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Evidence for integration of *Glyptapanteles indiensis* polydnavirus DNA into the chromosome of *Lymantria dispar* in vitro

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Abstract

Polydnaviruses replicate within calyx cells of the female ovaries of certain species of parasitic wasps and are required for the successful parasitization of lepidopteran hosts. These viruses, which have unusual double-stranded circular DNA segmented genomes, are integrated as proviruses into the genomes of their associated wasp hosts and are believed to be transmitted vertically through germline tissue. Here, by combined Southern hybridization, polymerase chain reaction (PCR) assays and viral sequence analyses we provide evidence that DNA originating from two distinct double-stranded circular segments of the polydnavirus genome from the braconid *Glyptapanteles indiensis* (GiPDV) integrates in vitro into the genome of cells derived from the natural host, *Lymantria dispar*. The *G. indiensis* polydnavirus DNA, as a result of its unique ability to be integrated in part into the chromosome of cells derived from its lepidopteran host, has potential to be developed as an in vitro cell transformation system. © 2000 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Polydnaviruses (PDVs) are unique insect viruses found in some parasitic wasps of the families Ichneumonidae and Braconidae and are characterized by unusual multi-segmented closed circular dsDNA genomes. PDVs are considered as non-traditional viruses because they do not replicate once outside specific calyx cells within the female reproductive tract. PDVs are injected along with eggs by parasitic wasps into their lepidopteran hosts during oviposition. Inside the larval host the PDVs do not replicate, but certain genes are transcribed and translated, presumably under the control of larval host factors that are not well understood. The PDV gene products function to suppress the larval host immune systems and regulate host development and physiology (Lawrence and Lanzrein, 1993; Stoltz, 1993;

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Lavine and Beckage, 1995; Strand and Pech, 1995; Asgari et al., 1997). Thus PDVs play an essential role in the life cycles of parasitic wasps and are required for successful parasitoid development (Stoltz, 1993).

There are two recognized PDV genera, ichnoviruses found in parasitoid wasps of Ichneumonidae and bracoviruses found in parasitoid wasps of Braconidae, which differ in their particle morphology, physiology, molecular characteristics and host range (Stoltz and Whitfield, 1992; Stoltz et al., 1995). Ichnoviruses, described as ovoid in shape and having a lenticular nucleocapsid surrounded by two unit membrane envelopes, are extruded by budding from calyx cells within the ovary (Krell et al., 1982; Stoltz and Whitfield, 1992). Bracoviruses, characterized as having one or more rod shaped nucleocapsids within a single unit membrane envelope, are believed to be released by lysis of calyx cells into the ovary (Stoltz and Whitfield, 1992). Genetic studies have indicated that PDVs of both groups are transmitted vertically through the germline (Stoltz et al., 1986; Stoltz, 1990). It is believed that this transmission takes place through integration into their respective wasp host genomes as proviruses (Stoltz et al., 1986; Fleming and Summers, 1990, 1991; Stoltz, 1990, 1993; Fleming, 1991; Gruber et al., 1996; Savary et al., 1997).

Evidence for transmission through genome integration has been effectively shown for ichnoviruses from *Campoletis sonorensis* (the ichnovirus type species) (Fleming and Summers, 1986, 1990) and *Hyposoter fugitivus* (Xu and Stoltz, 1991) and, more recently, for bracoviruses of *Chelonus inanitus* (Gruber et al., 1996) and *Cotesia congregata* (Savary et al., 1997). In ichnovirus genetic studies, off-size restriction fragments indicative of viral DNA integration were detected on Southern blots of restriction digested male and female wasp and virus genomic DNAs (Fleming and Summers, 1986, 1990; Fleming and Krell, 1990; Xu and Stoltz, 1991). Evidence for integration of the bracovirus of *Chelonus inanitus* in the female wasp's genomic DNA was shown by genetic and molecular methods (Gruber et al., 1996). A 12 kb DNA segment integrated in the wasp genomic DNA was shown to be excised and circularized in females in the later pupal and adult stages (Gruber et al., 1996). Most recently, sequences of circular and integrated forms of the PDV of *Cotesia congregata* containing the viral early protein 1 (EP1) were shown by molecular methods to be present in both male and female wasps and that the circular form detected in males and females was produced by excision of the linear integrated form (Savary et al., 1997). In this study, a model was proposed that strand exchange and excision of a PDV circle is catalyzed by binding of a DNA recombinase and that this event occurs at this recombination site on the PDV EP1 circle (Savary et al., 1997). Based on the combined evidence from PDV studies to date, integration of PDVs within the respective wasp host genome is highly indicated as the mechanism for viral transmission to progeny for both ichnoviruses and brachoviruses. In related studies, the occurrence of PDV segment nesting whereby a single PDV locus within the wasp genome gave rise to multiple nested viral segments has been shown and described for the ichnovirus *C. sonorensis* and suggested as a possible mechanism for propagating and increasing the copy number of important and functional PDV genes in parasitized insects (Cui and Webb, 1997).

Studies concerning the fate of PDVs within their natural lepidopteran hosts have primarily focused on virus-specific gene expression and the physiological changes associated with parasitism (Stoltz, 1993). Studies have shown that once injected into the hemocoel of the lepidopteran host, PDV genomes are transcriptionally active (Blissard et al., 1986; Strand et al., 1992) and that their gene products disrupt the normal immune activity and development of the host (Blissard et al., 1986; Stoltz, 1993; Lavine and Beckage, 1995; Summers and Dibb-Hajj, 1995; Asgari et al., 1997). Scientists in our laboratory recently described an unusual phenomenon among insect viruses exhibited by the braconid parasitic wasp *Glyptapanteles indiensis* PDV (GiPDV) DNA in the presence of lepidopteran host cells. It was reported that GiPDV DNA may be integrated, not only as a provirus within the parasitoid wasp genomic DNA as described, but also in vitro within the chromosomal DNA of cells derived

from the natural host of the parasitoid (McKelvey et al., 1996). Specifically, it was shown that partial PDV DNA from the braconid *G. indiensis* could persist in vitro in cells derived from natural host *Lymantria dispar* (McKelvey et al., 1996). The GiPDV is essential for successful parasitism of gypsy moth (*L. dispar*) larvae by the braconid wasp *G. indiensis*. *L. dispar* cells infected with GiPDV initially displayed cytopathic effects, from which cells gradually recovered over a 1–2 month period (McKelvey et al., 1996). Clones were obtained from the genomic DNA of the recovered GiPDV-infected cells that contained both PDV and cellular sequences in the same fragment, indicating GiPDV DNA sequences were present within the *L. dispar* host chromosome in vitro. The GiPDV DNA sequences in 'transformed' cells were stably maintained over the course of 250 weekly passages. A similar event suggesting possible in vitro integration of DNA from the ichnovirus of *Hyposoter fugitivus* into the chromosomal DNA of natural host *L. dispar* cells was also reported (Kim et al., 1996). In both cases, PDV DNA appeared to be stably maintained in the recovered *L. dispar* cell line(s).

The probable occurrence of PDV integration into the chromosome of cells of the natural insect host, the maintenance of PDV DNA within the host chromosome and the characteristics of the integrating DNA are of great interest to us from both biological and biocontrol perspectives. In this study, we examine the GiPDV and investigate and provide evidence for the phenomenon of integration of GiPDV DNA into the chromosomal DNA of *L. dispar* in vitro.

2. Materials and methods

2.1. GiPDV-transformed L. dispar cell lines

L. dispar cell lines derived from embryonic tissue (IPLB-LdEp and IPLB-LdEIta) were infected with GiPDV in vitro as previously described (McKelvey et al., 1996). Total genomic nucleic acids were extracted from non-infected and GiPDV-infected IPBL-LdEp (IPLB-LdEp/Gi) cells, after greater than 250 passages, non-infected and GiPDV-infected IPLB-LdEIta (IPLB-LdEIta/Gi) cells, after greater than 150 passages, by standard techniques (Ausubel et al., 1994).

2.2. Isolation and analysis of GiPDV DNA

Female braconid *G. indiensis* parasitic wasps were dissected, reproductive tracts isolated and fluid containing PDVs gently released from the calyx with dissecting forceps. The fluid was collected under dissecting scope avoiding eggs and ovarian tissues, filtered through a 0.45 μm filter and PDV nucleic acid extracted in an equal volume of extraction buffer (0.02 M Tris, pH 8, 0.04 M NaCl, 0.002 M $CaCl_2$) containing 500 μg/ml proteinase K, 0.5% SDS and incubating at 37°C. Nucleic acid was gently extracted by rocking in an equal volume of phenol/chloroform. GiPDV genomic DNA was analyzed by field inversion gel electrophoresis (FIGE). 0.5 μg of GiPDV DNA was loaded onto a 1% pulsed field certified agarose (BioRad Laboratories, Hercules, CA) gel and electrophoresed on a FIGE apparatus (BioRad) in 0.5 × TBE buffer at room temperature for 11 h using a 0.1–0.4 s switch time ramp with 180 forward voltage and 120 reverse voltage. The gel was then electrophoresed by standard methods an additional 12 h at constant 15 V. Molecular size standard digoxigenin-labeled linear λ DNA/*Hin*dIII digest (Boehringer Mannheim Biochemicals, Indianapolis, IN) was included. The gel was stained in ethidium bromide and DNA bands visualized using a UV transilluminator.

2.3. Isolation of GiPDV DNA clones

A GiPDV plasmid clone library was generated using the method described by Albrecht et al. (1994) in which GiPDV DNA fragments partially digested with *Eco*RI in the presence of ethidium bromide were inserted into the *Eco*RI site of the pSP64 vector (Promega, Madison, WI) by electroporation. This method allowed isolation of several plasmid clones containing complete GiPDV circular genomic segments. The library was screened for individual clones representing GiPDV DNA persisting in infected *L. dispar* cells in vitro, which were selected by dot hybridization at 48°C using

digoxigenin-labeled (Boehringer) total GiPDV DNA, IPLB-LdEp cellular DNA, and IPLB-LdEp infected with GiPDV (IPLB-LdEp/Gi) DNAs separately as probes and were identified by their ability to hybridize strongly with GiPDV and IPLB-LdEp/Gi DNAs while failing to hybridize with the IPLB-LdEp DNA. The annealed DNAs were visualized using CSPD (Boehringer).

2.4. Analysis of transformed L. dispar cell DNAs for the presence of GiPDV sequences by Southern hybridization

Digoxigenin-labeled plasmid DNA from GiPDV clones p157 and p384 (isolated as described above) were used as hybridization probes to analyze transformed IPLB-LdEp/Gi cell DNA for evidence of integrated GiPDV viral sequences. For Southern blot, IPLB-LdEp, IPLB-LdEp/Gi (5 μg each) and GiPDV (0.5 μg) genomic DNAs were digested with restriction endonuclease *Hin*dIII (Gibco/BRL, Gaithersburg, MD), electrophoresed through a 0.8% agarose gel, transferred to nylon membrane (Southern, 1975) and probed with labeled clone p157. Separately, IPLB-LdEp (5 μg), IPLB-LdEp/Gi, IPLB-LdEIta, IPLB-LdEIta/Gi (5 μg each) and GiPDV (0.5 μg) genomic DNAs were digested with restriction endonuclease *Bam*HI (Gibco/BRL), electrophoresed through a 0.8% agarose gel, transferred to nylon membrane as described (Southern, 1975) and probed with labeled clone p384. The temperature for hybridization was 48°C. Annealed DNAs were visualized using CSPD (Boehringer).

2.5. Analysis of transformed L. dispar cell line DNAs for the presence of GiPDV sequences by PCR

By restriction mapping and partial sequencing of GiPDV clones p157 and p214 (a polymorphic clone of the same GiPDV circular dsDNA genome segment) PCR primer pairs were designed to amplify different discrete regions of this circular viral segment (see approximate locations of primer sequences in Fig. 2). Primer pairs p157F1/p214R1 and p214F16/p214R16 were designed to amplify separated regions of the GiPDV genomic segment represented by p157 and have been described previously (Gundersen-Rindal et al., 1999). Primer pair p214F1/p214R21 was designed to position primers in the correct orientation upstream and downstream of the putative site of integration identified as described below, to analyze whether GiPDV sequences were maintained in transformed cellular DNA as an episome or integrated. The oligonucleotide sequences of these primers were:

p214F1: 5′-GTG TGT AAT ATA CCT ACA GC-3′

p214R21: 5′- GTG TCA CGA TTA GTT TCG TG-3′

For PCRs, total genomic nucleic acids extracted from the homologous clones, GiPDV and the non-infected and GiPDV-infected IPLB-LdEp cell line were diluted in sterile deionized water to a final concentration of 20 ng/μl. Each 50 μl PCR reaction was performed using AmpliTaq Gold (Perkin–Elmer Cetus, Norwalk, CT), 0.2 μM of each primer and 200 μM deoxynucleotide triphosphates. Thirty-five PCR cycles were conducted in an automated thermocycler (Hybaid, Teddington, UK). After a 10 min initial enzyme activation at 94°C, the following parameters were used: 1 min (2 min for the first cycle) denaturation at 94°C, 2 min annealing at 52°C and 3 min primer extension (10 min in final cycle) at 72°C. Control tubes without template were included in each experiment as negative controls. PCR products were analyzed by electrophoresis through a 0.8% agarose gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator.

2.6. Isolation of a site of GiPDV viral integration in IPLB-LdEp chromosomal DNA

Clones containing both viral and cellular sequences were isolated by generating a lambda phage library from transformed IPLB-LdEp/Gi cells. *Mbo*I-digested DNA fragments were inserted into the *Bam*HI site of the Lambda Dash II vector (Stratagene, La Jolla, CA). Individual clones from this library hybridizing to labeled GiPDV were isolated by plaque lift. These clones were analyzed by restriction digestion and South-

ern hybridization (Southern, 1975) using digoxigenin-labeled (Boehringer) total GiPDV and IPLB-LdEp DNAs separately as probes. Several clones were composed of sequences from both GiPDV and *L. dispar* insect cell genome as determined by hybridization. Several large (> 10 kb) clones from the λ phage library were sub-cloned in order to isolate an integration site in a clone sized suitably for sequencing. An 11 kb clone found to contain both viral and cellular fragments, λE/G 7, was sub-cloned by inserting its *Xba*I restriction digested fragments into the pGem7zf vector (Promega). Sub-clones were again screened by reciprocal hybridization with labeled total GiPDV and IPLB-LdEp cellular DNAs. A sub-clone of 4.5 kb, p7–54, containing viral and cellular sequences was isolated and analyzed by restriction digestion. A small (< 800 base pairs) sub-clone, pB17, was obtained by gel purifying and sub-cloning an *Alu*I digest fragment of clone p7–54 that contained a viral/cellular border into the pGem3zf vector (Promega). The sub-clone pB17 was restriction mapped as described above except restriction fragments were separated on polyacrylamide gel, and sequenced using SP6 and T7 promoter primers on an ABI310 automatic sequencer (Applied Biosystems, Norwalk, CT) (Fig. 5). GiPDV viral sequences from sub-clone pB17 were compared with GiPDV viral sequences obtained by partial sequencing of clone p157 using Lasergene software (DNASTAR, Madison, WI). The sequence of clone pB17 containing a GiPDV-LdEp integration junction was deposited in GenBank under accession number AF198385.

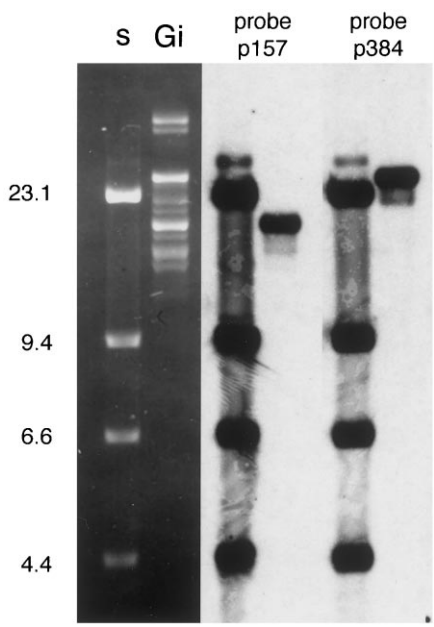

Fig. 1. Analysis of the GiPDV DNA genome by field inversion electrophoresis (FIGE). GiPDV DNA (lane Gi) isolated as described in the text and digoxigenin-labeled lambda phage DNA digested with *Hin*dIII (lane λ) (Boehringer) were separated by FIGE on a 1% pulsed field grade agarose gel. The Southern blotted DNA was probed separately with probes p157 and p384 to identify the circular genome fragment of origin. Molecular sizes in kilobase pairs for the linear DNA molecular weight standard is given.

3. Results

3.1. GiPDV genome

Like other PDVs, the GiPDV DNA genome comprised multiple large dsDNA circular segments and exhibited a high degree of complexity. The GiPDV genome consisted of approximately eleven visible circular dsDNA segments that ranged in size from ≈ 10 to over 30 kb (Fig. 1), of which four segments were present in higher molar concentration, as described for polydnaviruses (Fleming and Krell, 1990). The circular DNA fragments comprising the GiPDV genome were comparatively larger and less numerous than those comprising genomes of ichnoviruses and of other braconid PDVs, such as *C. congregata* PDV (Savary et al., 1997). There were no GiPDV circular ds DNA segments smaller than ≈ 10 kb. For the purpose of specifying particular dsDNA GiPDV genome segments, fragments were assumed to be predominantly superhelical in form, and specific segments of interest to this study were identified as unique, each corresponding to a single genomic circular segment (Fig. 1).

3.2. GiPDV-transformed L. dispar cell lines

Infection of *L. dispar* cell lines derived from embryonic tissue with GiPDV in vitro was previ-

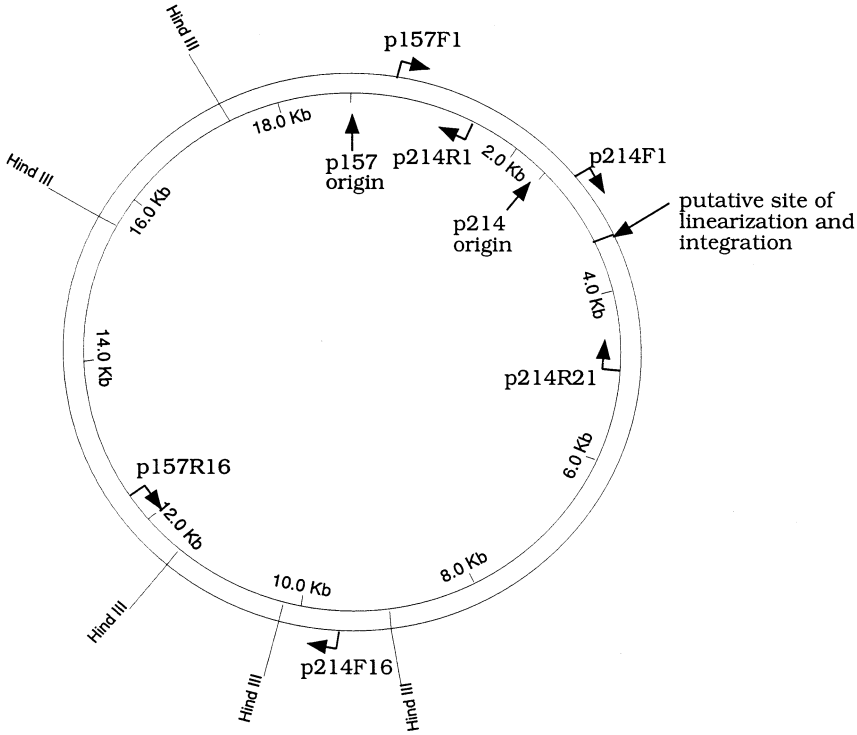

Fig. 2. Restriction map showing *Hin*dIII sites of a complete ds circular DNA segment of the GiPDV genome. Approximate locations of the origin of clones of this circular segment represented by p157 and p214 are shown. Approximate locations on the GiPDV circular fragment of binding sites for GiPDV-specific primers p157F1, p214R1, p214F1, p214R21, p214F16 and p157R16 are shown. The arrows indicate the 5′ to 3′ direction of synthesis on the leading (primers p157F1, p214F1, and p214F16) or complementary (primers p214R1, p214 R21 and p157R16) strand of the circular DNA. The approximate location of the putative site of GiPDV DNA segment linearization and integration is indicated by an arrow. Legend indicates approximate size and locations on the circular genome segment in kilobase pairs.

ously described (McKelvey et al., 1996). GiPDV-infected IPLB-LdEp (IPLB-LdEP/Gi) and LdEIta (LdEIta/Gi) cells utilized in these experiments had been passaged in the laboratory at least 250 and 150 times, respectively, at the time genomic DNA was extracted, indicating that the GiPDV viral sequences were stably maintained essentially as a permanent part of the replicating cells and that the cells were truly 'transformed'.

3.3. Isolation of GiPDV DNA persisting in transformed IPLB-LdEp/Gi in vitro

A GiPDV DNA clone, p157, was identified that hybridized strongly with GiPDV and IPLB-LdEp/Gi DNAs, but failed to hybridize with IPLB-LdEp DNA. Clone p157 was $\approx$18.7 kb in size as determined by restriction analysis and mapping (data not shown) and represented one complete dsDNA circular segment of the GiPDV genome (Fig. 2). This circular fragment, identified in Fig. 1, was one of the segments present in higher molar ratio in the GiPDV genome as determined by hybridization of labeled clone p157 to FIGE-separated GiPDV genome segments (Figs. 1 and 3). A second clone representing this same GiPDV circular segment, p214, was cloned at a different *Eco*RI site on the segment circle (Fig. 2).

A second GiPDV clone was identified that hybridized with GiPDV and IPLB-LdEp/Gi DNAs, but failed to hybridize with IPLB-LdEp DNA. This recombinant, p384, was over 25 kb in size as determined by restriction analysis (data not shown). Clone p384 represented one complete ds-

DNA circular segment of the GiPDV genome and represented a segment present in higher molar ratio in the GiPDV genome as determined by hybridization of labeled clone p384 to FIGE-separated GiPDV genome segments (fragment identified in Fig. 1). Clones p384 and p157/p214 represented unique GiPDV segments and did not have any homology by reciprocal cross hybridization (data not shown). In a prior study (McKelvey et al., 1996), off-size restriction fragments indicative of GiPDV integration were seen when restriction digested GiPDV and IPLB-LdEp/Gi DNAs were probed with labeled clone pTM145. Clone pTM145 is contained within the full circular GiPDV genome segment represented by clone p384 (data not shown).

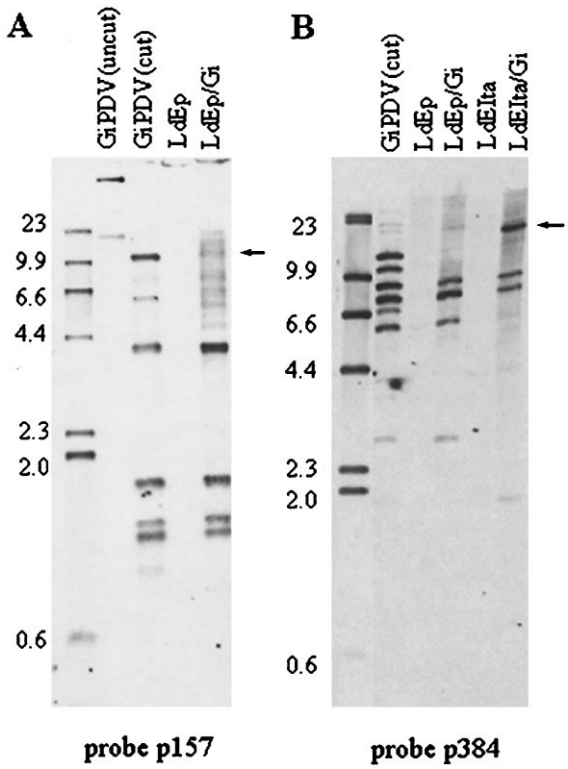

Fig. 3. Southern blot analysis of GiPDV sequences maintained in insect cell lines. (A) Genomic DNA from GiPDV virus, non-infected and GiPDV-infected cell line digested with *Hin*dIII, was electrophoresed through a 0.8% agarose gel and transferred to nylon membrane as described (Southern, 1975). Digoxigenin-labeled DNA (BMB, IN) from clone p157 was used as a hybridization probe; arrow denotes missing band. (B) Genomic DNA from GiPDV virus, non-infected and GiPDV-infected cell lines digested with *Bam*HI were electrophoresed through a 0.8% agarose gel and transferred to nylon membrane as described (Southern, 1975). Digoxigenin-labeled DNA (BMB, IN) from clone p384 was used as a hybridization probe; arrow denotes off-size fragment. Abbreviations are as given in text. Molecular sizes are indicated in kilobase pairs.

3.4. Evidence for in vitro GiPDV DNA integration in transformed L. dispar by Southern hybridization and PCR assays

Hybridization of clone p157 with *Hin*dIII-digested GiPDV, IPLB-LdEp, and IPLB-LdEp/Gi DNAs showed that transformed cellular DNA lacked the largest ($\approx$ 11 kb) GiPDV *Hin*dIII fragment (Fig. 3A). Interestingly, this largest GiPDV HindIII fragment contains the putative site of integration as identified below. There was no hybridization of this circular GiPDV segment to IPLB-LdEp cellular DNA. This and additional Southern hybridization results using various restriction enzymes for analysis (data not shown) indicated that much of the GiPDV circular segment represented by clone p157 was integrated in vitro in the IPLB-LdEp/Gi chromosome. Hybridization of clone p384 with *Bam*HI-digested GiPDV, IPLB-LdEp, and IPLB-LdEp/Gi DNAs showed the presence of an off-size restriction fragment in transformed cellular DNA indicative of integration, particularly evident in IPLB-LdEIta/Gi (Fig. 3B). There was no hybridization of this circular GiPDV segment to IPLB-LdEp or IPLB-LdEIta cellular DNA.

PCR primers were designed to amplify discrete regions of the p157 circular GiPDV viral segment (Fig. 2) to show that viral sequences were amplified in transformed cellular DNAs and to assess whether viral sequences were maintained in transformed cells extrachromosomally (as an episome) or integrated within transformed cellular DNA. PCR primer pair p157F1/p214R1 primed amplification of a 1.7 kb fragment from GiPDV clone p157, GiPDV, and from IPLB-LdEp/Gi transformed cell line DNAs (Fig. 4a). PCR primer pair p157F16/R16 primed amplification of a 2.8 kb fragment from GiPDV clone p157, GiPDV, and from IPLB-LdEp/Gi transformed cell line DNAs

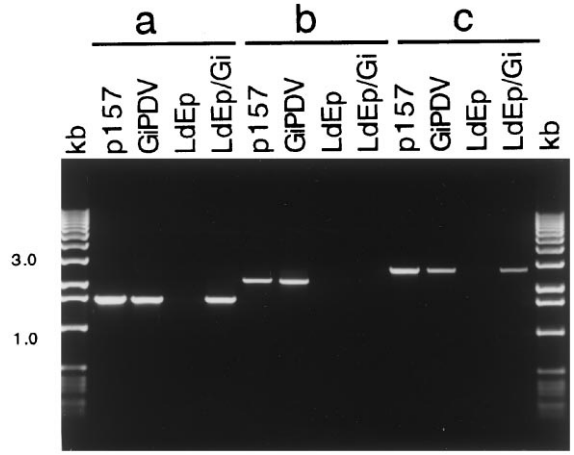

Fig. 4. PCR amplification of GiPDV sequences from infected and non-infected gypsy moth cell line DNAs using GiPDV-specific primer pairs (a) p157F1/p214R1, (b) p214F1/p214R21 and (c) p214F16/p157R16. Abbreviations are as given in text. Lanes 1 and 14 contain molecular size standard 1 kb ladder (Gibco/BRL) indicating size in kilobase pairs.

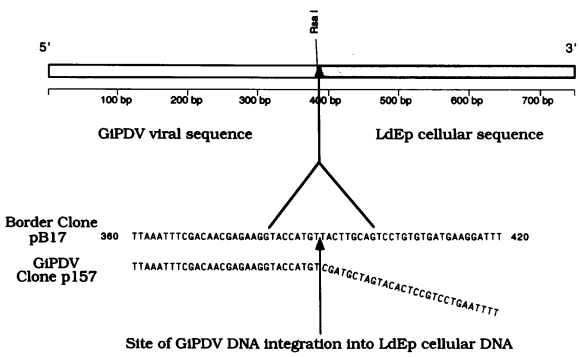

Fig. 5. Map of junction clone pB17 (*Alu*I fragment isolated from p7–54), indicating location of the integration junction between GiPDV viral and LdEp cellular DNAs as determined by sequencing. The putative junction site identified by comparing sequence of circular and integrated forms of GiPDV is indicated by an arrow. Legend indicates approximate locations in base pairs.

(Fig. 4c). These GiPDV-specific primer pairs did not prime amplification from non-infected IPLB-LdEp DNA (Fig. 4). The GiPDV DNA fragments amplified with these two primer pairs were from widely separated regions of the GiPDV circular genomic fragment (Fig. 2). PCR primer pair p214F1/p214R21 primed amplification of a 2.2 kb fragment from GiPDV clone p157 and GiPDV DNAs (Fig. 4b). This GiPDV-specific primer pair did not prime amplification from non-infected IPLB-LdEp or from transformed IPLB-LdEp/Gi DNAs. This primer pair was designed to amplify a region on the GiPDV circular segment containing a putative site of integration as identified by the following analysis of a GiPDV viral-LdEp cellular junction within the transformed cells. This primer pair and others (data not shown) designed to amplify across the region on the GiPDV circular segment containing a putative site of integration, were able to amplify GiPDV sequences of the circular form (native GiPDV or clone DNA) only. In all cases, amplification patterns using IPLB-LdEIta and IPLB-LdEIta/Gi DNA templates were identical to those using IPLB-LdEp and IPLB-LdEp/Gi DNA templates, respectively (data not shown).

3.5. Sequence analysis of a junction site of integrated GiPDV DNA

The clone pB17, which originated from a lambda phage clone containing both viral and cellular sequences and contained a putative integration junction, was sequenced. The approximate junction site of integrated GiPDV DNA was located by comparison to GiPDV clone p157 sequences. The precise junction between GiPDV and its flanking cellular sequences was identified by comparing sequence of the circular (p157) and putatively integrated (pB17) forms. The integrated GiPDV viral sequences of clone pB17 were homologous with those complementary to those the circular GiPDV clone p157 near a *Rsa*I site (Fig. 5). The DNA bases at the junction location of this site were identified, and the site of viral integration was presumed to occur where pB17 and viral sequences diverged (Fig. 5). It has been shown in studies of PDV integration in the respective wasp host chromosome as a provirus that inverted repeated sequences (sometimes lengthy) are present at integration junction sites (Savary et al., 1997). GiPDV sequences directly at the integration junction were 'CATG'. The other junction sites of GiPDV integration in the *L. dispar* chromosome must be isolated to identify potential inverted repeated sequences. Isolation of a viral integration

site from chromosomal IPLB-LdEp/Gi DNA provides evidence that GiPDV DNA is physically linked to *L. dispar* cellular DNA in transformed cells. The cellular sequences at the junction site were subjected to a BLAST (Altschul et al., 1990, 1997) search and had some homology to sequences of the gypsy moth gene for vitellogenin (Hiremath and Lehtoma, 1997). This homology will be further investigated.

4. Discussion

Changes occurring upon in vitro exposure of the braconid *G. indiensis* PDV to cells derived from natural lepidopteran host *L. dispar* are intriguing. *L. dispar* cells infected with GiPDV show signs of infection, such as reduced cell number and morphological changes, but over time are able to recover. The surviving recovered cells are 'transformed', because DNA derived from the GiPDV genome is stably maintained within these cells as an essentially permanent part of the chromosome in replicating cells. In an earlier study, from our laboratory, it was indicated that partial GiPDV genome DNA persists in transformed IPLB-LdEp cells. However, molecular events pertaining to the phenomenon of PDV maintenance within the lepidopteran host cell, as exhibited by GiPDV, have not been clarified.

There were several lines of evidence suggesting GiPDV DNA was integrated in *L. dispar* cells in vitro. The isolation of an integration junction containing GiPDV viral and IPLB-LdEp cellular DNA demonstrated that the two were physically linked in transformed cells. Southern hybridization results indicated by the absence of the 11 kb *Hin*dIII fragment containing the putative site of GiPDV integration in transformed cell DNA probed with GiPDV clone p157 and by the presence of off-size fragments in transformed cell DNA probed with GiPDV clone p384, that GiPDV DNA sequences were likely integrated in *L. dispar* chromosomal DNA in vitro. The amplification of GiPDV sequences by PCR from several loci on transformed, but not non-transformed, *L. dispar* cellular DNAs indicated that GiPDV viral sequences were present in transformed cells. The amplification of GiPDV sequences from the circular form, but not from the integrated form of the p157 circle, using PCR primers, designed to amplify the GiPDV region containing the putative site of integration, indicated that the GiPDV circular sequences were no longer circular (now linearized) in transformed cells. This also indicated that once exposed to host cells and passed over time, extrachromosomal circular GiPDV molecules were not able to be amplified and no longer existed, suggesting GiPDV sequences were not simply maintained within *L. dispar* cells as an independently replicating episome(s).

Southern and PCR results indicated that much of the GiPDV circle segment represented by p157 was integrated. This 18.7 kb circular GiPDV DNA could represent only part of the 25–27 kb or greater of GiPDV DNA estimated to persist in transformed IPLB-LdEp cells in our prior study (McKelvey et al., 1996). In that same study, GiPDV DNA clone pTM145 (7.5 kb), which is a part of larger GiPDV circular DNA segment represented by clone p384, was also shown to comprise part of the GiPDV capable of persisting in transformed IPLB-LdEp cells. This would lead us to believe that integrating GiPDV DNA comprises a large part of the p157 segment plus partial DNA from the GiPDV genome circle represented by clone p384. The estimation of 25–27 kb or greater GiPDV DNA persisting in IPLB-LdEp/Gi cells and isolation of GiPDV DNA from two different GiPDV genome circles indicated that DNA from at least these two segments of the GiPDV genome are likely involved in integration in vitro. It is possible that more than two GiPDV segments are involved in the integration event, which we are currently investigating. Based on cross-hybridization assays, the distinct GiPDV ds circular fragments represented by p157 and p384 represented unique PDV segments. Thus their hybridization characteristics were not related to the phenomenon of inter-segment nesting, described for segments of ichnovirus CsPDV from *C. sonorensis*. (Cui and Webb, 1997).

The integration of PDV sequences in proviral form with the respective wasp host chromosome has been investigated for several ichno- and

braco-viruses. It has been suggested, based on the combined evidence from ichneumonid and braconid PDVs, that integration of PDV sequences within the wasp genome is a common event for all PDVs. In a recent study by Savary et al. (1997) a possible model was proposed for excision of the *C. congregata* provirus from the wasp genome and circularization to form a PDV circular segment in which it was proposed that site specific recombination may occur by binding of recombinases to DRJ sites on the *C. congregata* EP1 sequences causing dimerization and strand exchange that resulted in both EP1 circle and an 'empty' locus on the wasp DNA. Such a mechanism for GiPDV excision and circularization to form viral particles in the *G. indiensis* wasp has not been shown. Based on the prevailing data for PDVs it can be assumed that GiPDV utilizes a mechanism suggested as common for PDVs in which proviral PDV DNA is excised from the wasp chromosome at a specific locus and circularized to form PDV circular segments. The GiPDV DNA integration event into the chromosome of cells derived from its natural lepidopteran host in vitro may be contrasted with putative events occurring in the wasp. While integration of PDV DNA in the wasp chromosome as a provirus likely involves excision at a particular site on the wasp chromosome and circularization to form viral particle(s), the integration event suggested by our data for GiPDV would require linearization at a certain site on a circular GiPDV genome segment and ligation to a chromosomal site in the host cell where the integrated GiPDV sequences would then be stably maintained and replicated as a part of the host cell.

It is certain that for integration some form of recombination must take place between GiPDV and *L. dispar* chromosomal sequences that allows GiPDV DNA sequences to be stably maintained in vitro. It is unclear why this phenomenon might happen or whether it would take place by host or viral machinery. Certainly, to establish a mechanism for the integration, sequences from the other GiPDV integration border(s) must be isolated to identify conserved cellular target sequence motifs, to identify potential direct or inverted repeated sequences within GiPDV itself and for comparison with other PDVs. This phenomenon requires further investigation to examine whether events in vitro in any way mirror natural events in vivo where PDV DNA is injected into the hemocoel along with parasitoid eggs, and to examine whether integration of PDV sequences could effect the interruption of gene(s) translation in *L. dispar*, potentially related to complex immune response. It is necessary to examine whether the phenomenon of integration into the natural host chromosome in vitro is a phenomenon exclusive to GiPDV or common to PDVs. Furthermore, because of the unique ability to be integrated in part into cells derived from the lepidopteran host, the potential of developing GiPDV as an in vitro cell transformation system should be explored.

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