
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Abstract. During oviposition the endoparasitic wasp *Campoletis sonorensis*, introduces a polydnavirus into parasitized insects where viral gene expression is required for endoparasite survival. The polydnavirus is integrated into wasp chromosomal DNA and replicates only in the ovary. Ecdysteroids regulate the developmental expression of many insect genes and may regulate polydnavirus replication. Direct verification of viral replication was performed by dot blot hybridization and by amplifying DNA sequences containing the viral integration site; this 'junction' fragment cannot be amplified from integrated virus. Thoracic ligations and in vitro ecdysteroid treatments of wasp ovaries support the hypothesis that polydnavirus DNA replication is regulated by ecdysteroid during parasite development.
Key words. Hymenopteran endoparasites; ecdysteroids; developmental regulation; virus replication; polydnaviruses.

The survival of endoparasitic wasps in their insect hosts requires the disruption of host immune and developmental systems. Accessory products (venom, virus and soluble proteins) from the female wasp's reproductive tract are injected into host insects with parasite eggs and inhibit the host immune response allowing successful endoparasite development. Venoms and polydnaviruses working alone, or together, may disrupt host physiological systems [1-4]. To date, polydnaviruses, an unusual family of symbiotic DNA viruses, have been described from two families of parasitic wasps where they replicate only in specialized cells of the female reproductive tract [5,6]. Polydnaviruses bud through the calyx cell membrane into the oviduct lumen and are injected with parasite eggs and venom into host insects during parasitization. The virus then enters the insect's cells [7] where a host-specific subset of viral genes is expressed [8-10]. In some wasp species, such as *C. sonorensis*, polydnavirus expression is necessary and sufficient to functionally inhibit host immune and developmental systems enabling the immature endoparasite to survive [11]. Since the polydnavirus genes which are expressed after parasitization apparently perform essential functions for the developing wasp, viral transmission between generations must be very efficient to insure that all females carry this symbiont. Molecular and genetic analyses have shown that some polydnaviruses are integrated into the genomes of every male and female of an affected species [12-14]; genomic integration of polydnaviruses would insure that all members of the species carry the virus. Polydnaviruses replicate only in the ovaries of pupal and adult parasitic wasps suggesting that viral replication may be controlled by the developmental program of the endoparasite.

Norton and Vinson [5] observed virions in pupal wasp ovaries coincident with the appearance of a specific pattern of melanization in the newly formed adult cuticle. Based on their morphological description and our observations of pupae at 2-h intervals over the 96 h from pupation to adult eclosion, we estimated that *Campoletis sonorensis* polydnavirus (CsPDV) replication begins between 48 and 66 h after pupal ecdysis at 27 °C. Morphological features such as apolysis of the antennae and changes in eye color were also identified which allowed prediction of the onset of melanization and, presumably, viral replication. During replication the segmented polydnavirus genomic DNA is packaged into virions as multiple circular superhelical DNA molecules [15-17]. Both the viral (superhelical) and proviral (integrated) forms of certain CsPDV DNA segments have been cloned and mapped to identify their integration sites [18]. Pupal development to the adult is initiated by the molting hormone ecdysone and its biologically active derivatives, primarily 20-hydroxyecdysone (20 HE). The rising hormone titer causes events such as apolysis and the deposition of a new cuticle, whereas later events such as cuticular pigmentation may depend on the subsequent decline of the hormone titer [19-21]. Melanization in tobacco hornworm larvae occurs in the later part of the molt during the declining phase of the ecdysteroid titer and is dependent on the increased synthesis of dopa decarboxylase which only occurs after an initital exposure to 20 HE followed by its disappearance [22-26]. In *Drosophila* dopa decarboxylase is induced by the increased ecdysteroid titer during pupariation [27,28]. In this study we examine the role of ecdysteroids in initiating the replication of a polydnavirus during *C. sonorensis* development.

Materials and methods

Heliothis virescens (Lepidoptera: Noctuidae) and *Campoletis sonorensis* (Hymenoptera: Ichneumonidae) were reared as previously described [8]. The pupal developmental stages of Norton and Vinson [5] were determined to be related to developmental time at 27 °C as follows: stage 1, 0–60 h after pupal ecdysis; stage 2, 60–66 h post ecdysis; stage 3, 66–72 h post ecdysis; stage 4, 72–96 h post ecdysis.

Total nucleic acid (TNA) was prepared from individual oviducts [29] and viral DNA was isolated [8]. Oviduct TNA blot hybridizations were performed using genomic CsPDV DNA or pgV1100 [30] as hybridization probes at

1×10^6 cpm/ml. Serial tenfold dilutions of control CsPDV DNA (1 µg) and TNA samples extracted from individual oviducts were blotted to nitrocellulose for hybridization experiments. Two 15-min washes in 2 × SSC (1 × SSC is 15 mM sodium chloride/1.5 mM sodium citrate, pH 7.0), 0.2% SDS at 47 °C followed by two 15-min washes (0.1 × SSC, 0.1% SDS) at 65 °C were performed. Blots were air dried and exposed to Kodak XAR-5 film for 16 h with an intensifying screen. Blot hybridizations were quantified densitometrically[31]. The 417 bp Bgl II fragment from viral segment B[32] was subcloned into the *BamH 1* site of Bluescribe (Stratagene) using previously described procedures[9]. The fragment was sequenced from plasmid DNA by the di-deoxy-chain termination method[33]. Amplification experiments[34] were performed by precipitating individual oviduct TNAs in a 500-µl eppendorf tube, drying the nucleic acid, adding 100 µl of the amplification mix and overlayering with 100 µl of mineral oil. The amplification mix was 1 × amplification buffer (50 mM Tris-HCl pH 9.0 at 25 °C, 10 mM $MgCl_2$, 200 µm dATP, dCTP, dTTP and dGTP) with 1 µg of each primer (B2R – CGGATCAAAGAGAGCAATC and B2F – GTCGTATAATATTTGCTATG) and 1 unit of *Thermus aquaticus* (taq) DNA polymerase (Promega)[34]. The substrate DNA was denatured at 94 °C for 2 min and 35 amplification cycles were performed (50 °C annealing 2 min, 74 °C extension 2 min, 94 °C denaturing 1.5 min) in a thermal cycler (Perkin-Elmer Cetus). Amplification products were removed from the reaction tube by extracting with 100 µl chloroform and collecting the aqueous layer and extracting with phenol: chloroform: isoamyl alcohol (48:48:1) 1 ×. A 20-µl aliquot was analyzed by gel electrophoresis on a 1% agarose gel. Amplified DNA was visualized with ethidium bromide under ultraviolet light. Representative data are illustrated from a minimum of three replications of all amplification reactions (figs 1, 3 and 4).

Results

To determine if the initiation of viral DNA replication coincided with the morphological changes which signal and predict the molt, TNA was isolated from individual staged pupal ovaries, blotted to nitrocellulose and probed sequentially with genomic CsPDV DNA (fig. 1 A) and with a viral genomic clone expressed only after parasitization (pgV1100)[30]. The results showed that the amount of viral DNA increased at times coincident with the onset of melanization. Hybridization analyses, however, do not differentiate between an increase in viral DNA caused by the initiation of viral DNA replication and an increase in the integrated viral DNA hybridization signal which would accompany cell division. To differentiate between these two possibilities requires the ability to distinguish between integrated and extrachromosomal viral DNA forms. During replication polydnavirus DNA is excised and/or copied from proviral DNA and then packaged into virions as multiple circular DNA molecules[15,16]. An assay was developed which utilized the integration site of viral segment B as an indicator of the initial appearance of the replicative or viral form of the CsPDV DNA segment during parasite development.

In the wasp, the integration site of viral segment B lies within a 417 bp sequence which is flanked by Bgl II sites (fig. 2)[18]. Two independent clones (pBB 6700 and pBE 6700) containing the entire viral segment B (shB) were isolated[32]. The Bgl II fragment from pBE 6700 was subcloned, sequenced and oligonucleotide primers flanking the integration site were synthesized for use in the polymerase chain reaction (PCR; fig. 2). Sequence analysis of the pBB 6700 Bgl II fragment has also been reported and contains minor differences in the junction region that may be related to the mechanism of viral replication[18]. Comparison of flanking genomic sequence with the 2 sh B clones suggest the inclusion of a short, imperfect inverted repeat from either the left (pBB 6700) or

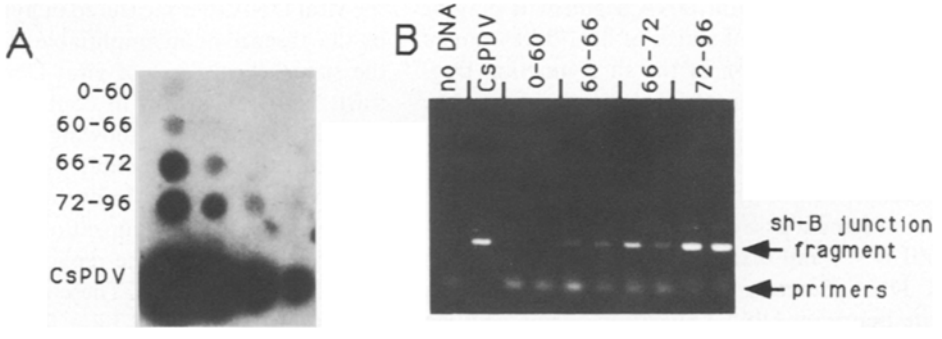

Figure 1. Detection of the initiation of viral DNA replication by dot blot hybridization (*A*) and junction fragment amplification (*B*). Total nucleic acid was isolated from individual staged oviducts and either blotted to nitrocellulose for hybridization experiments or used as the substrate for PCR amplification. Abbreviations are as follows: CsPDV – CsPDV DNA (1 µg blot hybridization experiments; 1 ng PCR amplification experiments), sh-B – superhelical DNA segment B, no DNA – mock amplification assay set up without template DNA. Timing of pupal developmental stages[5] at 27 °C: stage 1, 0–60 h after pupal ecdysis; stage 2, 60–66 h post ecdysis; stage 3, 66–72 h post ecdysis; stage 4, 72–96 h post ecdysis.

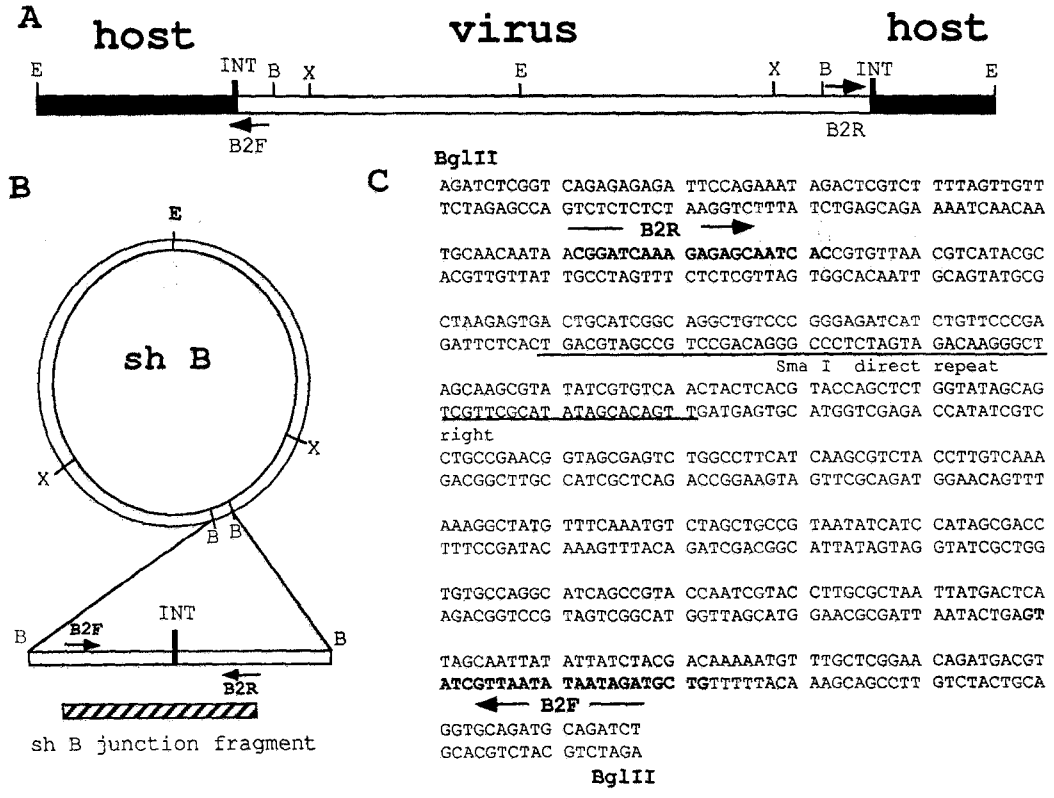

Figure 2. Integrated and superhelical forms of DNA B. *A* Schematic of the integrated form of DNA B. *B* Schematic of the superhelical form of DNA B. *C* Nucleotide sequence of the 417 bp Bgl II fragment of segment B which contains the integration site. The right direct repeat described by Fleming and Summers[18] is underlined and the Sma I site is indicated. Abbreviations are as follows: host – *Campoletis sonorensis* genomic DNA (non-viral); virus – integrated viral DNA; sh B – viral superhelical DNA segment B; B2F and B2R – location of oligonucleotide primers; E – EcoRI; B – Bgl II; X – XhoI; INT – integration site. Arrows indicate the direction of synthesis during the amplification cycle from the two primers. Boldface in the nucleotide sequence indicates the sequence selected for primer synthesis. The amplified junction fragment (hatched) has a molecular size of 311 or 316 bp. The solid bar represents non-viral flanking *C. sonorensis* DNA.

right (pBE 6700) integration site. The primers flanking the integration site are identical in both clones and allow for the specific amplification of a 311 bp (pBE 6700) or 316 (pBB 6700) sequence containing the integration site of viral DNA segment B. This 'junction' fragment is not amplified from the integrated segment B because DNA synthesis proceeds from the primers into flanking chromosomal sequences (fig. 2). Therefore, the appearance of the superhelical form of viral DNA segment B may be detected during the different stages of host development by the specific amplification of the sh B junction fragment. The superhelical form of DNA B, as indicated by amplification of the sh B junction fragment from individual pupal ovaries, first appeared after apolysis (60–66 h post pupal ecdysis, fig. 1 B). Together, the hybridization and junction fragment assays allowed quantitative detection of viral DNA and distinguished between viral and integrated DNA forms of segment B. These assays demonstrate that viral DNA replication begins between 48 and 60 h which correlates with the initial detection of virions by electron microscopy[5].

Since viral replication in *C. sonorensis* occurred just after apolysis and the initiation of cuticular melanization, we investigated whether replication may be regulated by changes in the ecdysteroid titer. 48-h pupae were ligated between the thorax and the abdomen to isolate the ovaries from the source of ecdysteroids which are produced in the prothoracic gland. Ovaries were dissected and assayed 24 h later for the initiation of replication. Ligation prevented the initiation of viral DNA replication as measured by both the failure to detect increases in the viral DNA titer measured in hybridization assays and by the absence of an amplifiable junction fragment from the superhelical form of viral DNA B (fig. 3). By contrast, viral replication in control, unligated larvae occurred as expected showing that thoracic ligation blocked the developmental signal which normally induces viral replication. Importantly, thoracic ligation after the initiation of melanization had no detectable effect on either the titer or the replication of viral DNA (unpublished data, n = 6). These results indicate that viral replication is regulated by a factor or factors released from the head or thoracic segments of the wasp and support the putative regulatory role for ecdysteroids.

To determine if ecdysteroids could induce the replication of viral DNA, ovaries were isolated from 48-h pupae

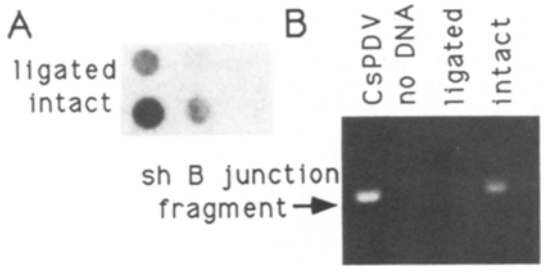

Figure 3. Effect of thoracic ligation on viral DNA replication. Pupae (48 h post-ecdysis) were either ligated to block endogenous hormone transport to the ovary or left intact and maintained in a humidified environment for 24 h before dissection of the ovary and analysis of the ovary TNAs by dot blot hybridization (*A*) or junction fragment amplification (*B*). Other abbreviations and experimental conditions are as in fig. 1.

(prior to the initiation of viral DNA replication, fig. 1) and cultured individually in vitro for 20 h in the presence or absence of 20-HE. Culture conditions were adapted from previously described procedures[29] and were not optimized for wasp tissues. However, the ovaries were motile and synthetically active after culture. TNA was isolated from cultured ovaries and analyzed either by blot hybridization or for the presence of an amplifiable sh B junction fragment. Ovaries cultured in the presence of 20-HE had significantly higher titers of viral DNA than did ovaries cultured without the hormone (fig. 4A). Furthermore, the sh B junction fragment was detected predominantly from ovaries cultured in the presence of 20-HE (fig. 4B).

Ovaries which were extracted 24 h after pupation did not consistently respond to the in vitro hormonal stimuli suggesting that the hormonal responsiveness of this tissue might be regulated. To test this possibility, ovaries were isolated 24 and 48 h after pupal ecdysis. These staged ovaries were assayed for the presence of the junction fragment after incubation in increasing concentrations of hormone (fig. 4C). Viral replication was not induced by exposure to 20-HE in 24-h ovaries (fig. 4C). Ovaries isolated from 48-h pupae responded to 20-HE treatment with the junction fragment first detected after exposure to 0.1 µg/ml 20-HE. Increased quantities of the junction fragment were consistently produced with increased ecdysteroid concentrations ranging to 2.0 µg/ml and presumably reflect increased viral template although these experiments were not designed to produce quantitative results.

Discussion

The initiation of viral replication in ovaries may be blocked in vivo by blocking endogenous hormone release and stimulated in vitro cultures with 20-HE strongly indicating that CsPDV replication in ovaries is regulated by the ecdysteroid titer. The active ecdysteroid concentrations lie within physiological ranges as established by both direct measurements of in vivo insect hormone titers

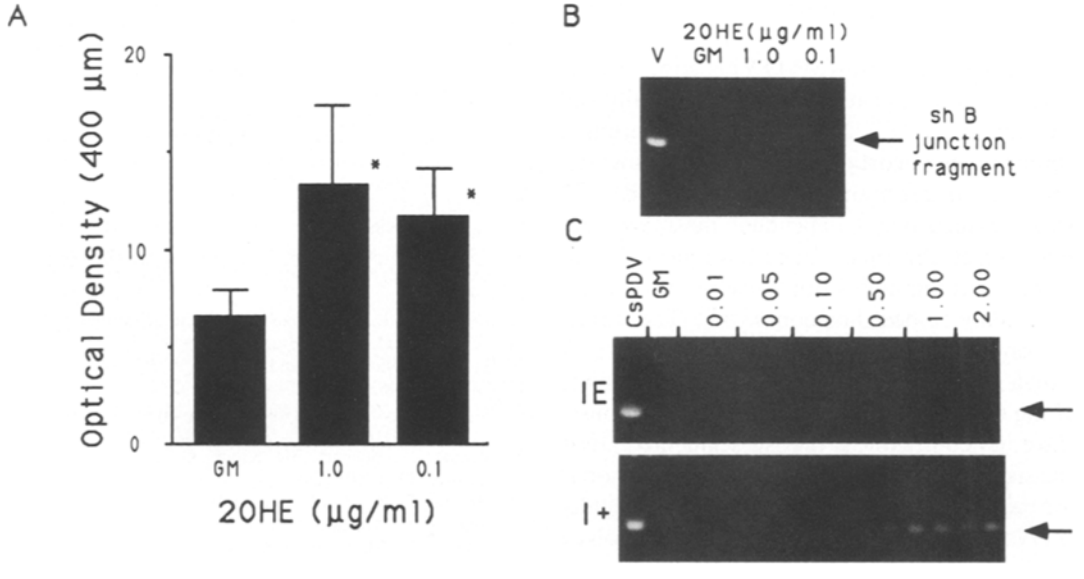

Figure 4. Effect of 20-hydroxyecdysone on viral replication in vitro. Ovaries were removed from 24- (IE) and 48-h (I+) pupae and cultured in 200 µl of Grace's medium (GM) in the presence or absence of 20-hydroxyecdysone (20-HE) at various concentrations. *A* After culture TNAs were extracted from the ovaries and analyzed for viral DNA in hybridizations as described in the methods. Blot hybridization signals from 6 replicates were quantified based on the optical density of the hybridization signal from a 16-h exposure of XAR-5 film with an ELISA plate reader[31] and compiled. Averages from the 6 replicates are shown graphically; * – indicates statistical significance at the 0.01 level using Student's t-test. *B* The presence of amplifiable sh B junction fragment was assayed in TNAs prepared from cultured ovaries on agarose gels as described in the methods. Ovaries were cultured in complete GM either with 20-hydroxyecdysone (1.0 and 0.1 µg/ml) or without hormone. *C* Because some variability was observed in the response of stage I oviducts to hormone a series of ovaries was timed from parasite ecdysis to the pupal stage. Oviducts were extracted from these timed stage I pupae at 24 and 48 h post-ecdysis and cultured in varying concentrations of hormone (0 (GM) 0.01, 0.05, 0.10, 0.50, 1.00 and 2.00 µg/ml 20-hydroxyecdysone). TNAs were extracted from cultured ovaries, junction fragments were amplified and analyzed on agarose gels as described above and in the methods.

and by tissue responses during in vitro cultures[25]. There are clearly other factors involved in the regulation of polydnavirus replication such as sex and tissue specific regulatory factors. During the first 48 h of pupal development in *C. sonorensis*, the ovary increases in size and the differentiation of ovariole, calyx and oviduct region of the organ becomes more pronounced. Variation in the responses of ovaries to ecdysteroids may be due to other factors which regulate viral replication and appear to be related to the developmental age of the tissue. The appearance of hormone receptors at a given stage of development may also be under developmental regulation and could limit the ability of the tissue to respond to the hormone[35]. Alternatively, other proteins such as substrate phosphoproteins which are phosphorylated in response to an extracellular signal may be developmentally expressed and limit the response to hormonal stimuli[36]. The developmental regulation of CsPDV replication allows for the initiation of stage and tissue specific viral gene expression in the ovary and for the replication of superhelical viral DNA. The precise mechanism for the control of viral replication is unknown but could be simply explained by the hormonal regulation of a viral 'replicase' which would control the excision or copy of the integrated virus to produce an extrachromosomal, superhelical form. Alternatively, small amounts of non-chromosomal, viral DNAs have been detected in all adult *C. sonorensis* tissues[12,13] and if these non-chromosomal viral DNAs are also present in larvae and pupae, viral replication could occur through amplification of these existing episomal viral DNA segments.

Mouse mammary tumor virus (MMTV) is another integrated virus for which replication is developmentally and hormonally regulated[37–39]. The MMTV long terminal repeats contain a glucocorticoid receptor binding site from which viral transcription may be induced[40,41]. Furthermore, glucocorticoid dependent maturation of MMTV envelope glycoproteins has been reported indicating that this virus responds both transcriptionally and post-translationally to host hormones[42,43]. The principal differences in these systems are that MMTV is a retrovirus that is not permanently integrated into every member of the species and viral replication deleteriously affects its host. By contrast, CsPDV is a segmented DNA virus and its successful replication and gene expression is required for the survival of the endoparasite. The regulation of CsPDV DNA replication appears to have evolved to produce the virus at the appropriate developmental time and in the appropriate tissue for transfer to the host insect during parasitization. The ability to induce viral replication in vitro could facilitate mechanistic studies of polydnavirus replication when additional experimental tools such as an endoparasitic, polydnavirus-containing cell line are available and may provide an inducible model system for the study of viral integration and replication.

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