

Characterization and transcriptional analysis of protein tyrosine phosphatase genes and an ankyrin repeat gene of the parasitoid *Glyptapanteles indiensis* polydnavirus in the parasitized host

D. E. Gundersen-Rindal and M. J. Pedroni

Correspondence

D. E. Gundersen-Rindal
gundersd@ba.ars.usda.gov

US Department of Agriculture, Agricultural Research Service, Insect Biocontrol Laboratory,
Bldg 011A, Room 214, BARC West, Beltsville, MD 20705, USA

Glyptapanteles indiensis (Braconidae, Hymenoptera) is an endoparasitoid of *Lymantria dispar*, the gypsy moth. Expression of *G. indiensis* polydnavirus (GiBV)-encoded genes within the pest host results in inhibition of immune response and development and alteration of physiology, enabling successful development of the parasitoid. Here, GiBV genome segment F (segF), an 18·6 kb segment shown to encode nine protein tyrosine phosphatase (PTP) genes and a single ankyrin repeat gene (ank), is analysed. PTPs have presumed function as regulators of signal transduction, while ankyrin repeat genes are hypothesized to function in inhibition of NF- κ B signalling in the parasitized host. In this study, transcription of each gene was mapped by 5'- and 3'-RACE (rapid amplification of cDNA ends) and temporal and tissue-specific expression was examined in the parasitized host. For polydnavirus gene prediction in the parasitized host, no available gene prediction parameters were entirely precise. The mRNAs for each GiBV segF gene initiated between 30 and 112 bp upstream of the translation initiation codon. All were encoded in single open reading frames (ORFs), with the exception of PTP9, which was transcribed as a bicistronic message with the adjacent ank gene. RT-PCR indicated that all GiBV segF PTPs were expressed early in parasitization and, for most, expression was sustained over the course of at least 7 days after parasitization, suggesting importance in both early and sustained virus-induced immunosuppression and alteration of physiology. Tissue-specific patterns of PTP expression of GiBV segF genes were variable, suggesting differing roles in facilitating parasitism.

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INTRODUCTION

Polydnaviruses (PDVs), unusual and complex segmented dsDNA viruses, have evolved in mutualistic symbioses with their primary hosts, ichneumonid and braconid parasitoid wasps. PDVs are injected during oviposition with eggs and ovarian proteins into their secondary hosts, lepidopteran larvae, where they exert pathogenic effects. No PDV replication occurs within the larval host, and PDVs are maintained by vertical transmission in the wasp population, integrated as provirus and are transmitted vertically to progeny (Fleming & Summers, 1986; Fleming, 1991; Gruber *et al.*, 1996; Savary *et al.*, 1997; Stoltz, 1990, 1993; Stoltz *et al.*, 1986; Wyder *et al.*, 2002). Replication from parasitoid genomic provirus DNA and packaging into virions occurs only within specialized calyx cells of the female parasitoid ovary (Theilmann & Summers, 1986; Norton & Vinson, 1983; Wyder & Lanzrein, 2003). Once injected into larval hosts as virions, PDVs infect host cells, where specific viral

genes are transcribed and translated. Although the manifestations of PDV–parasitoid manipulation of host varies according to parasitoid and host insect species (Summers & Dib-Hajj, 1995), PDVs and their gene products function to regulate the larval host to favour parasitoid survival in several interrelated ways; these include suppression of host immune systems (Blissard *et al.*, 1986; Theilmann & Summers, 1986; Asgari *et al.*, 1997; Lavine & Beckage, 1996; Li & Webb, 1994; Cui & Webb, 1998), the inhibition of host protein synthesis (Shelby & Webb, 1994; Pennacchio *et al.*, 1998; Vinson *et al.*, 1998) and the regulation of host development and physiology (Lawrence & Lanzrein, 1993; Stoltz, 1993; Strand & Pech, 1995). Multiple host-regulatory functions are exerted by PDVs such that expression of PDV-encoded gene products within the pest host results ultimately in inhibition of the host immune response and development and/or alteration of physiology, enabling the parasitoid to evade encapsulation and to complete development.

The GenBank/EMBL/DDBJ accession number for the sequence of GiBV segment F is AY871265.

The segmented genomes of both braconid and ichneumonid PDVs (BV and IV, respectively) encode genes and

gene groups or families, many bearing structural resemblance to insect genes. Expression of many predicted and characterized PDV genes involves splicing of two or more exons to generate full-length mRNAs, which are then processed and translated in a fashion characteristic of their integrated eukaryotic cellular origin. Some PDV genes are encoded in single exons or open reading frames (ORFs). Numerous studies have analysed IV and BV gene transcription in parasitized lepidopteran hosts. Transcripts, genes or gene products have been identified as being involved in immunosuppression and inhibition of encapsulation (Asgari *et al.*, 1996, 1997; Cui *et al.*, 1997; Glatz *et al.*, 2004; Hayakawa *et al.*, 1994; Li & Webb, 1994; Provost *et al.*, 2004; Strand *et al.*, 1992; Strand, 1994; Yamanaka *et al.*, 1996), and these gene transcripts tend to be present in largest quantities immediately upon parasitization and either decline thereafter or persist for days after parasitization. PDV genes are also involved in developmental regulation and alteration of host physiology (Béliveau *et al.*, 2000; Johner *et al.*, 1999; Johner & Lanzrein, 2002; Provost *et al.*, 2004), and these gene transcripts for the most part are produced early in parasitization, although a recent study has shown certain gene transcripts from *Chelonus inanitus* BV (CiBV) to be present at low levels early in infection and upregulated late in parasitization (Bonvin *et al.*, 2004). Gene transcription has also been examined for several genes of unknown function in the parasitized host (Chen *et al.*, 2003b; Chen & Gundersen-Rindal, 2003; Volkoff *et al.* 2002). Transcription of viral genes from the parasitoid itself, as opposed to those within the parasitized host, has not been well studied (Bonvin *et al.*, 2004).

Many BV genes and gene families are conserved across the BV genomes characterized to date. Our research focuses on the braconid parasitoid *Glyptapanteles indiensis*, a parasitoid of the lepidopteran pest *Lymantria dispar* (gypsy moth). The *G. indiensis* BV (GiBV) shares some genetic similarity with the *Cotesia congregata* BV (CcBV), the first BV genome to be fully sequenced (Espagne *et al.*, 2004). Recently, a member of the GiBV protein tyrosine phosphatase family (PTP) gene family, GiBV segF PTP2 (then called PDVPTP), was identified, characterized and shown to be expressed ubiquitously in parasitized host tissues from early parasitization through to at least 8 days after parasitization (Chen *et al.*, 2003a). Subsequently, Espagne *et al.* (2004) showed that PTPs comprise the largest multigene gene family in the CcBV genome, and Provost *et al.* (2004) characterized the family of PTPs encoded in both CcBV and *Toxoneuron nigriceps* (Tn) BV; these genes appear to be found in abundance in many bracoviruses. CcBV and GiBV PTPs exhibit some similarity in sequence and structure. PTPs, which are generally encoded in single ORFs in BVs, have been hypothesized to function in BVs as regulators of host signal transduction, likely having roles in alteration of host physiology (Provost *et al.*, 2004).

Here, the focus is on GiBV genome segment F (GiBV segF), a segment predicted on the basis of nucleic and amino acid sequences to encode numerous PTPs and a single gene containing four ankyrin repeats, termed ank (Espagne *et al.*,

2004) (also referred to as vankyrin; Kroemer & Webb, 2004). Ankyrin repeat genes have been hypothesized to have a functional role in inhibiting NF- κ B signalling in the parasitized host, as they have similarity to inhibitors of NF- κ B gene transcription factors (Kroemer & Webb, 2004). Because of our interest in analysing the roles in parasitization of existent GiBV genes versus putative or predicted GiBV genes, the validity of various gene predictors for GiBV genes was tested by analysing and mapping GiBV segF PTP and ank gene transcripts using 5' - and 3' -RACE (rapid amplification of cDNA ends) as they are transcribed early (2 h after parasitization) in the infected host. In addition, we examined temporal and tissue-specific GiBV segF gene expression by assessing transcription over time using non-quantitative RT-PCR in the parasitized host, including a time point after emergence of the parasitoid larva.

METHODS

Insect rearing and parasitization. *G. indiensis*, a solitary parasitoid of gypsy moth (*L. dispar*) larvae, was imported from India for culture at the USDA-ARS Beneficial Insects Introduction Laboratory (Newark, DE, USA) and fed on 30% honey water. Host *L. dispar* larvae were reared on a high-wheatgerm diet in 200 ml cups with paper lids. Both wasp and host larvae were maintained at 26 °C, 50% relative humidity and a 16 h light:8 h dark photoperiod according to the protocol established by Bell *et al.* (1981). Parasitization of gypsy moth larvae by *G. indiensis* was conducted by exposing an individual first instar *L. dispar* larva to a single *G. indiensis* female within a 35 × 10 mm Petri dish until oviposition was observed. After a single oviposition was observed, the parasitized larva was removed from the Petri dish and put into a 30 ml plastic cup with high-wheatgerm diet and incubated at room temperature. Times post-parasitization (p.p.) were calculated and recorded from the initiation of parasitization. The parasitoid embryo or larva was not removed from 2 h p.p., 24 h p.p. or 3 days p.p. individuals. The parasitoid larva was removed by dissection from 7 days p.p. individuals. The parasitoid larva had emerged from 13 days p.p. individuals. *G. indiensis* larvae were dissected from the parasitized host 10 days p.p. from parasitized *L. dispar* larvae and washed to remove host material.

Isolation and sequencing of GiBV segment DNA. Adult female *G. indiensis* were dissected, reproductive tracts were isolated and fluid containing PDVs was gently released from the calyx with dissecting forceps. The fluid was collected under a dissecting microscope, avoiding eggs and ovarian tissues, and filtered through a 0.45 µm filter and PDV nucleic acid was extracted in an equal volume of extraction buffer containing 500 µg proteinase K ml⁻¹ and 0.5% SDS and incubated at 37 °C. Nucleic acid was gently extracted by rocking in an equal volume of phenol/chloroform according to the method of Beckage *et al.* (1994). GiBV segF was cloned in its entirety as described previously (Gundersen-Rindal & Dougherty, 2000). Plasmid DNA was prepared in large scale using the Quantum Prep Maxi DNA kit (Bio-Rad). Plasmid clone DNA was sequenced by a combination of primer walking and random transposon insertion using the Genome Priming System (GPS-1; New England Biolabs) followed by cycle sequencing using N, S, SP6 or T7 promoter primers and analysis on an ABI310 automatic sequencer (Applied Biosystems, Inc.). Contigs were assembled using Lasergene software (DNASTAR, Inc.). The sequence of the GiBV circular genome segment was assembled at 2 × to 6 × coverage and validated. The GiBV segF sequence was deposited in GenBank under accession number AY871265. GiBV segF ORFs or coding regions

Table 1. Comparison of GiBV segF predicted genes

Locations indicate the beginning and end of exons on the genomic DNA sequence. Correctly predicted nucleotides that were verified by analysis of gene transcripts by 5'- and 3'-RACE and PCR as detailed in the text are underlined.

ORF	Gene	Location	Length (nt)
ORF Finder (predicted many organisms, standard genetic code)			
1		<u>492–1388</u>	897
2		<u>3922–4815</u>	894
3		<u>5739–6635</u>	897
4		<u>8565–9497</u>	933
5		<u>8242–7283</u>	960
6		<u>11009–10243</u>	867
7		<u>12613–11771</u>	843
8		<u>14125–13163</u>	963
9		<u>17147–16674</u>	474
10		<u>18189–17299</u>	890
FGENESH (predicted <i>D. melanogaster</i>)			
1	1	<u>492–1289</u>	798
2		<u>3976–4689</u>	714
3		<u>5956–6509</u>	552
4		<u>8784–9377</u>	594
5		<u>9593–9598</u>	6
6	2	<u>14116–13340</u>	777
7		<u>12649–12242</u>	408
8		<u>11875–11771</u>	105
9	3	<u>17147–16674</u>	474
FGENESH (predicted <i>Anopheles gambiae</i>)			
1	1	<u>492–1298</u>	807
2		<u>2172–2174</u>	3
3	2	<u>5739–6515</u>	777
4		<u>6758–6832</u>	75
5	3	<u>8242–7448</u>	795
6		<u>7347–7303</u>	45
7	4	<u>8604–9377</u>	774
8		<u>9593–9598</u>	6
9	5	<u>11109–10774</u>	336
10		<u>10498–10466</u>	33
11	6	<u>14116–13229</u>	888
12		<u>12649–12242</u>	408
13		<u>11452–11423</u>	30
14	7	<u>14993–14766</u>	228
15	8	<u>18189–17404</u>	786
16		<u>16940–16674</u>	267
FGENESH (predicted <i>Apis mellifera</i>)			
1	1	<u>492–1388</u>	897
2	2	<u>2484–2465</u>	18
3		<u>1834–1712</u>	123
4	3	<u>3922–4809</u>	888
5		<u>4882–4935</u>	54
6	4	<u>5739–6515</u>	777
7		<u>6758–6832</u>	75
8	5	<u>8242–7448</u>	795
9		<u>7347–7303</u>	45
10	6	<u>8565–9377</u>	813

Table 1. cont.

ORF	Gene	Location	Length (nt)
11		<u>9593–9598</u>	6
12	7	<u>11109–10243</u>	867
13	8	<u>12613–11771</u>	843
14	9	<u>14116–13172</u>	945
15		<u>13109–13047</u>	63
16	10	<u>15093–15116</u>	24
17		<u>15672–15800</u>	129
18	11	<u>18189–17410</u>	780
19		<u>17132–16674</u>	459
GENSCAN W (predicted human)			
1	1	<u>492–1290</u>	799
2		<u>3883–4815</u>	933
3	2	<u>5739–6635</u>	897
4	3	<u>8565–9497</u>	933
5	4	<u>8242–7283</u>	960
6	5	<u>11109–10243</u>	867
7	6	<u>12613–11771</u>	843
8	7	<u>14116–13163</u>	954
9	8	<u>14993–14766</u>	228
10	9	<u>17599–18189</u>	591
11		<u>17132–16674</u>	459

were predicted based on sequence analysis and genes were identified using several gene predictor programs: NCBI ORF Finder [prediction for standard genetic code, wide variety of organisms; <http://www.ncbi.nlm.nih.gov/gorf/gorf.html> (only large ORFs are shown)], FGENESH [ab initio gene prediction based separately on *Drosophila melanogaster*, *Anopheles gambiae* and *Apis mellifera* insect gene parameters; <http://www.softberry.com> (Salamov & Solovyev, 2000; Solovyev & Salamov, 1999)] and GENSCAN W [prediction based on human gene parameters; <http://genes.mit.edu/GENSCAN.html> (Burge, 1998; Burge & Karlin, 1998)] (Table 1). ORFs were searched against the sequence database (NCBI) using BLAST searches (Altschul *et al.*, 1990, 1997) based on the amino acid sequence translations of individual ORFs.

RNA isolation. Total RNAs from *L. dispar* larvae were isolated at various time points following parasitization by *G. indiensis*. Whole individual larvae were disrupted in the presence of 1 ml Trizol (Invitrogen) with the Fastprep FP 120 instrument using matrix 'A' tubes (Q-Biogene) for 30 s, setting 4.5. Supernatants were transferred to sterile 1.5 ml tubes and incubated for 5 min at room temperature. Samples were extracted in an equal volume (200 µl) of chloroform and incubated for 3 min at room temperature. Samples were centrifuged at 10 000 r.p.m. for 15 min at 4 °C and the aqueous phase was precipitated with 100 % isopropanol. Total RNA pellets were washed once with 75 % ethanol and resuspended in water; all solutions were DEPC-treated. RNAs were used immediately for RACE and RT-PCR or stored at –80 °C after addition of 40 U RNase inhibitor µl⁻¹ (Ambion). Tissue-specific RNAs were obtained by the same method from parasitized larvae. In practice, we found it difficult to dissect specific tissues consistently and obtain sufficient RNA from each tissue from parasitized first-instar larvae at early time points p.p. At 7 days p.p., specific tissues could be dissected accurately and sufficient quantities of RNAs were obtained.

5'- and 3'-RACE and cloning. To identify 5' and 3' ends of GiBV segF genes, first-strand 'RACE ready' cDNAs were synthesized separately for 5'- and 3'-RACE reactions from larval RNAs 2 h p.p.

Table 2. Primers used for amplification and analysis of GiBV segF-encoded genes

Primer	Sequence (5'→3')	Predicted amplicon (bp)
5'-RACE		
5'RACEptp1	AAATCGCTTTTCCGTCTTCTGTAGG	
5'RACEptp2	CTCGGAAGAACGACGCGGCTGTGAT	
5'RACEptp3	ATGCCATCCCCGTTTCTTCAGACTTATT	
5'RACEptp4p2	TTGGACTTTTATGGCCTTTGACAGATTC	
5'RACEptp5	TCTACGGGAATGGTGTGATGGAGTC	
5'RACEptp6	TTTTGAGCTGCGGACCGTAGTAAAGGAAT	
5'RACEptp7	CGTCAACAAAACCTGGCCTCTGGAATCAT	
5'RACEptp8	TTCTTGGGGGTTATCTTTACACAGATTG	
5'RACEank	TTCCCATTGTATTCTTGAGTTGTAA	
5'RACEankp2	ATCCACCAGCTCATAGTCCTTGTTTFTA	
5'RACEptp9	TCTTGATTGCGCTGCTTACTCTCCC	
5'RACEptp9p2	AATTACCGTTGACCCGCCTTATCATA	
3'-RACE		
3'RACEptp1	GCCGCTCCAAGAACTGCCCAAGAT	
3'RACEptp2	TTGATCACAGCCGCGTCGTTCTTC	
3'RACEptp3	GAACACAGCCGCGTATTTCTTTCAG	
3'RACEptp4	AAGCGGTTGAATTTATTTAGCAAGAAC	
3'RACEptp5	CCCTGGTGCCCTAATAACTGGAAGTGGT	
3'RACEptp6	TACGGTCCGCAGCTCAAAAATAATCAAG	
3'RACEptp7	CTGGCAAACGGTGTGGGATAACG	
3'RACEptp8	ACAGCCGCACTTTGGATCCTTTGAACAT	
3'RACEank	TACAAATGGGGAAACTTGCTTGCATGTTGC	
3'RACEptp9	GGGAGAGTAAGCAGCGCAATCAAGA	
RT-PCR		
ptp1F	CTTAAATTGGATCCATGGGAGTAACTTGTGCCA	929
ptp1R	ATACAATTTTGAATTCAAACCTACATCTTATCAGCAA	
ptp2F1	AAACCGCTGCTCGATTTATTTTC	751
ptp2R1	GTTCTTCTAAGTTGCGACACA	
ptp3F	GCTGCTATGGATCCATGGGTAGCTGGAATTCGA	929
ptp3R	AGACTAACAAGAATTCTGCTTACACATAATACGTAC	
ptp4F	GCCCGAATGGATCCATGATTTTGGAACTTTA	687
ptp4r	TATAATCGTGAATTCTCATTAAGTCAAGAAAGTCA	
ptp5F	ACAGCTTTGGATCCATGGTTGTGAGTACCAACT	704
ptp5R	TTAAATCTTAGAATTCTTCTTAATTAACGAAA	
ptp6F	GGAGCGCTGGATCCATGGCACTCTGCATCCCAA	899
ptp6R	TCACACAGAGGAATTCCGACTAAACTCTATCAAGAC	
ptp7F	CCTGCTTTGGATCCATGAATACTGACGACTTCC	875
ptp7R	TTATAAAAAAGAATTCACTAAATCTCATCAAGAT	
ptp8F	GCCGTATTGGATCCATGTACGAAATGCGTCTTA	995
ptp8R	AACTAACTTAGAATTCCAATTAACCTACATGTGATA	
ptp9R1	TCCCCTTGATAATGAAATGGTAC	690
ptp9F1	GCAATTGCGGCGAAGAGTACA	
ankF	TTCAATAAGGATCCATGTCTTTCTCGAAGGAAA	506
ankR	TAGAACACGTGAATCCACTCAGTCCTCAAGTGGTT	

using the BD SMART RACE cDNA amplification kit (BD Biosciences) with the supplied modified oligo(dT) and BD SMART II A oligonucleotide primers according to the manufacturer's instructions. Gene-specific primers were designed for 5'- and 3'-RACE second-strand cDNA synthesis reactions on the basis of NCBI ORF predictions for GiBV segF (Table 2). 5'- and 3'-RACE gene-specific products were generated with cycling conditions of 25 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min followed by 16 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 5 min and a

final elongation at 72 °C for 7 min, and examined by electrophoresis on 1% agarose gels containing ethidium bromide. 5'-RACE products having more than one product were cloned into the pCR2.1 TOPO vector according to the protocol of the TOPO TA Cloning kit (Invitrogen). Plasmid DNA was isolated using a Quantum Prep miniprep kit (Bio-Rad) as specified by the manufacturer.

Sequencing and transcription mapping. The 3'-RACE and most 5'-RACE reactions yielded strong single products that were

sequenced directly or cloned and sequenced. For sequencing, products were concentrated by speed vacuum, separated on 1.5% NuSieve agarose gel (FMC) in modified $1 \times$ TAE (0.04 M Tris/acetate and 0.1 mM EDTA) and excised for sequencing using the ABI BigDye version 3 kit (Applied Biosystems) with corresponding gene-specific primers (Table 2). Cloned products were sequenced without gel purification. Cycle-sequencing conditions were 35 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Automatic sequencing was carried out on an ABI 3100 (Applied Biosystems). 5'- and 3'-RACE product sequences were assembled (DNASTAR, SeqManII component) and compared with genomic sequences to identify features of each gene transcript.

RT-PCR. Primers were designed to amplify the translated region of each identified GiBV segF gene (Table 2), including those predicted by insect parameters (not shown). Total RNAs from *L. dispar* larvae at various time points p.p. and various tissues at 7 days p.p. were treated with DNA-free (Ambion) to eliminate DNA contamination and used as templates in non-quantitative RT-PCR, which was performed according to the manufacturer's protocol using the RETROscript kit (Ambion) for two-step RT-PCR. Cycling conditions were 95 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min with a final elongation at 72 °C for 7 min. Duplicate reactions were performed using RNA templates without reverse transcription to verify the absence of contaminating viral DNA in the RNA templates. Products were separated by electrophoresis on 1% agarose gels containing ethidium bromide and visualized on a UV transilluminator.

RESULTS

Gene prediction in GiBV: predicted versus existent in the parasitized host

In an effort to identify GiBV-encoded genes, we isolated and sequenced full GiBV circular genomic segments. GiBV segF has abundant coding regions and was previously of interest to us for study of its integration *in vitro* in infected host cells (Gundersen-Rindal & Dougherty, 2000; Gundersen-Rindal & Lynn, 2003). GiBV segF ORFs or coding regions were predicted based on the standard genetic code of a wide variety organisms, on *D. melanogaster*, *Anopheles gambiae* and *Apis mellifera* insect gene parameters and on human gene parameters (Table 1). ORFs were searched against the sequence database (NCBI) using BLAST searches (Altschul *et al.*, 1990, 1997) based on the amino acid sequence translations of individual ORFs. All GiBV segF ORFs identified as existent had conserved sequence similarity to characterized genes, nine PTP genes and one single ankyrin repeat gene (ank) (Fig. 1). These PTPs, similarly to CcBV PTPs, were predominantly predicted to be encoded as single exons. By contrast, those based on FGENESH *D. melanogaster*, *Anopheles gambiae* and *Apis mellifera* were predominantly predicted to contain introns, in many cases multiple introns (Table 1). FGENESH predictions based on *Drosophila* suggested only three encoded genes for GiBV segF, with two containing multiple introns. FGENESH predictions based on *Anopheles gambiae* suggested eight genes, with seven containing at least one intron. FGENESH predictions based on *Apis mellifera* suggested 11 genes, with eight containing one intron. Fairly accurate gene predictions were obtained based on a wide range of organisms using standard genetic code (NCBI) or

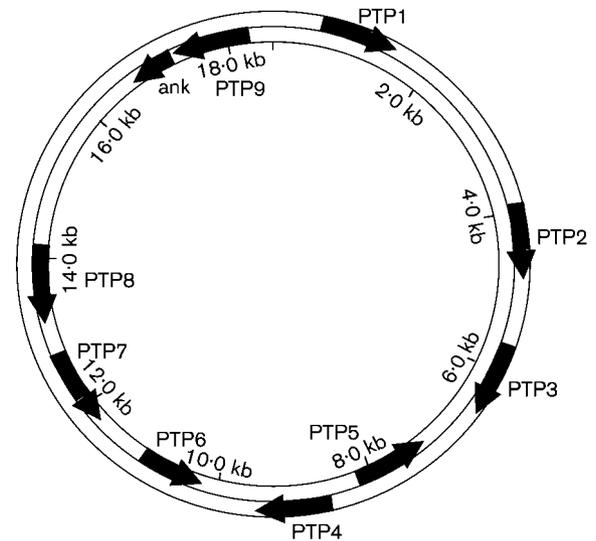


Fig. 1. GiBV segF validated gene map. Map structure of GiBV segF, validated and mapped as detailed in the text. Identities of genes (represented by large arrows) were determined based on BLAST searches for conserved homology with known genes. Arrows are orientated in the direction of transcription relative to the segment origin.

human (GENSCAN W) parameters for gene finding. GENSCAN W predictions based on human parameters predicted nine genes, two containing a single intron. A better prediction of GiBV genes, in terms of accurate gene start sites, was obtained using hymenopteran *Apis mellifera* parameters. However, using *Apis mellifera* parameters, introns were frequently predicted which did not exist in the parasitized host, as verified through RACE and RT-PCR analyses.

GiBV segF PTPs were characterized by a fairly high divergence in their amino acid sequences, even in the conserved core domain responsible for phosphatase activity (Table 3), with sequences of GiBV segF PTPs 1–9 ranging

Table 3. Amino acid sequences of catalytic core conserved 'HC' domains of GiBV segF PTPs

Generally invariant amino acid residues are underlined.

Gene product	Sequence
Consensus	PxxVHCSAGxGRTG
PTP1	PMVVHCSAGLNRTG
PTP2	PIVVHCSDGLERSM
PTP3	PIVVHCSDGLHRSM
PTP4	PMVHCTDGLERSM
PTP5	PILVHGKAGTGRTA
PTP6	PILVHCFSGLGSSQ
PTP7	PTLVHCLDGLGSSQ
PTP8	PIIVHCSAGVGRG
PTP9	PIVVHCSDGINKSG

from 17.9 to 63.4% identity at the amino acid level. GiBV genome segF was most similar in its sequence and gene organization to the characterized C1 circular genome segment of CcBV, which has been shown to encode eight PTPs and three EP1-like genes, but no ankyrin repeat genes (Espagne *et al.*, 2004; Provost *et al.*, 2004). At the amino acid level, GiBV segF PTP1 was most closely related to the CcBV C1 PTPB, GiBV segF PTP2 to CcBVC1 PTPQ, GiBVsegF PTP3 to CcBVC1 PTPP, GiBV segFPTP4 to CcBV C1 PTPP and PTPQ and GiBV segF PTP5 to CcBV C1 PTPM; GiBV segF PTP6 and PTP7 were both most closely related to CcBV C1 PTP1. The GiBV segF PTPs 8 and 9 were most closely related to CcBV PTPN and PTPC, respectively, which both originated from a different CcBV circular genome segment, C10.

Transcription mapping and gene organization

Gene transcription from GiBV dsDNA circular genome segments was bidirectional. To map GiBV segF genes and analyse GiBV gene transcription, gene-specific primers based on predicted genes were designed for nested amplification of transcripts by 5'- and 3'-RACE. Using RNA isolated from parasitized *L. dispar* larvae at 2 h p.p., specific products were detected for each gene (Fig. 2a). 5'-RACE products included the region from the nested 5' gene-specific primer to the gene translation start site plus the 5'-UTR or leader sequence and were of the approximate sizes expected. 3'-RACE products included the region from the nested 3' gene-specific primer to the translation stop site plus the 3'-UTR, terminating in a poly(A) tail, and were also of the approximate size expected. Occasional faint and likely non-specific products were observed in 5'-RACE reactions. 5'-RACE reactions for two predicted genes, PTP7 and ank, yielded two visible products of different sizes (Fig. 2a). 3'-RACE products yielded single amplification products for each predicted gene (Fig. 2b). All RACE products were sequenced, assembled and analysed for gene hallmarks.

By comparison of 5'-RACE sequences to the genomic sequence, the 5' sites for transcription initiation were identified for each gene. Transcription initiation sites for each gene were located between 30 and 112 bp from the gene translation start sites, with an average of 57 bp leader sequences for transcripts from this segment. For each, gene transcription was initiated at ATT, AAT or, most commonly, AGT sites (consensus ANT) (Figs 2b and 3b). For most GiBV segF genes, promoter sequences were predicted with high confidence within the 40 bp immediately upstream of the sites of transcription initiation (Neural Network promoter prediction; <http://www.fruitfly.org/>

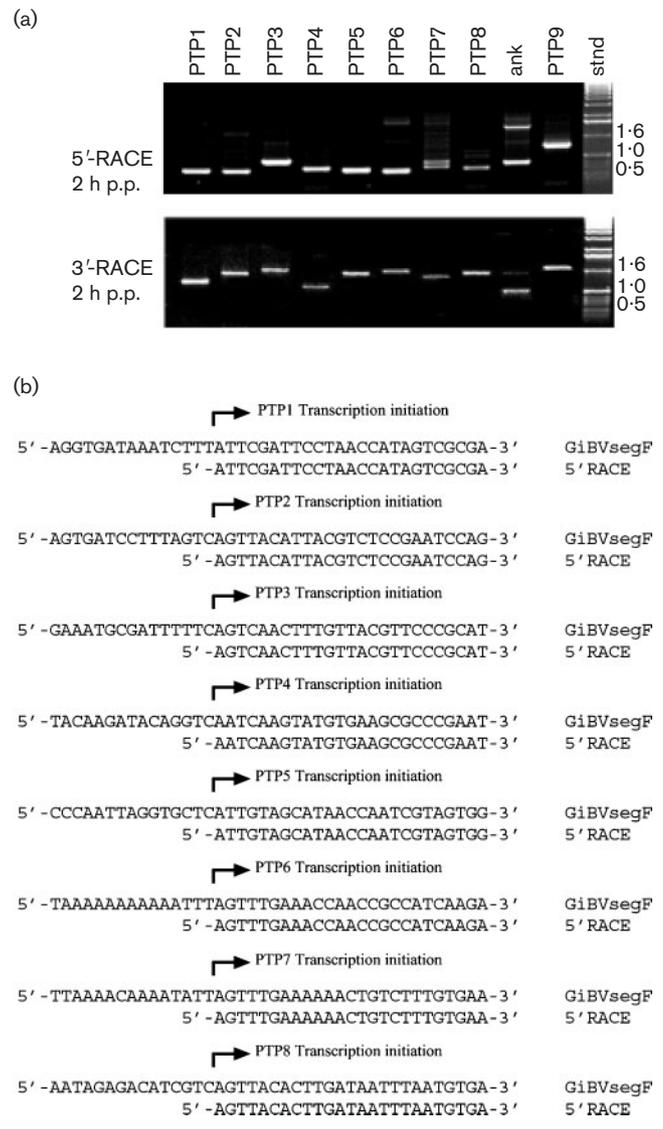
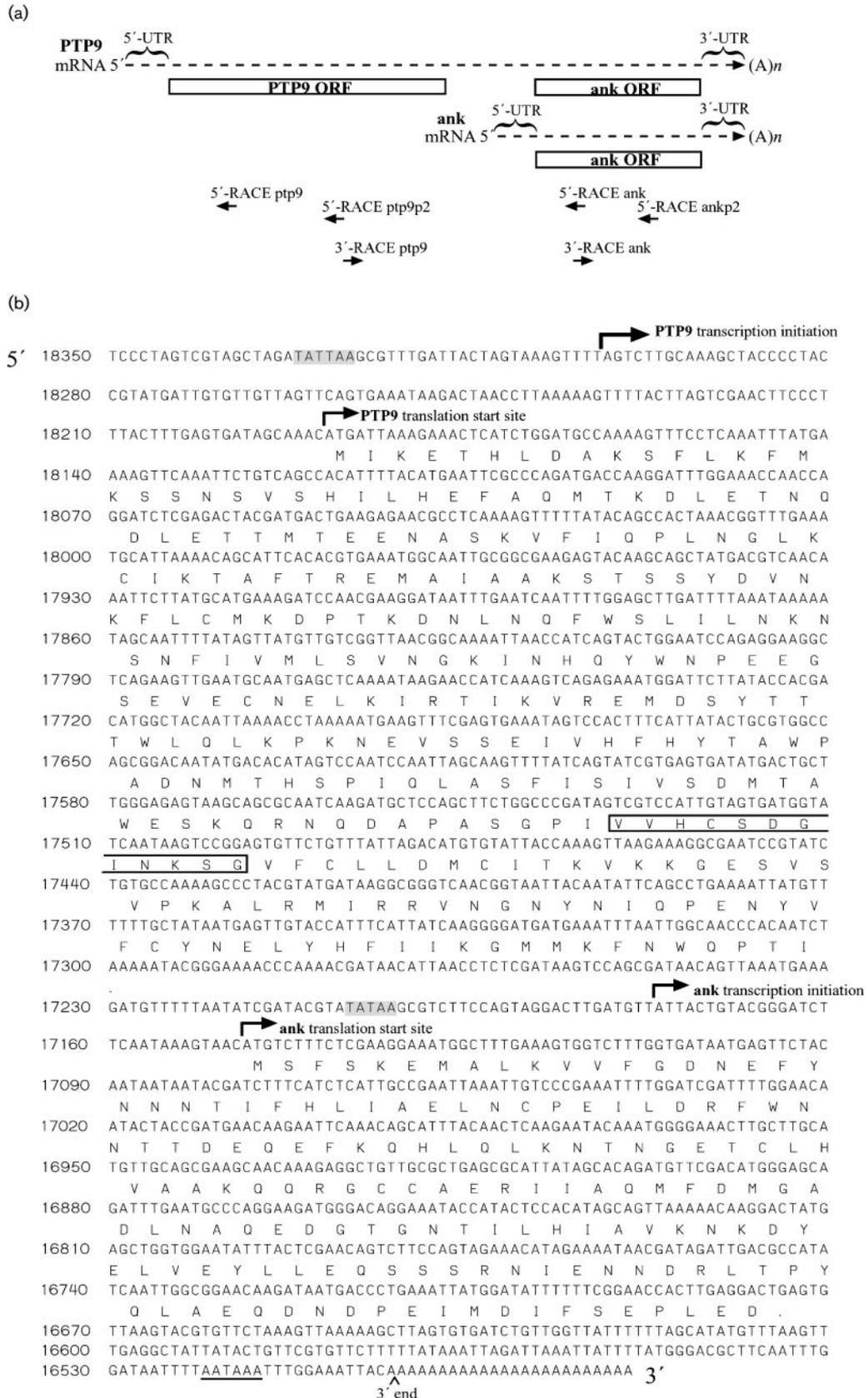


Fig. 2. Transcription and sequence analysis. (a) 5'- and 3'-RACE analysis of predicted GiBV segF ORFs yielded 5'- and 3'-RACE products generated from gene-specific primers and amplified from GiBV cDNA as described in the text. std, Size standards. (b) Sequencing of 5'-RACE products with comparison to transcription initiation sites identified in the GiBV genomic sequence as indicated by arrows.

seq_tools/promoter), although promoter sequences were not predicted for every gene within such close proximity of the transcription initiation sites.

Fig. 3. Mapping of GiBV segF PTP9 and ank genes based on 5'- and 3'-RACE. (a) Organization of the PTP9 gene region of GiBV segF. The mRNAs for both transcript variants are shown (dotted lines). Locations of gene-specific primers (see Table 2) used for 5'- and 3'-RACE analysis and sequencing are indicated. (b) Corresponding nucleotide sequences of PTP9 and ank genes of GiBV segF, shown together, with the 5'-UTR of PTP9, the locations of the mapped transcription initiation sites (TIS) and the 3'-UTR and 3' end used for both mRNA variants. Amino acid translations of the genes are given. The imperfect conserved P-loop 'HC' motif of the catalytic loop of PTPs is boxed. Putative TATA box sequences for both PTP9 (30 bp upstream of the TIS) and ank (30 bp upstream of TIS) genes are shaded. Nucleotide sequences corresponding to the poly(A) signal (AATAAA) are underlined. Corresponding base locations for nucleotide sequences are shown on the left.



By comparison of 3'-RACE sequences to genomic sequences, the 3' ends of the transcripts were determined. For each, the 3' end was located 12–20 bp downstream of a readily identifiable consensus poly(A) signal. Sequencing of 3'-RACE products generated from 3'-RACEank and 3'-RACEptp9 primers revealed that the two 3'-RACE products mapped to 3' ends at the same site, indicating that ank and PTP9 transcripts share a common end (Fig. 3a, b) located 12 bp downstream of the consensus poly(A) signal. Data from 5'- and 3'-RACE indicate the PTP9 and ank are transcribed together before processing. RACE data using multiple gene-specific primers indicate that the ank repeat gene is also transcribed independently (Fig. 3a, b), whereas PTP9 is transcribed only as a bicistronic message with ank. GiBV segF PTP genes 1–8 were all mapped as described, but details are shown only for PTP9.

The existence of predicted gene 7 (14993–14766), a 76 amino-acid-encoding gene predicted by FGENESH based on *Anopheles gambiae* (and also by NCBI ORF Finder), and predicted gene 10 (15093–15116 joined to 15672–15800), a 51 amino acid-encoding gene predicted by FGENESH based on *Apis mellifera*, were shown to be non-existent in the parasitized host by 5'- and 3'-RACE analysis using primers specific for these regions (data not shown). Products of the incorrect size were amplified from parasitized *L. dispar* RNA in 3'-RACE, the sequences of which were not GiBV-related and were likely the result of non-specific amplification. Overall comparison of actually transcribed GiBV segF genes with those predicted by the various gene-finding programs showed that no predictor of GiBV genes was entirely precise. It is quite possible that predicted genes are existent but transcribed only in the parasitoid.

GiBV segF gene temporal and tissue-specific expression patterns

In a previous study (Chen *et al.*, 2003a), we used quantitative RT-PCR to detect GiBVsegF PTP2 gene expression in tissues of parasitized *L. dispar* larvae within 2 h of parasitization, followed by a decline after 8 days. Here we used non-quantitative RT-PCR with gene-specific RT-PCR primers to look at expression patterns for the nine existent GiBV segF PTPs and the single ankyrin repeat gene. Using gene-specific primers designed for RT-PCR to amplify all or most of each gene identified in GiBV segF, temporal expression patterns in the parasitized host were assessed (Fig. 4). The absence of contaminating DNA in RNA samples was verified by inclusion of templates that were DNase-treated but not reverse-transcribed (shown only for 2 h p.p.). Amplified product was generated for GiBVsegF PTPs 1–9 and ank throughout parasitization, with the exception of PTP4, for which product was not detected at 7 and 13 days p.p. The presence of product 13 days p.p. and after emergence of the parasitoid suggested that GiBV gene transcripts remain at a very low level in the parasitized host even after their role has been served in parasitization. Amplification with specific *L. dispar* actin gene primers verified the presence of sufficient template for non-parasitized samples. Because gene predictors

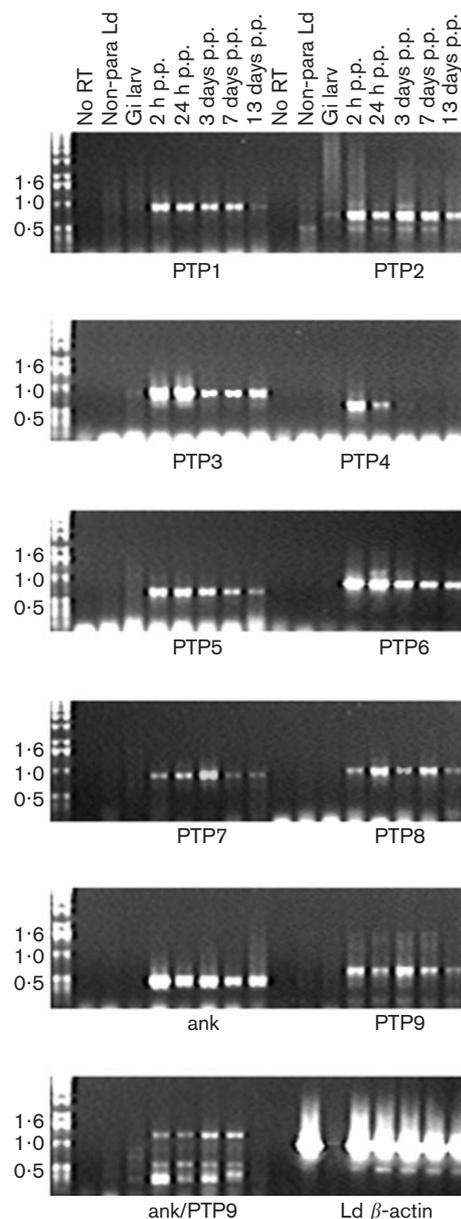


Fig. 4. Expression of GiBV segF genes in the parasitized host assessed by RT-PCR. Non-quantitative RT-PCR of each identified GiBV segF gene was performed using specific primers based on each identified ORF and total RNAs from *L. dispar* larvae parasitized by *G. indiensis* and isolated various times p.p. (non-parasitized and 2 h, 24 h, 3 days, 7 days and 13 days p.p.). No RT, No RT control; Non-para Ld, non-parasitized *L. dispar* larvae; Gi larv, *G. indiensis* larvae dissected from parasitized *L. dispar* at 10 days p.p.

for GiBV segF based on *Anopheles gambiae*, *Apis mellifera* and human parameters had predicted PTP1/PTP2 and/or ank/PTP9 to be spliced genes containing two exons with an intervening intron, we used specific primers for RT-PCR in combinations ptp1F/ptp2R1 and ptp9F1/ankR to assess the presence of the larger transcripts. No product was amplified

for ptp1/ptp2R1 (data not shown). The larger product was amplified using ptp9F1/ankR, indicating the presence of detectable longer transcript in the parasitized host, too large to possess the intron of 278 bp predicted using *Apis mellifera* parameters. No product was detected in control reactions using tissue from non-parasitized hosts or in reactions using RNA templates that were not reverse-transcribed (not shown).

The GiBV segF PTP genes showed variable tissue-specific expression patterns when assessed at 7 days p.p. (Fig. 5). Only GiBV segF PTP3 and the ank gene yielded detectable product from haemocytes at 7 days p.p. GiBV segF PTPs 1,

2, 3 and 7 yielded detectable product from midgut at 7 days p.p. All GiBV segF PTPs except PTP1 and PTP4 were detected from fat body at 7 days p.p., while all but PTP4 were detected from nervous tissue.

DISCUSSION

The need for a microgastroid parasitoid full genome sequence and comprehensive PDV gene transcription, processing and expression models means there are currently no ideal parameters that can be applied for accurate PDV gene prediction. Accurate PDV gene prediction is essential to identify viral genes that may be transcribed transiently or at very low levels, but may serve important roles in host regulation. Analysis of predicted versus existent gene identification for GiBV segF genes points out these deficiencies. The recent availability of a gene predictor based on the genome of the honey bee, *Apis mellifera*, the first hymenopteran genome to be fully sequenced, enables PDV gene prediction using parameters from an insect more closely related to PDV parasitoid wasp primary hosts. *Apis mellifera* gene predictors were shown to be useful for accurately predicting many GiBV segF gene start sites, although introns which did not exist were predicted for several of these genes. Accurate predictions for several GiBV genes expressed in the parasitized host were obtained based on predictors from several organisms (NCBI) and human (GENSCAN W). Human gene parameters more accurately predicted the presence or absence of introns, which occur in some gene families of PDVs. In spite of their insect origin, *Drosophila* and *Anopheles* parameters predicted GiBV genes with less overall accuracy. Gene prediction algorithm comparisons in transcription studies for two CiBV genes of unknown function upregulated late in parasitization (Johner & Lanzrein, 2002) found that human parameters predicted exons and introns of one CiBV gene (CiV14g1) with 100% accuracy, but did not recognize another (CiV14g2). Collectively, the few BV gene transcripts mapped and verified to date indicate that gene-prediction and gene-calling accuracy based on currently available parameters is gene-dependent. Until PDV-trained gene predictors are available, gene existence in PDVs will need to be verified in parasitized host and parasitoid, especially since, unlike most viral genes, large percentages of PDV genes (69% of CcBV genes reported by Espagne *et al.*, 2004) are predicted to contain introns.

The existence of predicted genes in GiBV segF was verified by transcript mapping using 5'- and 3'-RACE, which also identified sites of transcription initiation for the ten expressed genes, nine members of the PTP gene family and one ankyrin repeat gene, and defined their parameters. The nine PTPs were diverse in sequence, as evidenced by their catalytic core amino acid sequences (Table 3). Gene transcription occurred bidirectionally from the circular segment, with PTPs 1–4 transcribed in clockwise orientation, and the remaining genes in anti-clockwise orientation relative to the segment origin. The absence of introns was confirmed for each GiBV segF PTP, with the exception of PTP9, which was

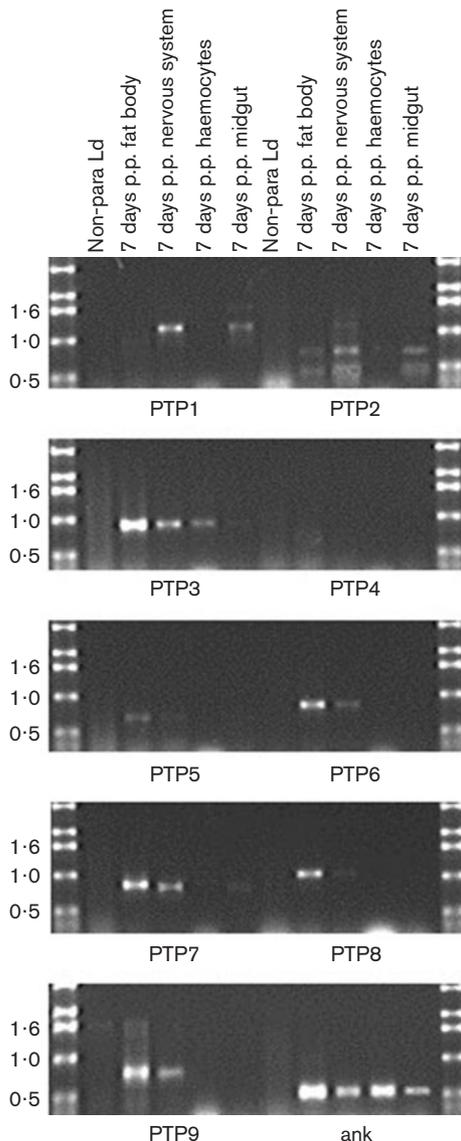


Fig. 5. Tissue-specific expression of GiBV segF genes in the parasitized host by RT-PCR using tissue-specific RNAs from *L. dispar* larvae parasitized by *G. indiensis* isolated 7 days p.p. as template. Non-para Ld, Non-parasitized *L. dispar* larvae.

transcribed as a bicistronic message with the ankyrin repeat gene, ank. Ank was also transcribed independently of PTP9. The presence of transcript for each GiBV segF gene was assessed in the parasitized host using non-quantitative RT-PCR, where amplified product was detected at 2 h p.p. and over the course of parasitization and parasitoid development for each, with the exception of PTP4, which was detected only up to 3 days p.p., indicating both early and sustained expression of these genes throughout the course of parasitism.

Tissue-specific expression of the GiBV segF PTP and ank genes in the parasitized *L. dispar* host was examined at 7 days p.p. CcBV tissue-specific PTP expression patterns in the parasitized host *Manduca sexta* at 12 or 24 h p.p. in infection and parasitization had previously been assessed, with the finding that certain CcBV PTPs were expressed ubiquitously, while others were expressed in certain host tissues (Provost *et al.*, 2004). At 7 days p.p., much later in the course of parasitism than was assessed in the CcBV system, GiBV gene tissue-specific expression patterns were somewhat variable. Very little GiBV gene expression was detected in host haemocytes or in midgut tissue. This suggested perhaps that any involvement of these particular GiBV PTPs in signal transduction pathways controlling haemocyte immune cells and the encapsulation processes of the host immune system functions may have been required immediately and through early infection, but may not have been sustained once the parasitoid embryo reached the 7 day p.p. time point. For PDVs in general, midgut has not usually been regarded as a site for functional PDV host-impairing activity, although certain PTPs may function at some level in this host tissue. For most PTPs, early and sustained expression was expected, as had been observed in the previous study using quantitative RT-PCR to detect sustained expression of GiBVsegF PTP2 in several tissues of parasitized *L. dispar* larvae within 2 h p.p., which started to decline by 8 days p.p. Several GiBV segF PTP genes (PTP2, 3 and 7) were expressed ubiquitously and expression was sustained for longer than 7 days p.p. The tissue-specific expression patterns of GiBV segF PTPs were sometimes, but not always, consistent with the tissue-specific expression patterns of their most closely related CcBV PTP, although differing time points p.p. for assessment, differing methods for assessment and differing BV–host systems could easily have accounted for discrepancy. The unexpected presence of some detectable PTP RT-PCR products 13 days p.p. (a time point occurring after emergence of the parasitoid) suggested that certain GiBV gene transcripts may have remained at low levels in the parasitized host even after their physiological role(s) in parasitism had been served. It is tempting to speculate that sustained detection of GiBV segF transcripts could relate to potential integration of this genome segment *in vivo*, as GiBV segF DNA has previously been demonstrated by this laboratory to integrate into chromosomal DNA of cultured lepidopteran cells. It is also interesting to note that certain GiBV PTPs possessed imperfect core catalytic (HC motif) regions, for example GiBV segF PTP5,

this region being essential for protein tyrosine phosphatase activity. The CcBV PTP to which GiBV PTP5 is most closely related, CcBV PTPM, is also mutated in the catalytic core region and was shown to lack phosphatase activity (Provost *et al.*, 2004). Since in both BV systems these PTPs are expressed in host tissues during the course of parasitism, it is likely that they do serve a function in parasitism, which may be quite distinct from other BV PTP activities.

The high allocation of coding sequence of GiBV and other BVs to members of the PTP gene family is undoubtedly related to their importance in signal transduction pathways used to control cellular protein phosphorylation in the regulation of host physiological processes, protein synthesis and immunosuppressive functions that ensure parasitoid survival. These roles may be wide-ranging and variable and may occur at various regulatory levels, including gene transcription and cell division. Dephosphorylation of proteins is a common mechanism among eukaryotes for regulation of cellular processes, where PTPs play critical roles in the reversible phosphorylation of proteins involved in several biochemical and signalling pathways and regulate tyrosine phosphorylation during cellular events such as proliferation, cell signalling and oncogenic transformations (Neel & Tonks, 1997). While BV PTPs will undoubtedly be found to be critical to host regulation, no precise functional role has been assessed or demonstrated to date for any identified PTP in any BV–host system. Certainly, collective expression data suggest that BV-encoded PTP genes are important regulators for early events in parasitized hosts and may serve a role(s) throughout the course of endoparasitoid development. PTPs may be equally important in disrupting signal transduction pathways controlling immune encapsulation and other vital processes. The precise, and perhaps multiple, functional roles for BV PTPs in the alteration of host processes remain to be explored.

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