

Polydnavirus replication: the EP1 segment of the parasitoid wasp *Cotesia congregata* is amplified within a larger precursor molecule

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Polydnaviruses are unique viruses: they are essential for successful parasitism by tens of thousands of species of parasitoid wasps. These viruses are obligatorily associated with the wasps and are injected into the host during oviposition. Molecular analyses have shown that each virus sequence in the segmented polydnavirus genome is present in the wasp DNA in two forms: a circular form found in the virus particles and an integrated form found in the wasp chromosomes. Recent studies performed on polydnaviruses from braconid wasps suggested that the circular forms were excised from the chromosome. The different forms of the EP1 circle of *Cotesia congregata* polydnavirus during the pupal–adult development of the parasitoid wasp were analysed. Unexpectedly, an off-size fragment formerly used to diagnose the integration of the EP1 sequence into wasp genomic DNA was found to be amplified in female wasps undergoing virus replication. The EP1 sequence is amplified within a larger molecule comprising at least two virus segments. The amplified molecule is different from the EP1 chromosomally integrated form and is not encapsidated into virus particles. These findings shed light on a new step towards EP1 circle production: the amplification of virus sequences preceding individual circle excision.

Introduction

Polydnaviruses (PDVs) are characterized by having a large segmented genome composed of multiple double-stranded DNA circles. They have been classified as a virus family, the *Polydnaviridae*, on the basis of the molecular features of their genome and their obligate association with endoparasitoid wasps (Stoltz, 1993; Summers & Düb-Hajj, 1995; Webb, 1998; Drezen *et al.*, 2000). Mature virions are produced exclusively in the ovaries and are injected by female wasps during oviposition. Once delivered, virus particles enter host cells and virus genes are expressed, causing major physiological alterations, which include disruption of the immune defence (Shelby & Webb, 1999; Beckage, 1998), retarded growth and inhibition of metamorphosis (Dushay & Beckage, 1993; Soller & Lanzrein, 1996).

Members of the family *Polydnaviridae* are divided into two different genera: *Bracoviruses* and *Ichnoviruses*, associated with

the Braconidae and Ichneumonidae family of wasps, respectively. Bracoviruses and ichnoviruses differ greatly in the morphology of virus particles and in the way in which they are released from cells undergoing virus replication (Webb, 1998). Ichnovirus particles are released through a budding process (Volkoff *et al.*, 1995), while cells producing bracoviruses most probably undergo lysis (De Buron & Beckage, 1992; Hamm *et al.*, 1990).

PDVs are captives of the hymenopteran wasps and are inherited exclusively through the chromosomal transmission of their sequences integrated into wasp DNA (Fleming & Summers, 1991; Gruber *et al.*, 1996; Xu & Stoltz, 1991; Savary *et al.*, 1997). Unlike most viruses, they are unable to replicate into the cells they have infected (Theilmann & Summers, 1986). Virus replication is restricted to specialized cells of the wasp ovaries, the calyx cells. The transmission of virus DNA is thus strictly dependent upon the success of the parasitoid, which, in turn, depends on the expression of virus genes for successful parasitism.

Very little is known about the mechanism of polydnavirus replication. However, the relationship between the integrated

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forms of the virus present in the wasp chromosomes and the circular forms injected into the parasitized host was analysed in wasps belonging to two different braconid subfamilies: *Cotesia congregata* (Microgastrinae) and *Chelonus inanitus* (Cheloninae) (Savary *et al.*, 1997; Gruber *et al.*, 1996). The chromosomally integrated form of a virus circle was cloned in each species (EP1 from *C. congregata* and CiV12 from *C. inanitus*). Interestingly, specific PCR products were obtained using DNA extracted from ovaries undergoing virus replication and primers flanking the integration site in the wasp DNA. These products are thought to result from PCR amplification of molecules where non-virus flanking sequences have become contiguous following virus sequence excision. It was therefore suggested that virus sequences are excised leaving an 'empty locus' on the chromosome and that the produced circles are amplified using a rolling-circle mechanism, as described for many circular DNA viruses (Savary *et al.*, 1997).

In this study, we present new data that modify our model of bracovirus replication. Production of virus particles was observed by ultrastructural analysis during pupal–adult development and adult life of *C. congregata* female wasps and the different forms of the virus EP1 sequence were monitored using a combination of molecular analyses. Strikingly, we found that in female wasps undergoing virus replication, the *C. congregata* polydnavirus (CcPDV) EP1 sequence is amplified within a molecule larger than the EP1 circle, a situation not predicted by the previous model (Savary *et al.*, 1997). Southern blot analysis shows that the amplified molecule does not correspond to the chromosomal form, since the map of the two molecules differs downstream of the EP1 sequence. The amplified molecule is detected earlier than the EP1 circle during pupal–adult development, suggesting that it is a precursor of EP1 circle production.

Methods

Insects. *C. congregata* (Hymenoptera, Braconidae, Microgastrinae) is a gregarious larval endoparasitoid of *Manduca sexta* larvae (Lepidoptera, Sphingidae). Adult female wasps are easily distinguished from males in that they have a prominent ovipositor on the posterior end of the abdomen. Females lay eggs into the fourth instar of the host. Larvae develop during approximately 12 days, then they emerge and spin their cocoons on the back of the caterpillars. Using our rearing conditions, the wasps emerge between 5 and 7 days after cocoon spinning and males generally tend to emerge earlier than females. Some animals from the same brood differ slightly in the developmental stage at a given time; these animals were not taken into account to define the morphological aspect characteristic of a given developmental stage nor used for DNA extraction.

The parasitic wasps were reared on their natural host, the tobacco hornworm, *M. sexta*. Host larvae were reared first collectively in cohorts of 15 individuals in Petri dishes for 4–5 days, then individually, as described previously (Harwood *et al.*, 1994).

Extraction of DNA from wasps and virus particles. DNA was extracted from adults, pupae and dissected ovaries using the Easy DNA kit (Invitrogen). Virus DNA was prepared following purification of virus particles by filtration, as described previously (Beckage *et al.*, 1994).

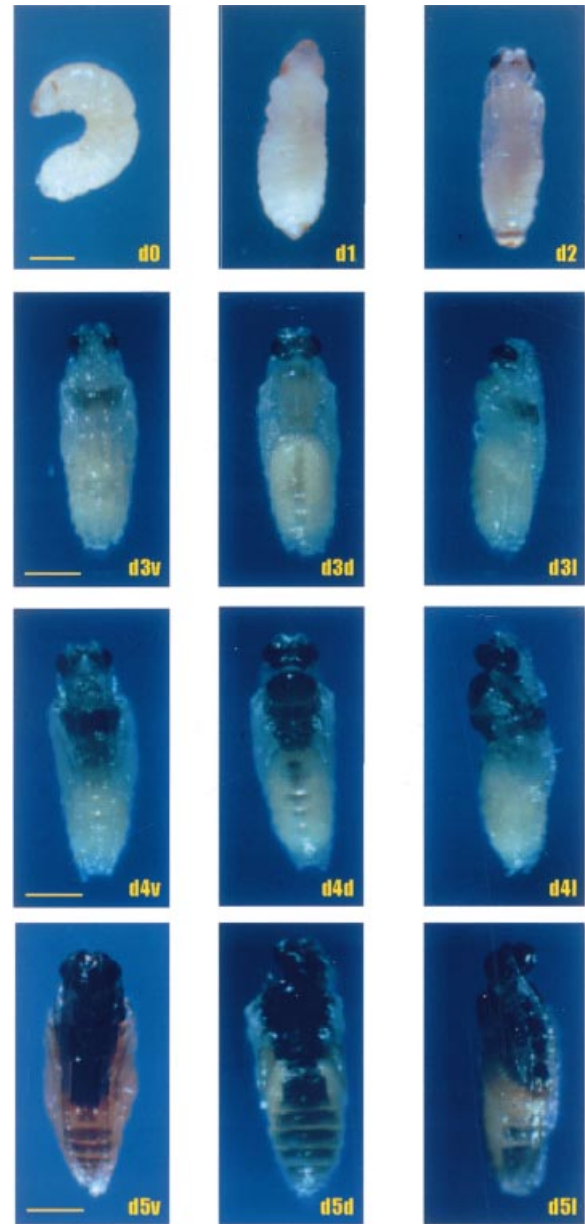


Fig. 1. *C. congregata* pupal–adult development. Photographs of the different pupal stages used in the analysis are represented (d0 to d5). Numbers indicate the number of days after the emergence of the parasitoid larvae from the parasitized *M. sexta* larvae (v, d, l: ventral, dorsal and lateral views). Bars, 1 mm.

Determination of pupal stages. The silk of the cocoons was cut using ophthalmologic scissors and the pupae were carefully removed. Pupae from the same brood were examined and sorted according to their melanization pattern. The different stages that were defined ranged from day 0 to 5 after cocoon spinning (day 0) as shown in Fig. 1. Females were identified either by the presence of developing ovaries, assessed by dissection, or by the pattern of their stripes once they had appeared. In addition, several substages of d4 pupal development were defined in order to characterize more precisely the onset of virus replication; the following morphological criteria were used: d4.1 white abdomen, stripes begin to appear on the top of the abdomen, white legs; d4.2 white

abdomen, extension of stripes, white leg; d4.3 light grey abdomen, complete black stripes, white legs; and d4.4 grey abdomen, grey legs.

■ **Quantitative PCR.** For quantitative PCR (QELS PCR), the QELS PCR competitor plasmid was first constructed. The pGEM-T-ELS plasmid, described previously by Savary *et al.* (1997), contains the 327 bp EL PCR product corresponding to the native empty locus fragment, inserted at the cloning site of the pGEM-T vector (Promega). There is a unique *Pst*I restriction site in the EL PCR product. This *Pst*I restriction site was used to introduce an arbitrarily chosen 200 bp *Pst*I fragment of λ DNA into the pGEM-T-ELS plasmid. A positive colony was arbitrarily chosen and the corresponding plasmid DNA, QELS PCR competitor, was used in all competition experiments. It was verified that the level of amplification of C 'competitor' (527 bp) and EL 'empty locus' (327 bp) products was similar when the same amount of QELS PCR competitor and pGEM-T-ELS plasmids were used in the same experiment.

QELS PCR was performed using DNA extracted from different developmental stages: from pupae, respectively, 2, 3, 4 and 5 days after larval emergence from parasitized *M. sexta* and from adult females 0, 1, 2 and 3 days after emergence from the cocoons. Adult females were not allowed to oviposit. A sample of 1 μ l QELS PCR competitor was introduced in increasing concentrations in a series of PCRs containing 3 μ g DNA to assess (corresponding approximately to half of the DNA extracted from a wasp). Amplified products were checked on 4% Nusieve (FMC Bioproducts). All quantitative PCR experiments were performed at least twice using two different DNA samples for each developmental stage.

■ **Southern blotting.** Digested DNA (10 μ g genomic DNA and 80 ng virus DNA) was electrophoretically separated on a 0.8% agarose gel and transferred onto a nylon membrane (Biotrans ICN), as described in the manufacturer's protocol. The filters were incubated for 30 min in hybridization solution (7% SDS, 0.5 M sodium phosphate buffer, pH 7.2, and 1 mM EDTA). The ³²P probes, labelled using random priming, were then added and hybridized at 65 °C overnight. Membranes were washed twice in 2 \times SSC containing 0.1% SDS and twice in 0.2 \times SSC containing 0.1% SDS for 20 min at 65 °C before autoradiography.

The EP1 probe corresponds to the *Hinc*II 634 bp fragment of the EP1 cDNA described already (Harwood *et al.*, 1994). The non-virus probe is a 1 kb long PCR product encoding a hypothetical protein with no significant homologies in GenBank. The immediate downstream (IDP) probe and the downstream (DP) probe are *Eco*RI–*Pst*I fragments, 1.2 and 1 kb long, respectively, isolated from a λ phage harbouring the EP1 sequence (λ 121) (Savary *et al.*, 1997). They correspond to sequences located 30 bp and 5 kb downstream of the EP1 3' end, respectively, in the 3' wasp-specific region of the EP1 locus.

■ **Ultrastructural analysis.** For transmission electron microscopy (TEM), ovaries were fixed in 1% paraformaldehyde, 3% glutaraldehyde and 0.1 M sucrose in 0.1 M cacodylate buffer, pH 7.4, for at least 5 h. After washing in the same buffer, samples were post-fixed in 2% osmium tetroxide for 1 h in the dark, dehydrated in a series of ethanol solutions (50, 70, 90 and 100%) and propylene oxide. Samples were then embedded in Epon, polymerized for 24 h at 60 °C and cut on Reichert Ultracut E. Ultrathin sections were cut in the lower calyx where the ovary is the largest. Then they were collected on grids coated with 2% collodion in amyl acetate, contrasted with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope.

Results

Characterization of *C. congregata* pupal development

The excision of virus segments A and EP1 from the wasp chromosome was previously shown to be developmentally

regulated (Savary *et al.*, 1999), beginning between the last larval and pharate adult stages. Characterization of the pupal development of *C. congregata*, never performed to date, was undertaken to obtain a more precise analysis of the pattern of virus replication during this early time frame. Photographs of pupae at different developmental stages are shown in Fig. 1. At the end of their development within the lepidopteran host *M. sexta*, *C. congregata* larvae emerge and spin cocoons on the back of the caterpillars (see Methods). Emerging animals have a larval shape (Fig. 1, d0) but upon dissection they reveal discernable developing organs such as eyes and antennae buds. Within the cocoons, the external larval appearance persists for approximately 38 h and the eyes become progressively orange (Fig. 1, d1). During moulting, the larval skin is eliminated, revealing an uncoloured pupa except for the eyes and the ocelli (Fig. 1, d2). At this stage the 'white pupae' have a fully formed body with legs and antennae on the ventral side. Morphogenesis of the ovaries also occurs during this time frame: the ovaries have the same shape in the white pupae as in the adults but their size is much smaller (data not shown). Afterwards, the most prominent developmental feature is a wave of melanization of the cuticle from the anterior to the posterior part of the animal, leading to the completion of the colour pattern of the adult (Fig. 1, d3–5). This wave of melanization allows precise characterization of the developmental stages occurring during late pupal development (see Methods).

Characterization of the onset of CcPDV replication using ultrastructural analysis

To study CcPDV replication during pupal–adult development, an ultrastructural analysis of the wasp ovaries was undertaken. Ultrathin sections were performed on the lower part of the calyx from individual pupae from d3, d4 ($n = 4$: d4.1 to d4.4 substages) and d5 stages (see Methods). The lower calyx is known to support PDV replication from previous studies on *Cotesia* species (De Buron & Beckage, 1992; Hamm *et al.*, 1990). Sections were observed using TEM (Fig. 2).

No sign of virus replication was observed in sections of the calyx from d3 pupae (data not shown). In contrast, we observed some modifications that could correspond to early steps in PDV replication (Fig. 2, left panel) in the youngest d4 pupa analysed (substage d4.1). The nuclei contained widespread patches of dense material, suggesting that chromatin reorganization was occurring. The cytoplasm of many cells contained black or white lysosomal vesicles as well as structures resembling apoptotic bodies. The typical features of PDV replication (De Buron & Beckage, 1992; Hamm *et al.*, 1990) were observed in a slightly older pupa (substage d4.2). In the calyx cells, chromatin patches were no longer visible and the nuclei were enlarged and filled with PDV envelopes that were empty or contained some nucleocapsids (Fig. 2, right panel). Moreover, typical PDV ring-shaped, electron-dense, virogenic stromata were observed, as described previously (De

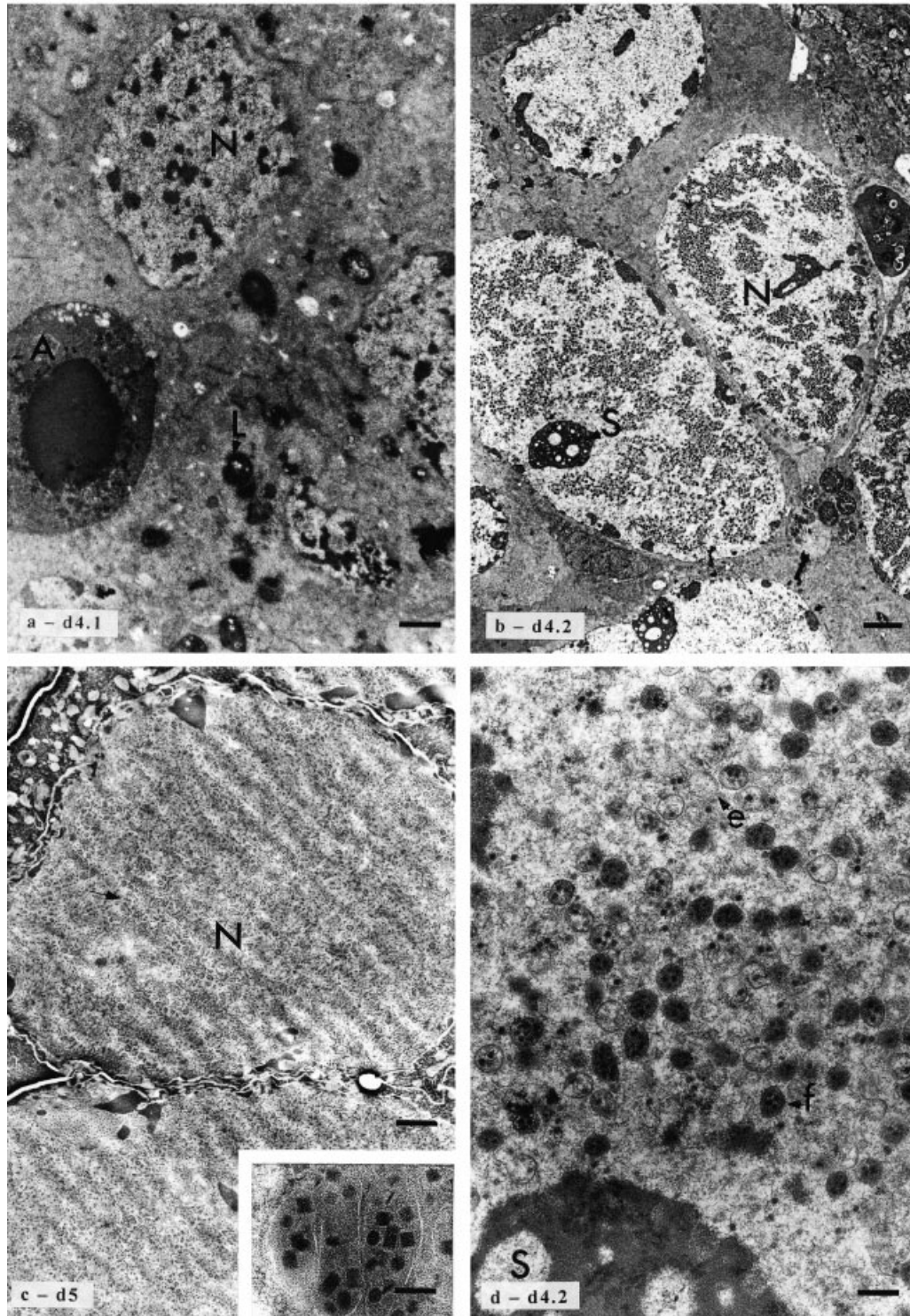


Fig. 2. Ultrastructural analysis of CcPDV replication in calyx cells of *C. congregata* pupae (sections were observed under TEM). (a) Calyx cells at the d4.1 stage: patches of dense material widespread in the nuclei (N) are visible. Lysosomes (L) and structures resembling apoptotic bodies (A) are observed in the cytoplasm (bar, 800 nm). (b) Calyx cells at the d4.2 stage: nuclei (N) are enlarged and contain variable quantities of PDV particles (arrowhead) (bar, 1600 nm). Moreover, typical virogenic stromata (S) are observed in the nuclei. (c) Calyx cells at the d5 stage: nuclei (N) are greatly enlarged and take up most of the volume of the cell. All the nucleoplasm is filled by PDV particles (arrow) (bar, 1000 nm). The insert shows a detail of three virus particles filled with nucleocapsids corresponding to an enlargement of the position indicated by the arrow (75000X). (d) Detail of the calyx cells at the d4.2 stage showing virogenic stromata (S) and empty CcPDV particles (e) or filled with nucleocapsids and protein matrix (bar, 250 nm).

Buron & Beckage, 1992). At day 5, most of the calyx cells had greatly enlarged nuclei occupying most of the cell volume and were densely packed with fully formed virus particles (Fig. 2, bottom panel).

This ultrastructural analysis allowed us to correlate the onset of PDV replication with the pigmentation pattern and was useful for comparison with results from molecular analysis of virus DNA molecules (see below).

The EP1 empty locus is an abundant molecule in female wasps

To analyse precisely the timing of virus replication by a molecular method, we developed a quantitative PCR approach. In a former study, a specific PCR product was obtained using DNA extracted from ovaries undergoing virus replication and primers flanking the EP1 sequence in the wasp DNA. Following production of the virus circle through excision of the EP1 virus sequence, the former 5' and 3' flanking sequences become adjacent and a PCR product can be obtained using primers within these sequences. The amplification of this fragment was used to monitor the excision of the EP1 virus sequence (Savary *et al.*, 1999). In the quantitative PCR approach, amplification of this fragment competes with the amplification of an artificial molecule, the QELS PCR competitor plasmid (Fig. 3a and see Methods).

According to the former model of EP1 circle production (Savary *et al.*, 1997), this quantitative PCR experiment was thought to measure the excision of EP1 sequence leaving an empty locus on the wasp chromosome. The data obtained using QELS PCR for pupae and adults of different ages are reported in Fig. 3(c) with a photograph of a representative experiment (Fig. 3b).

PCR products were observed using DNA extracted from d4 pupae and not with DNA from d3 pupae. This confirms that EP1 excision is developmentally regulated and that the onset of EP1 replication occurs during the d4 stage, as found using ultrastructural analysis. The amount of PCR product increased dramatically from d4 to d5 and remained at a high level in the adult females, indicating that cells undergoing PDV replication are continually present in the adult.

The quantity of QELS PCR competitor (Fig. 3) plasmid that had to be used to compete with the amplification of the empty locus was relatively high (up to 1 ng) considering that cells undergoing replication represent only a subset of ovary cells (Webb, 1998). This suggests that the empty locus does not correspond to a single copy on chromosomes that would have lost the EP1 sequence but to an amplified molecule.

Southern blot analysis on DNA from female wasps compared to signals obtained using purified virus DNA and male wasps

The fact that the empty locus might be amplified questions the nature of the molecule from which the EP1 circle is produced. The EP1 circle might be produced by excision from

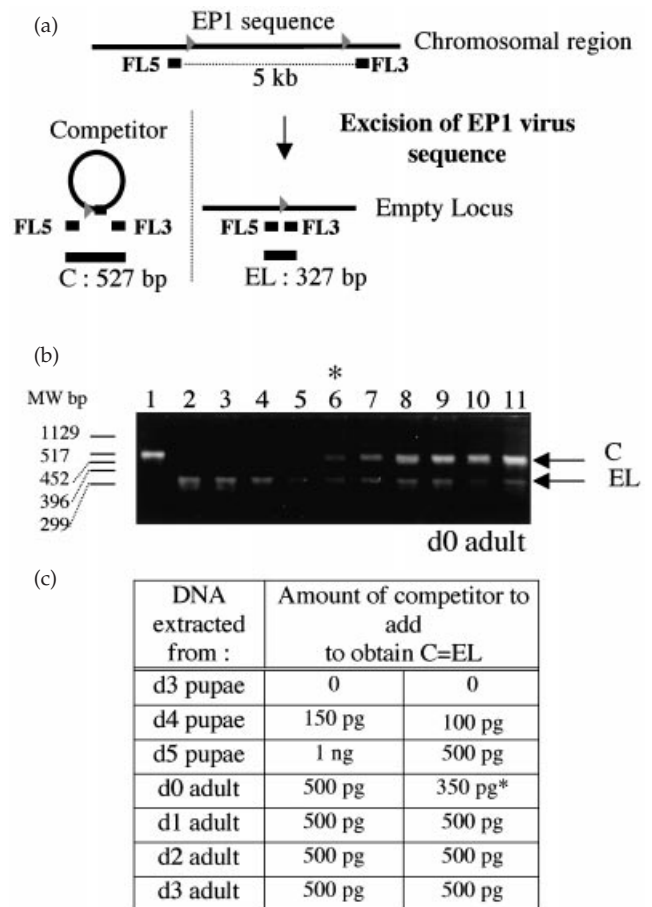


Fig. 3. Analysis of EP1 sequence excision during pupal development using QELS PCR. (a) Schematic representation of the PCR products obtained using QELS PCR; FL5 and FL3, primers used for PCR amplification; C, PCR product corresponding to the competitor molecule; EL, PCR product amplified from the empty locus. (b) Representative experiment corresponding to one of the QELS PCR performed on DNA extracted from female wasps. Lanes 1, 100 ng QELS competitor plasmid; 2, 100 ng wasp DNA; 3-11, 3 µg wasp DNA added with 100, 150, 200, 350, 500, 750, 1000, 1500 and 2000 ng QELS competitor plasmid, respectively. The quantity of competitor required to titrate the empty locus in the experiment shown (lane 6) is shown by an asterisk. (c) Table indicating the competitive PCR results obtained with DNA extracted from the different pupal developmental stages (d3-5) and from adult female wasps (d0-3) in two independent experiments using different DNA samples for each stage.

an amplified precursor. If such a precursor is produced during virus replication, it should be detected using DNA extracted from female wasps.

In a previous study intended to characterize the chromosomally integrated form of the EP1 virus segment, we have compared the different forms of this sequence both in virus particles and in male wasps. However, the different forms of the EP1 sequence were never analysed in female wasps where virus replication occurs. To complete the former study, we performed a series of Southern blot analyses.

The EP1 chromosomal region, cloned from a male wasp genomic library, has been described previously in detail (Savary

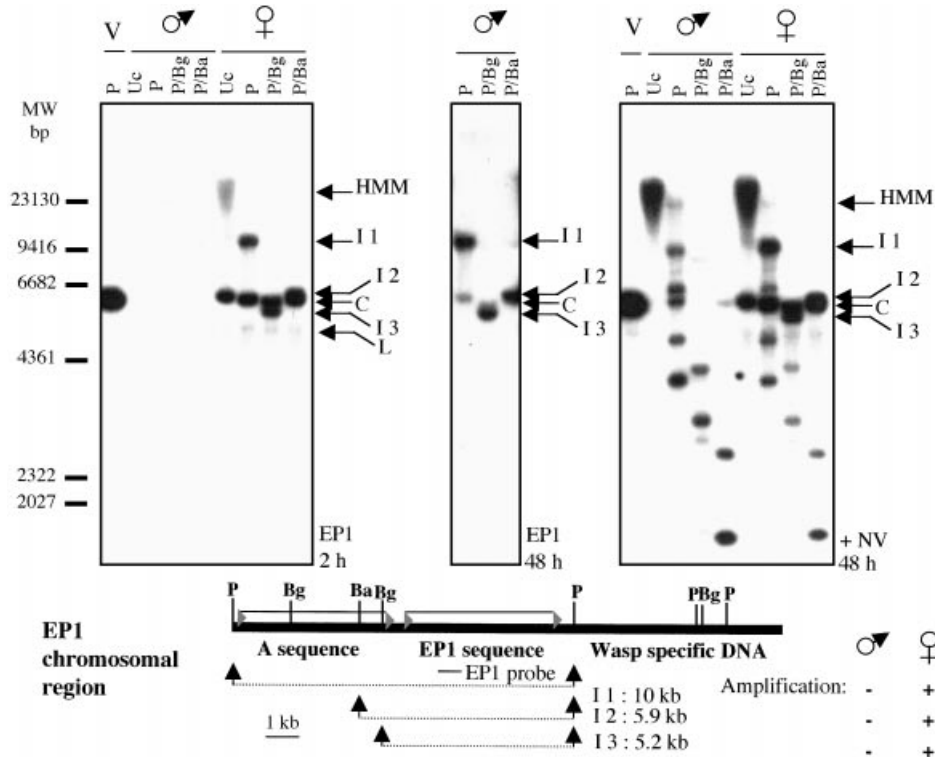


Fig. 4. Southern blot analysis of amplified EP1 molecules in DNA extracted from virus particles, adult female wasps undergoing virus replication and male wasps. The maps of the EP1 circle and of the EP1 integrated form (described in a previous study) are represented. The experiment was performed using DNA extracted from purified virus particles (V, 80 ng), female and male wasps (10 µg), either uncut (Uc) or restriction-digested (P, *PstI*; P/Ba, *PstI/BamHI*; P/Bg, *PstI/BgIII*) and hybridized with the EP1 probe or the non-virus probe as indicated. Left panel, 2 h exposure time autoradiography allowing detection of the amplified EP1 molecules. C, signals corresponding to the EP1 circle; L, signals corresponding to linearized circles due to damage during DNA extraction; I1–3, signals corresponding to fragments amplified in the females (the fragments corresponding to I3 and C are very close but can be identified separately using very short exposure time autoradiography); HMM, hybridization to high molecular mass DNA. Middle panel, detail of long exposure time autoradiography showing the lanes containing DNA extracted from male wasps comprising diploid individuals. A very low amount of EP1 circle is detected using male wasp DNA (see P and P/Bg digestions). The other signals correspond to the EP1 chromosomally integrated form, as described previously. Note that their positions match perfectly with those of the fragments amplified in female wasp DNA. Right panel, hybridization of the Southern blot with a *C. congregata* non-virus probe to control the amount of DNA transferred onto the blot. Hybridization to the amplified EP1 molecules remained after stripping (arrows). The other signals correspond to the hybridization of the non-virus probe to several related genes.

et al., 1997) and the map of this molecule is reported at the bottom of Fig. 4. Briefly, the integrated form of the EP1 sequence is flanked upstream by another virus molecule, the A sequence, and downstream by a sequence not found in virus particles (wasp-specific DNA). After 48 h of exposure, fragments of the integrated form (I1–3) can be visualized in male wasp DNA (Fig. 4, middle panel). In addition, a very low amount of signal corresponding to EP1 circle (C) was also detected, as reported previously (Fig. 4, lane P).

The EP1 and the A segments are coamplified in females

Using an EP1 probe to hybridize DNA extracted from virus particles and restricted by *PstI* (which does not cut in the EP1 circle), a unique intense signal corresponding to the migration of the circle was obtained (Fig. 4, left panel, lane V). After only

2 h exposure, signals corresponding to the integrated and circular form of the virus were very intense in female wasp DNA, while signals were barely detectable in males. Therefore, fragments corresponding to the integrated form of the virus appear to be amplified in female wasps (tenfold, as assessed by Instant Imager scanning). Furthermore, using undigested DNA extracted from female wasps (Fig. 4, left panel, lane Uc), two signals were obtained: one corresponds to the circle, while the other corresponds to hybridization with high molecular mass DNA (HMM). This indicates that the amplified molecule is very large. It was verified using a wasp-specific probe that the amount of male and female wasp DNA transferred onto the blot was similar (Fig. 4, right panel).

Similar results suggesting amplification of fragments of the integrated form of the virus were obtained using a series of restriction enzymes (data not shown). The fact that the I1 fragment – formerly used to diagnose EP1 sequence integration

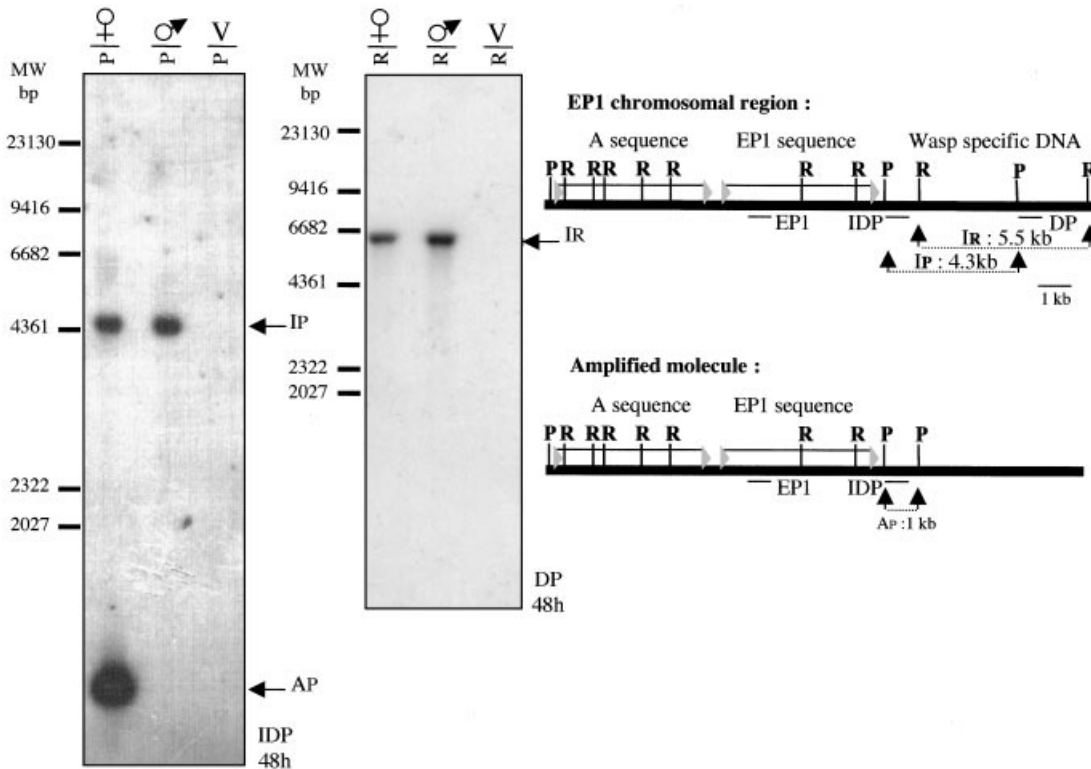


Fig. 5. Southern blot analysis of the EP1 chromosomal locus using probes located downstream in the wasp-specific region. Southern blots were performed using DNA extracted from female and male wasps (10 μ g) and from purified virus particles (V, 80 ng), restriction-digested (P, *Pst*I; R, *Eco*RV) and hybridized with the downstream probes. The maps of the chromosomal region containing the EP1 sequence and of the amplified molecule are shown on the right. Left panel, pattern of hybridization of the IDP probe. IP, fragment corresponding to the chromosomal form obtained using *Pst*I digestion (note that the signals are equally intense in DNA from female and male wasps); AP, hybridizing signal corresponding to a molecule specifically amplified in females. Right panel, pattern of hybridization of the downstream probe DP. IR, unique signal detected using *Eco*RV digestion corresponding to the chromosomal form. Note that the signal is not amplified using DNA from female wasps.

into the wasp chromosome (Savary *et al.*, 1997) – is amplified is particularly interesting because it contains all the A and EP1 sequences, indicating that at least two polydnavirus segments are coamplified in female wasp DNA. The I1 fragment also includes a short sequence located downstream of the direct repeat element (or direct repeat junction) that constitutes the border of the EP1 sequence.

Thus, in female wasps undergoing virus replication, in addition to the EP1 circle, a series of fragments corresponding to the map of the chromosomally integrated form (I fragments) are amplified, suggesting that the EP1 virus sequence might be chromosomally amplified.

The map of the amplified region is different from that of the EP1 locus

Chromosomal amplification such as that described in *Drosophila* in the salivary glands (polytene chromosomes) and in the ovaries (chorion genes regions) does not modify the restriction map of the amplified region. The level of amplification decreases progressively over several dozens of kilobases from the centre of the amplified region (Bostock, 1986; Osheim *et al.*, 1988; Spradling, 1981). To further analyse the

map of the EP1-amplified region and the level of amplification at different positions of the EP1 locus, we used several wasp-specific probes located downstream of the EP1 sequence in the chromosomally integrated form. Using the IDP probe on *Pst*I-digested female wasp DNA, two fragments were detected: a 4.3 kb fragment corresponding to the integrated form (IP) and a smaller fragment (AP, approx 1 kb) not present in male wasp DNA (Fig. 5, left panel). With virus DNA, no signal was obtained, as expected for the hybridization of a downstream non-virus probe. Interestingly, the signal corresponding to the chromosomally integrated form IP was equally intense using female and male wasp DNA, indicating that this DNA fragment was not amplified in females. In contrast, the female-specific signal AP was very intense, thus corresponding to an amplified molecule. A similar result was obtained using six different restriction enzymes: the expected chromosomal DNA fragments are not amplified in female wasp DNA but a unique signal of variable size was obtained corresponding to an amplified molecule (data not shown). Altogether, the results indicate that the map of the amplified molecule is different from the map of the wasp chromosome containing the EP1-integrated DNA.

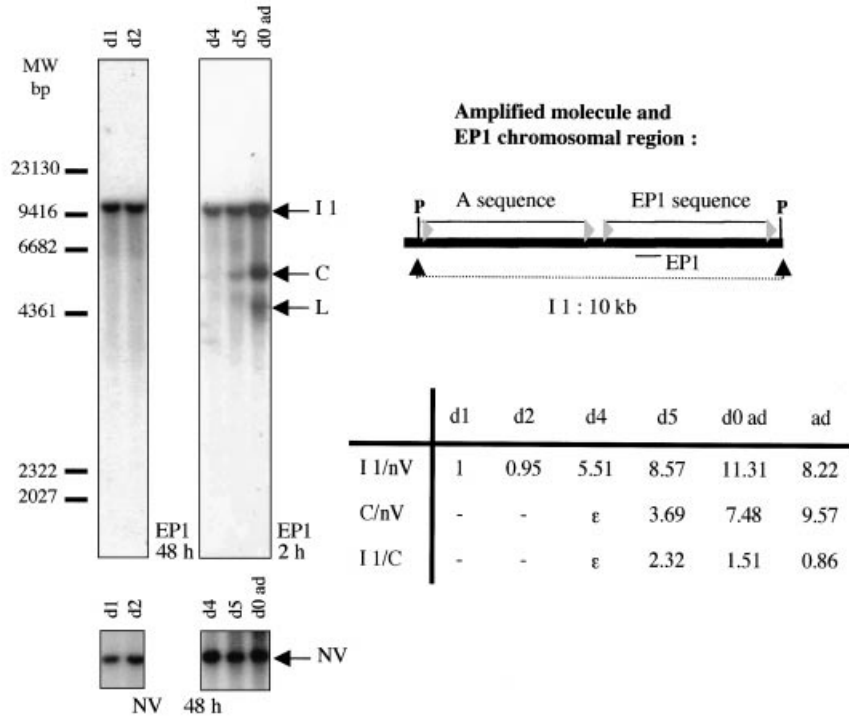


Fig. 6. Analysis of the timing of amplification of the EP1-containing molecules during *C. congregata* pupal development. A Southern blot was performed using DNA extracted from female pupae of different stages of development, and from adult female wasps, digested with *Pst*I. It was hybridized with the EP1 probe and the non-virus probe (NV). d1–5, DNA extracted from larvae 1 to 5 days after cocoon spinning; d0 adult, wasps newly emerged from the cocoon; ad, female wasps of different ages. Only 5 µg DNA was used for d1 and d2 (because of the difficulty to obtain DNA from these early stages), while 10 µg DNA was used for the other stages. Left panel, detail of a short exposure time autoradiography showing the amplified EP1 molecules. Bottom left panel, hybridization signal obtained using the NV probe to assess the amount of DNA hybridized onto the blot. The table indicates the ratios of the intensity of the signals obtained after Instant Imager scanning of the Southern blot. Values have been normalized: I1/NV = 1 for d1; –, no C signal obtained; ε, C signal too low for accurate quantification.

To confirm that the chromosomal form of the EP1 locus was not amplified, a Southern blot was hybridized with the DP probe, located further downstream on the EP1 locus (Fig. 5, right panel). Using Instant Imager scanning, the intensities of the hybridization signals obtained were similar using female and male wasp DNA, indicating that the corresponding region is not amplified in female wasps undergoing virus replication. The DP probe did not detect an amplified molecule.

Southern blot analysis of EP1 amplification during development

Amplified A and EP1 sequences are present in two forms in female wasp DNA: a form in which their sequences are physically linked and a circular form where they are separated (Savary *et al.*, 1997). In virus particles, the end product of virus replication, the A and EP1 sequences are found exclusively separated. Thus, it seems likely that the coamplification of A and EP1 virus sequences precedes the production of individual circles.

In order to determine if the amplification of the larger molecule precedes EP1 circle production, Southern blot analysis was performed with DNA extracted from different pupal stages, including the d4 stage where the first typical features of PDV replication were detected by ultrastructural analyses (d4.2 substage). DNA samples were digested by *Pst*I and hybridized with both the EP1 probe and the non-virus probe. The ratios of the intensities of the signals corresponding to EP1-containing sequences and the control probe are presented in Fig. 6 (Instant Imager scanning of the blot). The signals corresponding to the I1 fragment were detected only after a long exposure in DNA extracted from early stages (d1 and d2), as expected for the non-amplified chromosomal form (Fig. 6 left panel, for quantitative data see table). The amplified molecules detected in this analysis are shown in a short exposure time autoradiography of the blot (Fig. 6, right panel). Interestingly, the signal corresponding to the I1 fragment was amplified in DNA extracted from d4 larvae in contrast to the EP1 circle signal (Fig. 6, C, table), which was barely detectable at this stage (data not shown), and became intense only at d5

(Fig. 6). This result strongly suggests that the coamplification of A and EP1 sequences precedes the production of the individual EP1 circle during virus replication. Moreover, the level of the I1 signal was always higher than that of the EP1 circle whatever the developmental stage used, except in the adult where they were comparable (table of Fig. 6). Thus, the EP1 circle does not appear to be amplified following excision.

Taken together, these results suggest that a large precursor molecule is amplified prior to EP1 circle production and that the EP1 circle itself is not amplified further.

Discussion

The CcPDV EP1 sequence is integrated as a provirus into the wasp genome and found as a circular form in the virus particle (Savary *et al.*, 1997; Belle *et al.*, 2002). Very little is known about the mechanisms that allow the production of the PDV circular DNA present in the virus particles from the chromosomal provirus sequences. To improve the understanding of PDV replication, we have analysed the different forms of the EP1 sequence, the smallest virus CcPDV genome segment, during pupal–adult development of the parasitoid wasp *C. congregata*. Results of ultrastructural and PCR analyses are consistent and indicate that CcPDV replication begins late during pupal development, 4 days after the larvae have emerged from the parasitized *M. sexta* larva and continues throughout pupal and adult life.

In the ichneumonid *Campoletis sonorensis* and the braconid *Chelonus inanitius* (a species belonging to a different braconid subfamily than *Cotesia congregata*), PDVs have also been shown to begin replication late during pupal–adult development (Albrecht *et al.*, 1994; Webb & Summers, 1992). Despite the important differences between the two polydnavirus genera, PDV replication follows a similar temporal specificity. The association between wasps and viruses is thought to have occurred independently in the braconid and ichneumonid lineages (Whitfield, 2000). Therefore, it is likely that the common temporal pattern of ichnovirus and bracovirus PDV replication is due to a convergent evolution. Indeed, females of these short adult life species should be ready for oviposition shortly after emergence. It was recently proposed that all bracoviruses derived from the same virus captured by an ancestor braconid wasp (Dowton & Austin, 1998; Whitfield, 1997, 2000). The common temporal regulation of bracovirus replication might have been inherited from this ancestor.

Beyond the characterization of the onset of CcPDV replication, this study reveals that an amplification of virus sequences of the EP1 locus precedes the excision of the individual circles. We have shown that several fragments corresponding to the map of the EP1 chromosomally integrated form (I fragments) were unexpectedly amplified in female wasps, including a 10 kb off-size fragment formerly used to diagnose the integration of the EP1 sequence into wasp genomic DNA. The level of amplification is approximately

tenfold and is most likely considerably higher in the specialized cells of the ovaries undergoing virus replication. It will be of major interest to determine whether the off-size fragments used to diagnose the integration of PDV sequences in parasitoid wasp genomic DNA are similarly amplified in the females of other species (Fleming & Summers, 1991; Gruber *et al.*, 1996; Xu & Stoltz, 1991). It should be noted that the demonstrations that polydnavirus sequences are integrated into parasitoid wasp genomes all rely on the fact that off-size fragment virus sequences are physically linked to non-virus sequences, i.e. sequences not present in the virus particles. The fact that sequences not present in the virus particles might also be amplified questions their non-virus nature and thus potentially weakens the demonstrations that polydnavirus segments are integrated into wasp DNA.

The restriction fragments corresponding to the chromosomally integrated form are amplified; one could therefore argue that the amplified molecule is the chromosomal form of the EP1 sequence. Chromosomal amplifications in *Drosophila* salivary glands (polytene chromosomes) and ovaries have been studied extensively (Bostock, 1986; Osheim *et al.*, 1988; Spradling, 1981). In the ovaries, two clusters of chorion genes undergo amplification in specialized cells prior to their expression late in oogenesis. Chromosomal amplification is achieved through additional rounds of replication specifically initiated within a central region. The highest level of amplification is observed in the centre of the region containing the chorion genes with a gradual decrease on both sides over 50 kb (Spradling, 1981). In Southern blot analysis using DNA extracted from female wasps, a very intense signal of hybridization of the EP1 probe to high molecular mass DNA was obtained, in addition to the signal corresponding to the EP1 circle, thus suggesting chromosomal amplification. However, this hypothesis was excluded after a detailed Southern blot analysis using probes located in the wasp-specific region of the EP1 chromosomal locus (non-virus region). Indeed, using a probe located immediately downstream of the EP1 sequence, the I fragments were not detected as amplified in the females. Using the DP probe, which hybridizes further downstream, no evidence for any amplification was found. The fact that the downstream sequence linked to EP1 is not amplified confirms the physical link between virus and non-virus sequences and thus the integration of EP1 sequence into chromosome DNA. In addition, according to the models of chromosomal amplification described to date, it seems very unlikely that the level of amplification can decrease so dramatically between EP1 and this downstream sequence. Moreover, amplified signals that did not correspond to the fragments of the integrated form could be detected using the IDP probe and a series of restriction enzymes, which indicates that the map of the amplified molecule is different from that of the EP1 chromosomal locus. The direct visualization of the amplified molecule will require pulse field electrophoresis because it is most probably very large, as suggested from the

hybridization of the EP1 probe to high molecular mass DNA in Southern blot analysis. However, this technique requires a large amount of wasp DNA, which is very difficult to obtain with *C. congregata*. It was suggested that a recently identified gene encoding a structural protein of *Campoletis sonorensis* PDV, the p44 gene, was chromosomally amplified based on the hybridization of the P44 probe to high molecular mass female wasp DNA, using Southern blot analysis. Strikingly, this gene is not encapsidated within virus particles (Deng *et al.*, 2000). Our results suggest that hybridization to high molecular mass DNA is not sufficient to conclude that p44 is chromosomally amplified. The p44 gene, like the downstream sequence of the EP1 gene, might be amplified within a large virus precursor molecule but not encapsidated.

Close to the onset of virus replication, the large amplified molecule can be observed, whereas the EP1 circle is not yet detected. This indicates that coamplification precedes the excision of individual circles. Following excision, EP1 flanking sequences become adjacent and a specific product can be obtained using PCR analysis. This product corresponds to the amplification of an abundant molecule. After excision, the double-stranded DNA circles could be further amplified by a rolling-circle mechanism. However, this may not be necessary since the level of amplification of the precursor molecule is comparable to the amount of EP1 circle measured in the female wasps and could be therefore sufficient for EP1 circle production. Taken together, our new model of EP1 sequence replication involves the excision of a very large molecule (comprising at least A, EP1 and downstream sequence), the amplification of this molecule and the excision of the individual circles.

In ichnoviruses, smaller circles are generated from larger circles through alternative recombination pathways, a mechanism called 'nesting' (Cui & Webb, 1997). As a result, large and small circles are both found in the virus particles. Similarly, the EP1 and A circle appears to be nested in a large amplified molecule. Contrarily to ichnoviruses, however, the larger molecule is not found in the virus particles.

If one allows a speculative hypothesis, a precursor PDV molecule might contain several virus sequences separated by spacers that are not encapsidated within virus particles, such as the sequence separating the A and EP1 segments and the small part of the downstream sequence amplified along with EP1. The DNA enclosed in the virus particles would thus represent only a subset of virus sequences, with the 'true' virus genome corresponding to all the sequences amplified during virus replication.

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