

Excision of the polydnavirus chromosomal integrated EP1 sequence of the parasitoid wasp *Cotesia congregata* (Braconidae, Microgastinae) at potential recombinase binding sites

Stéphane Savary,¹ Nancy Beckage,² Frances Tan,² Georges Periquet¹ and Jean-Michel Drezen¹

¹Institut de Recherche sur la Biologie de l'Insecte – UPRESA CNRS 6035, Faculté des Sciences, Parc de Grandmont, 37200 Tours, France

²Department of Entomology, 5419 Boyce Hall, University of California at Riverside, Riverside, CA 92521-0314, USA

Cotesia congregata polydnavirus (CcPDV) is essential for successful parasitism of *Manduca sexta* larvae by the braconid wasp *Cotesia congregata*. To determine the molecular mechanisms for the vertical transmission of CcPDV in the wasps, we analysed the different forms of the virus sequences containing the gene encoding the early parasitism-specific protein 1 (EP1). By a detailed molecular analysis, we demonstrated that the EP1 sequences are present in wasp DNA in two forms: a circular form as seen in the virus particles and a form integrated into the wasp genome. Moreover, we showed that the integrated form of the EP1 sequences is able to excise in the ovary cells. A fragment corresponding

to an EP1 'empty locus' (without the viral sequence) was PCR-amplified from ovarian DNA. Comparison of the sequences isolated from the EP1 circle, the integrated form and the empty locus revealed that the extremities of the EP1 genomic sequences constitute a direct repeat. Strikingly, these sequences contain a potential binding site for a recombinase of the Hin family located in close vicinity to the position where the DNA strand exchange occurs. Thus, the data bear upon the possibility that the bracovirus circles are excised via a mechanism related to the Hin mediated Conservative Specific-Specific Recombination (CSSR) of prokaryotes.

Introduction

Replicating in the ovaries of ichneumonid and braconid species of parasitic hymenoptera, the polydnaviruses (PDVs) are designed for export into parasitized host larvae (reviewed in Whitfield, 1990; Stoltz, 1993; Summers & Dib-Hajj, 1995). Once within the host insect, the expression of viral genes leads to numerous physiological changes associated with parasitism, including suppression of the host immune response (Edson *et al.*, 1981; Lavine & Beckage, 1995, 1996) ensured by altering haemocyte functions or inducing their apoptosis (Strand & Pech, 1995; Davies & Vinson, 1988). Artificial introduction of parasitoid eggs into the host, with or without virus particles,

has shown that PDVs are necessary for successful development of the parasite (Edson *et al.*, 1981). Moreover, the viruses are strictly dependent on the wasp as they only replicate in a specialized ovarian cell type (the calyx cells) (de Buron & Beckage, 1992; Volkoff *et al.*, 1995 and reviewed in Fleming & Krell, 1993).

PDVs are also characterized by their segmented genome which is composed of multiple double-stranded DNA circles. PDVs have been classified in two groups based on significant differences in the shape of their particles and in their physiology. The ichnoviruses are found exclusively in parasitoid species belonging to the Ichneumonidae and are characterized by an ascovirus-like morphology (Federici, 1991; Bigot *et al.*, 1997) with an ovoid shape, two membranes and a large lenticular nucleocapsid (reviewed in Stoltz & Whitfield, 1992). They are released in the ovary lumen by budding from calyx cells. The bracoviruses, found in Braconidae, have rod shaped nucleocapsids localized in a protein matrix surrounded

Author for correspondence: Jean-Michel Drezen.

Fax +33 2 47 36 69 66. e-mail drezen@univ-tours.fr

The GenBank/EMBL accession numbers of the sequences reported in this paper are AF006205–AF006208.

by a single unit envelope. Multiple nucleocapsids are frequently present in a single virion. In contrast to the ichnoviruses, virions are released during lysis of the calyx cells (Stoltz & Whitfield, 1992).

Previous genetic analyses have shown that the viral sequences are transmitted as Mendelian traits, suggestive of chromosomal transmission via the germline. Furthermore, molecular analyses performed on the ichneumonid species *Camponotus sonorensis* and *Hyposoter fugitivus* (Hymenoptera, Ichneumonidae, Campopleginae) have indicated that the sequences of PDV circles found in the virus particles are also present in a linear form in the genome of the wasps. This was deduced from Southern blot analyses of genomic DNA (Fleming & Summers, 1986; Xu & Stoltz, 1991). The sequences of the viral DNA circles were detectable as off-size restriction fragments in male and female somatic tissues indicating the integration of viral DNA in a high molecular mass structure. The cloning of one of the integrated sequences in *Camponotus sonorensis* has demonstrated that the flanking region corresponds to wasp genomic DNA (Fleming & Summers, 1991).

The reported data on bracoviruses are not as consistent as those obtained on ichnoviruses. On the one hand, studies performed on *Cotesia melanoscela* have shown the presence of the circular form of a viral segment but not the corresponding integrated form (Stoltz *et al.*, 1986). On the other hand, a recent study carried out on a PDV circle of the solitary braconid parasitoid wasp *Chelonus inanitus* has strongly suggested that it is integrated in the wasp genome (Gruber *et al.*, 1996). However, the circular form was not detected in DNA of male wasps. These differences could reflect the differences in experimental approaches used in the studies, or the fact that the bracovirus group is polymorphic.

To answer the question, we have analysed the relationship between the gregarious braconid wasp *Cotesia congregata* and its PDV. The *Cotesia congregata* PDV (CcPDV) is essential for successful parasitism of tobacco hornworm (*Manduca sexta*) larvae by the wasp *Cotesia congregata* (Lavine & Beckage, 1995, 1996). Earlier studies focused on the virus sequences containing the CcPDV EP1 gene (early protein 1) encoding the EP1 parasitism-specific protein (Harwood & Beckage, 1994; Harwood *et al.*, 1994). The CcPDV EP1 product is expressed at a high level in *Manduca sexta* 24–48 h after oviposition and is hypothesized to play a role in impairing the ability of the tobacco hornworm to encapsulate the parasitoid's eggs. The haemocytes undergo apoptosis soon after the host is parasitized, within 24–48 h post-oviposition; then the host's haemocyte population gradually returns to normal over the next 6–8 days (Lavine & Beckage, 1995, 1996).

We show here that both the circular and integrated forms of EP1 sequences are present in male wasps, and that the circular form is produced by excision of the integrated sequences. Strikingly, the sequence data suggest that the excision of the EP1 PDV sequences might occur by a molecular mechanism related to that involving the conserved protein

family of site-specific recombinases from enteric bacteria (the Hin invertase protein family) (Hughes *et al.*, 1992).

Methods

■ **Insects.** *Cotesia congregata* (Hymenoptera, Braconidae, Microgasterinae) is a gregarious larval endoparasitoid. Adult female wasps are easily distinguished from adult males by the presence of a prominent ovipositor on the posterior abdomen. They lay their eggs in first instar larvae of the host, then their progeny emerge 10–12 days later from fifth instar hosts. The parasitic wasps were reared on their natural host, the tobacco hornworm, *Manduca sexta* (Lepidoptera, Sphingidae). Host larvae were reared individually in separate containers supplied with artificial diet as previously described (Harwood & Beckage, 1994).

■ Preparation of wasp genomic DNA and virus particles DNA.

The sex of each individual *Cotesia congregata* wasp was visually checked twice under a binocular dissecting microscope. A volume of 500 µl of wasps was stored frozen at –20 °C in a 1.5 ml Eppendorf tube. DNA was extracted using the 'easy DNA' kit (Invitrogen) essentially as described in the supplier's protocol. For further purification, after resuspension of the samples in distilled water, a proteinase K treatment followed by several phenol and chloroform–isoamyl alcohol extractions were performed. Nucleic acids were then precipitated with ethanol. Virus DNA was prepared following purification of the virus particles by filtration as previously described (Harwood & Beckage, 1994).

■ **Genomic library screening.** The genomic library was constructed by Stratagene from 100 µg of male wasp genomic DNA. After partial digestion using *Bam*HI, the inserts were cloned in a Lambda Dash II vector. A total of 1.2×10^6 p.f.u. were obtained.

After amplification of the library to 10^9 p.f.u., filters corresponding to 50000 p.f.u. per plate were hybridized under stringent conditions using the 634 bp *Hinc*II EP1 cDNA probe (Harwood & Beckage, 1994). Five clones were obtained comprising overlapping 13–15 kb inserts. Restriction mapping of the locus and subcloning of specific regions prior to sequence analysis were carried out from the clone 1.2.1.

■ Sequencing strategy: subcloning of DNA fragments and PCR products

(a) **Junctions between EP1 and flanking sequences.** The junctions between EP1 and its flanking sequences were determined by comparing the restriction maps of the circular and integrated forms. The 5' junction region has been mapped to the 1.5 kb *Hind*III–*Bgl*III fragment of the 1.2.1 clone. The 3' junction region has been mapped to the 2 kb *Hind*III–*Eco*RI fragment. These fragments were subcloned into a pBlue-script KS vector.

(b) **The EP1-containing viral circle.** The primers A1EP1c (AGAACTACTATGTGGGTGAT) and A2EP1c (CAGAAATAGTACCTCCGAGA) were synthesized by Genset. They correspond respectively to positions 472–492 and 447–466 on the EP1 cDNA sequence previously described (Harwood & Beckage, 1994) (GenBank/EMBL accession no. D29821). These primers were used for amplification of the near full-length EP1 circle sequence by reverse PCR using the expanded long template PCR system (Boehringer Mannheim). Amplification was performed from 40 ng of virus DNA. The temperature programme was 94 °C for 2 min followed by 94 °C for 10 s, 48 °C for 90 s, 68 °C for 5 min, repeated for 25 cycles in the PCR reaction.

(c) **Characterization of the junction between EP1 extremities on the circle.** In order to sequence the region corresponding to the junction of

EP1 extremities on the circle, a series of deletions was performed on the EP1 circle-KS Bluescript plasmid linearized by *SacI*/*Bam*HI, using the Erase-a-Base deletion kit (Promega). By comparing the locus and the circle restriction maps, the junction point was expected to be located in a *XhoI*-*XbaI* 429 bp fragment. A fragment corresponding to 5 min 30 s exonuclease III digestion was found to contain the junction point and was sequenced on both strands.

In addition, to determine if the junction region was the same on all the virus circles, four PCR products (272 bp) amplified from virus DNA were sequenced. They were obtained using the primers cirep1A1 (CTACCCACATCTAAAATCCT) and cirep1A2 (TATAGCAACTGATGGGTAC), designed from the first junction region sequence determined. The PCR experiment was carried out in the presence of 5 units of *Taq* polymerase (Promega), 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 9, 0.1% Triton X-100, 0.2 mM NTPs and 250 pmol of each primer. After a 3 min denaturation step at 94 °C, amplification was performed for 30 cycles with the following steps: 30 s at 94 °C for denaturation, 1 min at 55 °C for annealing and 1 min at 72 °C for extension. PCR products were purified on Wizard minicolumns (Promega) and cloned in KS Bluescript linearized at the *SmaI* site and T tailed using *Taq* DNA polymerase.

■ Characterization of the EP1 'empty locus'. The PCR product corresponding to the empty EP1 locus was amplified using DNA extracted from ovaries and primers WbglII (CCTTTGCTATGTGGATTCC) and WpstI (ACACTACAAGTACCCCGACT), designed from the 5' and 3' flanking sequences, respectively. The PCR was carried out as described previously. After a 3 min denaturation step at 94 °C, amplification was performed for 30 cycles with the following steps: 30 s at 94 °C for denaturation, 1 min at 55 °C for annealing and 1 min at 70 °C for extension. The specificity of the PCR amplification was verified by Southern blotting of the PCR product and hybridization under stringent conditions with the oligonucleotide amima2 (GCTATGTGATACTAAGAAC) whose sequence is closer to the 5' junction site than that of the WbglII primer. Prehybridization, hybridization and washings were performed in 6 × SSC, with 0.5% SDS added. The membrane was prehybridized for 30 min, hybridized for 5 h at 48 °C and washed, first for 10 min at 48 °C and then for 3 min at 54 °C. After purification on Wizard minicolumns (Promega), PCR products were cloned in KS Bluescript linearized at the *SmaI* restriction site and T tailed. Eight independent clones containing the empty EP1 locus were sequenced.

■ DNA sequencing reactions. All the DNA fragments destined for sequencing were cloned in a pBluescript KS vector. Plasmids were isolated and purified using the protocol of alkaline lysis followed by PEG purification (Sambrook *et al.*, 1989). Dideoxy reactions were performed on double-stranded templates using the Sequitherm long read cycle sequencing kit (Epicentre Technologies) and labelled T3 and T7 primers IRD41. The temperature programme was 94 °C for 2 min (first denaturation step) followed by 94 °C for 30 s (denaturation), 55 °C for 25 s (annealing), 70 °C for 25 s (elongation), for 30 cycles. The sequence data presented are based on sequencing of both strands. The nucleotide sequence data reported in this paper appear in the GenBank sequence database under accession nos AF006205–AF006208.

Results

Presence and physical structure of CcPDV DNA in male wasps

To determine whether male wasps (that do not produce

virus particles) contain viral DNA, the ³²P-labelled EP1 CcPDV specific probe (Harwood *et al.*, 1994) was hybridized on Southern blots of high molecular mass DNA extracted from pooled male wasps and compared to hybridization with DNA extracted from purified virus particles. Hybridization signals were detected using the EP1 probe on male genomic DNA indicating that male wasps contain viral sequences (Fig. 1, left panel). Two signals were obtained in each lane. First, the 5 kb signals correspond to the native EP1 circular DNA molecules (see the second lane: uncut DNA) found using DNA extracted from virus particles (see the first lane: virus UC). The EP1 circle does not contain any recognition site for the restriction enzymes used in the experiment and therefore remains uncut in digested male genomic DNA (see the lanes *Bgl*III, *Eco*O109I and *Pst*I). The second signal corresponds to molecules of higher molecular mass (8–9 kb using *Bgl*III, *Eco*O109I and *Pst*I). This suggests that the EP1 sequences are integrated in high molecular mass DNA, the restriction sites being provided by flanking regions. In the ichneumonid studies, such offsize fragments were interpreted as being diagnostic of the integration of viral sequences into the wasp genome (Fleming & Summers, 1986; Xu & Stoltz, 1991). This interpretation is supported for EP1 sequences by the hybridization of the probe on high molecular mass DNA of the wasp (lane wasp UC left and right panels). Thus, male genomic *Cotesia congregata* DNA appears to contain EP1 sequences in two forms: episomic circles similar to the viral DNA itself and genomic sequences.

In order to pursue the analysis of the different form of the EP1 sequences in the wasp tissues that do not support extensive replication, a Southern blot was carried out using DNA extracted from different dissected body parts (head, thorax and abdomen) of wasps of both sexes. After hybridization with the EP1 probe, the result shows first that the offsize EP1 fragment obtained with *Pst*I is present in the female DNA (Fig. 1, right panel, F H, F T), indicating that viral DNA is integrated in the genomic DNA of female wasps as well as males. In addition, the signal corresponding to the EP1 circular form is found in all body parts of both males and females. Thus, both the integrated and the circular form of EP1 sequences are ubiquitous. However, the level of the circular form varies depending on the sex and the body part of the animal. The ratio of circular versus integrated form determined from phosphorimager scanning of the Southern blot ranges from 0.26 in the DNA extracted from male heads to 3.7 in the DNA from the thorax of females.

Cloning of the EP1 integrated locus

To confirm the integration and to further analyse the EP1 integrated form, a *Cotesia congregata* male genomic library was screened with the EP1 probe and five independent clones containing 13–15 kb inserts were purified. The physical map of the genomic clone 1.2.1 is shown in Fig. 2. The map is consistent with the Southern blot data indicating that no major

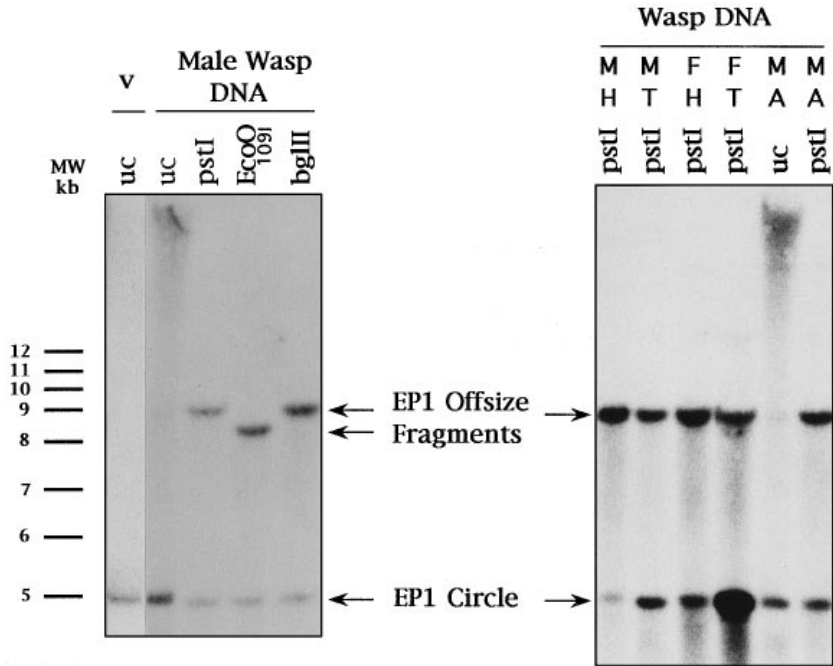


Fig. 1. Presence of circular and integrated forms of EP1 CcPDV DNA in male wasps. Southern blot of DNA extracted from male *Cotesia congregata* and from purified virus particles hybridized with the EP1 probe. Left panel: first lane, 5 ng of uncut virus DNA; following lanes, respectively: 20 µg of male wasp DNA, uncut, or digested with *BglII*, *EcoO109I* or *PstI*. These restriction enzymes do not cut in the EP1 circle. Right panel: DNA digested with *PstI* or uncut (uc) extracted from head (H), thorax (T) or abdomen (A) from male (M) or female (F) wasps. The lane female wasp abdomen DNA is not shown because the level of virus molecules in the ovaries was too high.

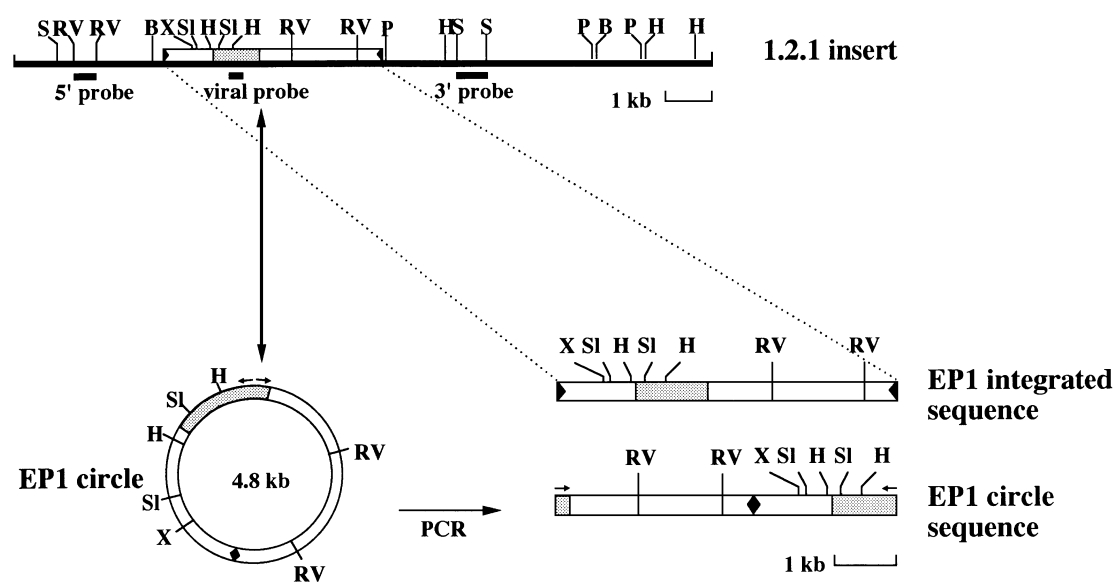


Fig. 2. Restriction maps of the EP1 integrated locus (clone 1.2.1) and of the EP1 circle cloned using PCR. The half diamond represents the junction between EP1 and flanking sequences; the complete diamond corresponds to the circle junction (region where the EP1 extremities come into contact to form the circle). The arrows show the position of the primers used for PCR amplification of the EP1 circle. EP1 coding sequence is shown in grey. S, *SacI*; RV, *EcoRV*; B, *BglII*; X, *XhoI*; SI, *Sall*; H, *HindIII*; P, *PstI*.

recombination occurred during the cloning process. To compare genomic and circular EP1 sequences, the EP1 circle was cloned by PCR using primers in reverse orientation designed from the sequence of the EP1 gene (see Methods). As shown in Fig. 2, and as far as it can be concluded from restriction analysis, the EP1 sequences of the integrated form and of the circle are identical. The data suggest that the

genomic copies of PDV molecules are used for circle production.

Characterization of the flanking sequences of the EP1 integrated locus

To determine the nature of the flanking sequences of the EP1 locus, several probes were subcloned from the 5' and 3'

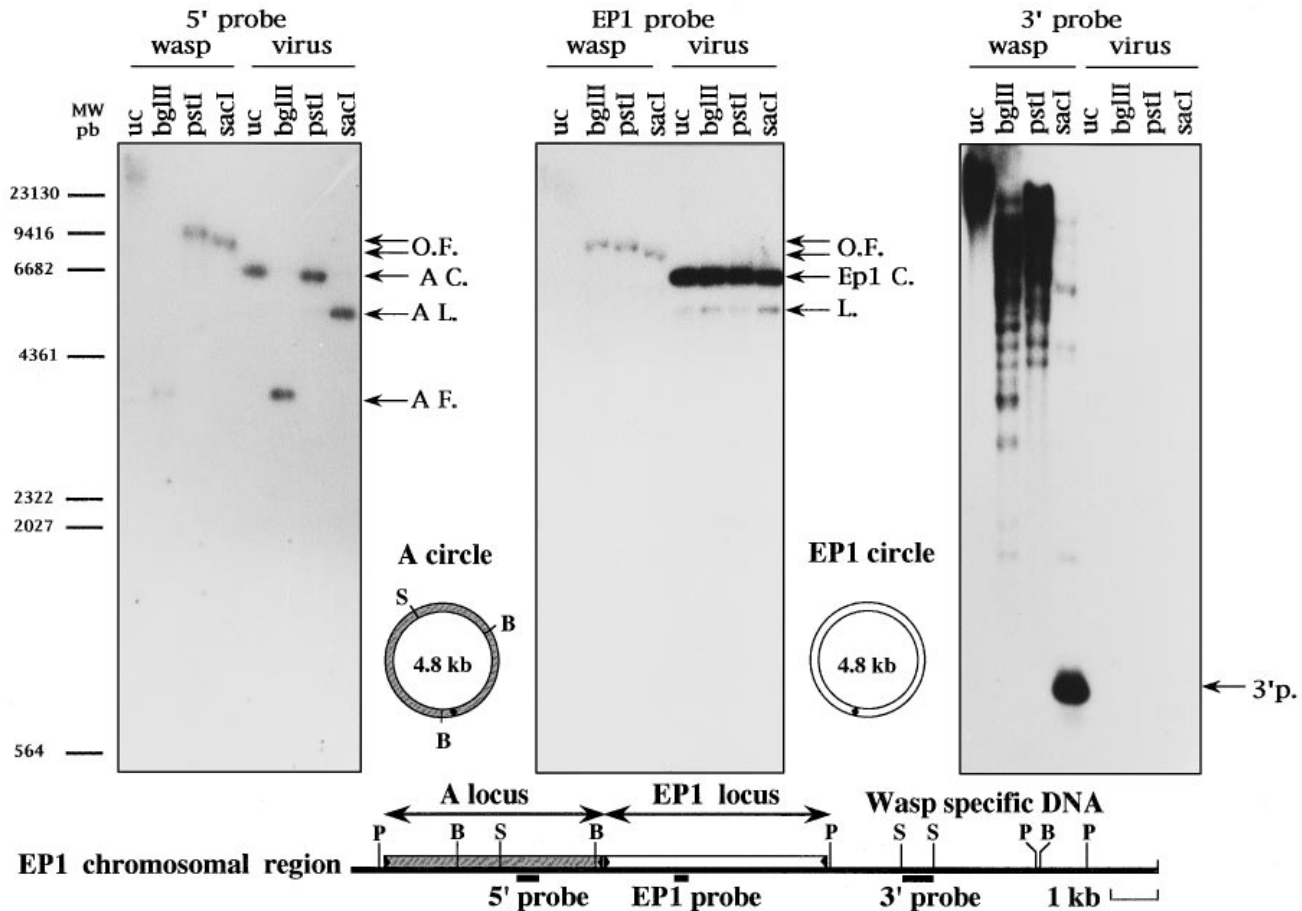


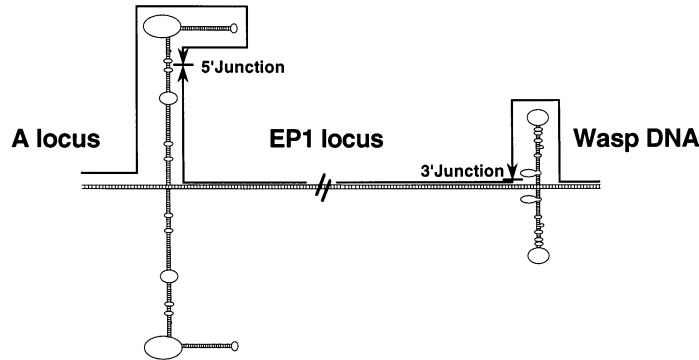
Fig. 3. Physical linkage between EP1 sequences and non-viral wasp sequences in the 3' region of the EP1 locus. Southern blot triplicates performed with DNA extracted from male *Cotesia congregata* (wasp) and from purified PDV particles (virus) were hybridized with 5', EP1 and 3' probes to analyse the nature of the flanking region of EP1 sequences (10 µg of genomic DNA and 80 ng of virus DNA were used per lane). The lanes correspond either to uncut DNA (uc) or to DNA digested with *Bgl*III, *Pst*I or *Sac*I. Centre panel: EP1 hybridization to virus DNA gives a strong hybridization signal (5 kb) corresponding to the closed relaxed EP1 circle (EP1 C.) and a faint signal corresponding to the size of linearized EP1 molecules (L.) (4.8 kb), probably produced by physical damage during DNA extraction. Offsize fragments (O.F.) were obtained with the three restriction enzyme (that do not cut in the EP1 circle). The signal corresponding to the circular form was not detectable in this experiment due to the lower amount of genomic DNA used. For the same reason, the signals obtained using uncut genomic DNA were very low except with the 3' probe, which contains a repetitive sequence. Left panel: hybridization of the 5' probe with virus DNA indicates that the corresponding DNA constitutes another virus circle, the A circle (A C.: A circle uncut; A L.: A circle linearized; A F.: 3 kb *Bgl*III A circle fragment hybridizing with the 5' probe). The same offsize fragments were obtained as with the EP1 probe, except with *Bgl*III because of the *Bgl*III restriction site located between the 5' and 3' probe. Right panel: the lack of 3' probe hybridization with virus DNA indicates that the corresponding sequences are non-viral, while the pattern of hybridization on male wasp genomic DNA is characteristic of a probe constituted by a moderately repetitive sequence (3'p, 600 bp *Sac*I fragment corresponding to the 3' probe). The map below summarizes the data obtained from the restriction analysis of the 1.2.1 locus and from the results of the Southern blot analysis for the relevant restriction enzymes (B, *Bgl*III; P, *Pst*I; S, *Sac*I). The 5' part of the diagram (the left part of the A locus) which extends over the 5' extremity of the 1.2.1 clone and the putative A circle map are deduced from the Southern blot analysis.

regions of the 1.2.1 insert. These probes were hybridized to Southern blots of male wasp genomic DNA and to DNA extracted from virus particles.

The 5' flanking region of the EP1 locus was shown to contain viral sequences. Nonetheless, the hybridization pattern obtained with the 5' probe on the virus DNA was different from that of the EP1 circle. The 5' flanking sequence thus corresponds to a different viral molecule. As a PDV sequence, this molecule is most probably circular in virus particles: thus

we called it the 'A circle' (Fig. 3). The hybridization pattern of the 5' probe on male genomic DNA is that expected from the map of the genomic DNA region. However, it should be noted that the intensity of hybridization of the 5' probe to genomic DNA is similar to that obtained to virus DNA, while with the EP1 probe the signal obtained is considerably higher with virus DNA (Fig. 3). This indicates that, unlike the EP1 circle, the A circle is very weakly represented in virus particles, a result confirmed using a second specific A probe (data not shown).

A) Overview of the putative cruciform structures surrounding EP1 sequences :



B) Junction sequences and potential intrastrand base pairing :

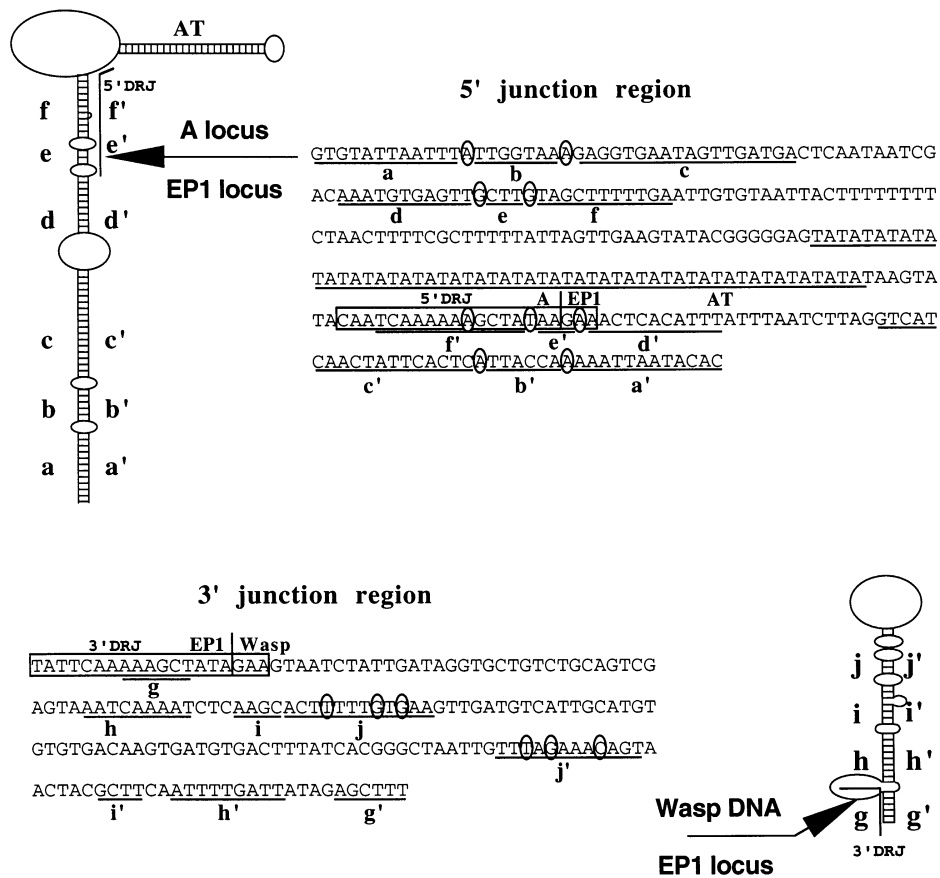


Fig. 4. Junction regions between EP1 and flanking sequences adjacent to the EP1 locus. (A) Schematic representation of the EP1 region with the potential cruciform structures deduced from the sequence around the junction between the EP1 locus and its flanking sequences. (B) Sequences of the 5' and 3' junction regions and schematic representations of the respective potential intrastrand base pairings. Each rung of the ladder represents a perfect pairing (TA or GC); bubbles indicates misalignments. The arrows indicate the position where the EP1 sequence begins (5' region) and terminates (3' region). Next to the schemes are shown the corresponding sequences where inverted repeats sequences are underlined. The sequences a, b, c, d, e, f (5' region) and g, h, i, j (3' region) are supposed to form intrastrand base pairing respectively with the sequences a', b', c', d', e', f', g', h', i', j'. The large AT region is indicated (AT).

Contrary to the 5' probe, the 3' probe did not hybridize to virus DNA, indicating that the 3' region does not contain viral DNA. This probe showed a complex pattern of hybridization with male genomic DNA characteristic of a moderately

repetitive sequence. This result demonstrates that the EP1 CcV sequences are physically linked in 3' to wasp genomic DNA of the braconid wasp *Cotesia congregata*. In conclusion, the EP1 sequences were shown to be integrated in a complex locus,

flanked upstream by other viral sequences and downstream by wasp DNA.

Analysis of the junction regions of the EP1 genomic locus and on the EP1 circle

To investigate the mechanism of EP1 circle production from the genomic copy, we sequenced the regions around the left and right junctions between EP1 sequence and flanking DNA. The sequences were determined after subcloning the fragments containing respectively the 5' and 3' junction (see Methods and Fig. 2). The EP1 circle sequence was also determined around the site where the left and right ends of EP1 sequences are joined by circularization (see Methods). The same sequence was found for four independent clones containing a 272 bp PCR product amplified from virus DNA. Comparison of the junction sequences on the locus and the circle allowed us to precisely determine the boundaries of EP1 virus sequences on the chromosome.

Strikingly, as shown in Fig. 4, the 5' and 3' junction regions are both characterized by the presence of several inverted repeats which constitute a large palindromic structure overlapping the EP1 sequence boundaries and involving 282 bp and 177 bp, respectively.

For such palindromic sequences in a double-stranded DNA, it has been shown that the interstrand base pairs might convert to intrastrand ones giving a pair of hairpinned structures known as a cruciform. The potential 5' and 3' junction cruciforms are shown in Fig. 4. The hairpin potentially formed by each strand in the 5' cruciform comprises as much as 62 bp separated by only five small bubbles of non-alignment. We have not shown that these structures actually form *in vivo* or have a physiological function. However, it seems unlikely that such a complementarity arose by chance and suggests that a selection process actively maintains the structure.

It is noteworthy that in addition to the inverted repeats, the 5' and 3' regions contain a direct repeat (Direct Repeat Junction) precisely located at the junction site in both 5' and 3' structures (Fig. 4). The 5' and 3' DRJ might thus be involved in the circularization of EP1 sequences.

Analysis of the direct repeat junctions

The motif (TTATTCAAAAAGCT) in the 3' DRJ sequence and, to a minor extent, the corresponding motif in the 5' DRJ, are strikingly similar to the *hixC* half site sequence (TTATCAAAAACCT) which constitutes the DNA binding motif of the Hin recombinase of *Salmonella typhimurium*. Hin recombinase belongs to a family of closely related prokaryotic proteins that can substitute functionally for one another in performing Conservative Site-Specific Recombinations (CSSR). Altogether, the comparison of the various native DNA recognition sites, the mutant analysis and the crystallographic experiments have allowed the determination of the key positions in protein–DNA interaction allowing Hin binding to DNA

(Hughes *et al.*, 1992; Feng *et al.*, 1994). Fig. 5 shows DNA motifs in *Cotesia congregata* DRJs that might constitute recombinase binding sites. The *Cotesia congregata* sites are located symmetrically in the 5' and 3' DRJs, close to the position where a DNA strand exchange leading to the excision of PDVs sequences might occur (see also Fig. 6). The *Cotesia congregata* sites are similar to the natural Hin site and do not contain any base pair preventing Hin binding. Depending on the respective orientation of the recognition sites for recombinases, CSSRs can result in the inversion of the DNA intervening sequence or in their excision as a circle. The presence of Hin-like sites at the extremities of EP1 integrated sequences suggest that they might be excised by CSSR thus forming the EP1 circle. A model of the mechanism of excision of *Cotesia congregata* EP1 integrated sequence is shown in Fig. 6. According to this model, in addition to the circle, an EP1 'empty locus' is formed by ligation of the former EP1 5' and 3' flanking regions. It should be noted that after examination of the CiV12 PDV sequence of *Chelonius inanitus*, we found that the right junction also contains a site obeying the rule for Hin binding while the left junction is only slightly divergent from a Hin binding site; a G residue (boxed) within the left junction region constitutes the only significant difference from the canonical Hin binding site (Fig. 5) (Gruber *et al.*, 1996).

Excision of the EP1 integrated form

To determine whether EP1 sequences were actually excised in a subset of *Cotesia congregata* cells, primers were designed from 5' and 3' flanking sequences in order to allow amplification of the putative EP1 'empty locus'. In the case of precise excision of EP1 sequences without any base pair loss, the primers were expected to allow the amplification of a 687 bp fragment. PCR reactions were carried out using two independent samples of DNA extracted from ovaries. Ovaries were chosen because they support virus replication: the rate of excision of EP1 integrated sequences is likely to be higher in this tissue. PCR was also performed on DNA from parasitoid third instar larvae that do not produce virus particles. A fragment of appropriate size and specifically hybridizing with an internal 5' flanking sequence oligonucleotide (amima2) was strongly amplified with ovarian DNA and not with larval DNA (data not shown). After cloning, eight individual clones were sequenced. All the sequences determined were identical and contained contiguous 5' and 3' EP1 locus flanking regions. Surprisingly, the actual size of the PCR product is 686 bp and not 687 bp. Indeed there is no A–T base pair of the 5' DRJ either on the sequence of the empty locus or on the circle junction. The data suggest that this base pair is lost during the excision process (Fig. 6B). The missing base pair probably constitutes the signature of the position where the DNA strand exchange occurs. The EP1 'empty locus' PCR product was also obtained (in a much lower amount) from male wasp tissue DNA (data not shown), which contains some EP1 circular molecules (Fig. 1).

Discussion

Our results show that *Cotesia congregata* EP1 viral sequences are integrated in the wasp genome and excised in the ovaries which produce the virus particles. The analysis of the EP1 locus cloned from a genomic library indicates that the EP1 sequences are flanked at the 5' end by another virus sequence, whose corresponding circle is found at a low level in virus particles. In *Hyposoter fugitivus* PDV, two different circles, HfPV-U and HfPV-L, are produced from the same locus; however, the L circle sequence is composed of a subset of the larger U circle sequence (Xu & Stoltz, 1993). Here the situation is quite different since the EP1 and A sequences do not share any similarity and are contiguous at the same locus. The function of the A circle in the virus is questionable as it is present in much lower amounts in virus particles than the EP1 circle.

Southern blot analysis using the 3' probe shows that the EP1 viral sequences are flanked in 3' by wasp cellular DNA. Previously, this result was obtained in a single case, for *Campoletis sonorensis* ichnovirus (Fleming & Summers, 1991). Our data constitute the first demonstration of the collinearity of PDV and wasp genomic DNA made in a braconid wasp. The recent study on *Chelonus inanitus* CiV12 conducted by Gruber *et al.* (1996) involved cloning a CiV12 offsize fragment but did not analyse the nature of the flanking DNA.

In the Southern blot analysis of EP1 sequences in male wasp DNA, a single EP1 offsize fragment was obtained for each restriction enzyme digest analysis. This indicates that the EP1 locus is unique. Furthermore, only one copy of the EP1 sequence is present at the EP1 locus as determined by cloning; thus, the integrated EP1 sequence represents a unique copy per haploid genome.

Altogether, the data obtained in both ichneumonids and braconids strongly suggest that the integration of virus sequences in the wasp genome is a common characteristic of all PDVs. The case of *Cotesia melanoscela*, in which the presence of the integrated form of CmV circle was not detected by molecular analysis whereas genetic studies showed a Mendelian mechanism of transmission of viral sequences (Stoltz *et al.*, 1986) is exceptional. Moreover, in more recent papers, the authors state that the PDV sequences are integrated in the *Cotesia melanoscela* genome but have not yet published data which directly address this question (Xu & Stoltz, 1993).

Interestingly, in addition to the virus-specific DNA detected as offsize restriction fragments, male wasps also contain a form of CcV DNA identical to the DNA isolated from purified virus. This result extends those obtained on the ichneumonid *Campoletis sonorensis* (Fleming & Summers, 1986) and *Hyposoter fugitivus* (Xu & Stoltz, 1991), and on the braconid *Cotesia melanoscela* (Stoltz *et al.*, 1986), showing the presence of such an extrachromosomal form in male wasp DNA. The detailed analysis of the EP1 sequences in the different body parts of *Cotesia congregata* shows that this circular form is ubiquitous. Either a variety of tissues produces a low level of

circular molecules or those molecules produced in a particular tissue (such as the ovaries for females) diffuse through the haemolymph.

By contrast, the extrachromosomal form of the braconid *Chelonus inanitus* CiV12 was not detected in male wasps (Gruber *et al.*, 1996). It could reflect a more stringent control of virus physiology in that species or, as suggested by the authors, a limit in the sensitivity of detection linked to their experimental approach.

Cloning and sequencing of the junction region on the genomic locus between EP1 and flanking sequences have shown that the 5' and 3' regions both contain large palindromic sequences that could promote intrastrand base pairing leading to cruciform structure formation. Such structures have been shown to be involved in transcriptional regulation and replication. The formation of cruciforms *in vivo* has been demonstrated for prokaryotic (Dayn *et al.*, 1992; Panayotatos & Fontaine, 1987), mammalian (Todd *et al.*, 1995) and viral (SV40) DNA (Hsu, 1985). Such palindromic structures were not described for the PDV sequences analysed to date [namely *Chelonus inanitus* CiV12 (Gruber *et al.*, 1996) and *Campoletis sonorensis* DNA B (Fleming & Summers, 1991)]. We did not show that the structure forms or play a physiological role. However, the low degeneracy of the repeats is striking and studies of the *in vivo* organization of the EP1 locus in calyx cells would be interesting to try to decipher their function. The structure of the chromatin in the different cell types might contribute to the specificity of virus gene expression and virus particle production in the calyx cells. For example, cell type-specific chromatin structure has recently been shown to determine the targeting of V(D)J recombinase activity toward immunoglobulin recombination in B cells and TcR in T lymphocytes (Stanhope-Baker *et al.*, 1996).

The cloning of the predicted EP1 'empty locus' from ovarian DNA demonstrates that the EP1 circle is actually excised in this tissue. A similar result was found for the bracovirus *Chelonus inanitus* CiV12 circle. The CiV12 excision was shown to be developmentally regulated, occurring at the pupal stage (see Gruber *et al.*, 1996 and Albrecht *et al.*, 1994 for a description of the wasp developmental stages). The analysis of the timing and cell specificity of excision of EP1 sequences led us to the same conclusion for *Cotesia congregata* PDV (unpublished). However, contrary to Gruber, we obtained the EP1 'empty locus' PCR product from male DNA, a result expected from the presence of some EP1 circular molecules in male wasp tissues. The developmentally regulated excision of virus sequences probably constitutes a general feature of PDVs which should be analysed in ichnoviruses as well as bracoviruses.

Interestingly, a DNA motif that resembles a Hin recombinase recognition site is located in close vicinity to the DNA strand exchange point in the two *Cotesia congregata* DRJs. A putative model of excision of EP1 sequences drawn from the Hin mediated inversion of *Salmonella typhimurium* (Hughes *et*

al., 1992) is shown in Fig. 6. The proposed mechanism leads both to the circularization of virus sequences and to the ligation of the flanking sequences. As *Chelonus inanitus* CiV12 also has similar sites close to the position where the DNA strand exchange occurs (Gruber *et al.*, 1996 and see Fig. 5), such a mechanism might be general for the braconid wasps.

We thank Yves Bigot for helpful discussions. This research was supported by NSF grant IBN-9420638 (USA) to NEB and grants from ACC-SV of MESER (France).

References

- Albrecht, U., Wyler, T., Pfister-Wilhelm, R., Gruber, A., Stettler, P., Heiniger, P., Kurt, E., Schümperli, D. & Lanzrein, B. (1994). Polydnavirus of the parasitic wasp *Chelonus inanitus* (Braconidae): characterization, genome organization and time point of replication. *Journal of General Virology* **75**, 3353–3363.
- Bigot, Y., Rabouille, A., Doury, G., Sizaret, P.-Y., Delbost, F., Hamelin, M.-H. & Periquet, G. (1997). Biological and molecular features of the relationships between *Diadromus pulchellus* ascovirus, a parasitoid hymenopteran wasp (*Diadromus pulchellus*) and its lepidopteran host, *Acrolepiopsis assectella*. *Journal of General Virology* **78**, 1149–1163.
- Davies, H. D. & Vinson, S. B. (1988). Interference with function of plasmatocytes of *Heliothis virescens* in vivo by calyx fluid of the parasitoid *Campoplex sonorensis*. *Cell and Tissue Research* **251**, 467–475.
- Dayn, A., Malkhosyan, S. & Mirkin, S. M. (1992). Transcriptionally driven cruciform formation in vivo. *Nucleic Acids Research* **20**, 5991–5997.
- de Buron, I. & Beckage, N. E. (1992). Characterization of a polydnavirus (PDV) and virus-like filamentous particle (VLFP) in the braconid wasp *Cotesia congregata* (Hymenoptera: Braconidae). *Journal of Invertebrate Pathology* **59**, 315–327.
- Edson, K. M., Vinson, S. B., Stoltz, D. B. & Summers, M. D. (1981). Virus in a parasitoid wasp: suppression of the cellular immune response in the parasitoid's host. *Science* **211**, 582–583.
- Federici, B. A. (1991). Viewing polydnaviruses as gene vectors of endoparasitic hymenoptera. *Insect Parasitoids* **3**, 387–392.
- Feng, J.-A., Johnson, R. C. & Dickerson, R. E. (1994). Hin recombinase bound to DNA: the origin of specificity in major and minor groove interactions. *Science* **263**, 348–355.
- Fleming, J.-A. G. W. & Krell, P. J. (1993). Polydnavirus genome organization. In *Parasites and Pathogens of Insects*, vol. 1, pp. 189–225. Edited by N. E. Beckage, S. N. Thompson & B. A. Federici. San Diego: Academic Press.
- Fleming, J.-A. G. W. & Summers, M. D. (1986). *Campoplex sonorensis* endoparasitic wasps contain forms of *C. sonorensis* virus DNA suggestive of integrated and extrachromosomal polydnavirus DNAs. *Journal of Virology* **57**, 552–562.
- Fleming, J.-A. G. W. & Summers, M. D. (1991). Polydnavirus DNA is integrated in the DNA of its parasitoid wasp host. *Proceedings of the National Academy of Sciences, USA* **88**, 9770–9774.
- Gruber, A., Stettler, P., Heiniger, P., Schümperli, D. & Lanzrein, B. (1996). Polydnavirus DNA of the braconid wasp *Chelonus inanitus* is integrated in the wasp genome and excised only in later pupal and adult stages of the female. *Journal of General Virology* **77**, 2873–2879.
- Harwood, S. H. & Beckage, N. E. (1994). Purification and characterization of an early-expressed polydnavirus-induced protein from the hemolymph of *Manduca sexta* larvae parasitized by *Cotesia congregata*. *Insect Biochemistry and Molecular Biology* **24**, 685–698.
- Harwood, S. H., Grosovsky, A. J., Cowles, E. A., Davis, J. W. & Beckage, N. E. (1994). An abundantly expressed hemolymph glycoprotein isolated from newly parasitized *Manduca sexta* larvae is a polydnavirus gene product. *Virology* **205**, 381–392.
- Hsu, M.-T. (1985). Electronic microscopic evidence for the cruciform structure in intracellular SV 40 DNA. *Virology* **143**, 617–621.
- Hughes, K. T., Gaines, P. C. W., Karlinsky, J. E., Vinayak, R. & Simon, M. I. (1992). Sequence-specific interaction of the *Salmonella* Hin recombinase in both major and minor grooves of DNA. *EMBO Journal* **11**, 2695–2705.
- Lavine, M. D. & Beckage, N. E. (1995). Polydnaviruses: potent mediators of host insect immune dysfunction. *Parasitology Today* **11**, 368–378.
- Lavine, M. D. & Beckage, N. E. (1996). Temporal pattern of parasitism-induced immunosuppression in *Manduca sexta* larvae parasitized by *Cotesia congregata*. *Journal of Insect Physiology* **42**, 41–51.
- Panayotatos, N. & Fontaine, A. (1987). A native cruciform DNA structure probed in bacteria by recombinant T7 endonuclease. *Journal of Biological Chemistry* **262**, 11364–11368.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Stanhope-Baker, P., Hudson, K. M., Shaffer, A. L., Constantinescu, A. & Schlissel, M. S. (1996). Cell type-specific chromatin structure determines the targeting of V(D)J recombinase activity in vitro. *Cell* **85**, 887–897.
- Stoltz, D. B. (1993). The polydnavirus life cycle. In *Parasites and Pathogens of Insects*, vol. 1, pp. 167–187. Edited by N. E. Beckage, S. N. Thompson & B. A. Federici. San Diego: Academic Press.
- Stoltz, D. & Whitfield, J. B. (1992). Viruses and virus-like entities in the parasitic hymenoptera. *Journal of Hymenoptera Research* **1**, 125–139.
- Stoltz, D. B., Guzo, D. & Cook, D. (1986). Studies on polydnavirus transmission. *Virology* **155**, 120–131.
- Strand, M. R. & Pech, L. L. (1995). *Microplitis demolitor* polydnavirus induces apoptosis of a specific haemocyte morphotype in *Pseudoplusia includens*. *Journal of General Virology* **76**, 283–291.
- Summers, M. D. & Dibb-Hajj, S. (1995). Polydnavirus-facilitated endoparasite protection against host immune defences. *Proceedings of the National Academy of Sciences, USA* **92**, 29–36.
- Todd, A., Landry, S., Khoury, V., Pearson, C. E. & Zannis-Hadjopoulos, M. (1995). Deletion analysis of minimal sequence requirements for autonomous replication of ors8 early-replicating DNA sequence. *Journal of Cellular Biochemistry* **57**, 280–289.
- Volkoff, A.-N., Ravallec, M., Bossy, J.-P., Cerutti, P., Rocher, J., Cerutti, M. & Devauchelle, G. (1995). The replication of *Hyposoter dydimator* polydnavirus: cytopathology of the calyx cells in the parasitoid. *Biological Cell* **83**, 1–13.
- Whitfield, J. B. (1990). Parasitoids, polydnaviruses and endosymbiosis. *Parasitology Today* **6**, 381–384.
- Xu, D. & Stoltz, D. (1991). Evidence for a chromosomal location of polydnavirus DNA in the ichneumonid parasitoid *Hyposoter fugitivus*. *Journal of Virology* **65**, 6693–6704.
- Xu, D. & Stoltz, D. (1993). Polydnavirus genome segment families in the ichneumonid parasitoid *Hyposoter fugitivus*. *Journal of Virology* **67**, 1340–1349.

Received 30 May 1997; Accepted 20 August 1997