Expression of a *Tranosema rostrale* polydnavirus gene in the spruce budworm, *Choristoneura fumiferana*

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The endoparasitic wasp *Tranosema rostrale* (Ichneumonidae) transmits a polydnavirus (PDV) to its host, *Choristoneura fumiferana*, during oviposition. Unlike most other PDVs examined, the virus of *T. rostrale* (TrPDV) does not appear to play an important role in suppressing the host cellular immune response. However, it inhibits host metamorphosis. In the present study, TrPDV gene expression was examined in parasitized and virus-injected last-instar caterpillars. Northern analysis with viral DNA as a probe revealed only one detectable mRNA, of about 650 bp. The corresponding cDNA, termed TrV1, was cloned and sequenced and found to encode a protein of 103 amino acids which, following cleavage of the putative signal peptide, has a predicted molecular mass of 9.3 kDa. This protein displays limited similarity to the VHv1.4 cysteine-rich protein from the PDV of *Campopleis sonorensis*, mostly within the signal peptide region. By using a TrV1-specific probe, the TrV1 gene was localized to segment G of the TrPDV genome. The cuticle and fat body were identified as the principal sites of TrV1 transcription, with little transcription observed in haemocytes and midgut. Western analysis of proteins extracted from selected tissues of parasitized insects suggested that the TrV1 protein is secreted in the haemolymph. As observed for other PDVs, injection of TrPDV did not suppress transcription of the gene that encodes juvenile hormone esterase, the activity of which is inhibited by the virus. We speculate that the TrV1 protein may play a role in the inhibition of *C. fumiferana* metamorphosis.

**Introduction**

Many endoparasitic wasps are capable of inducing developmental arrest in their lepidopteran hosts, apparently as a means of prolonging the period of suitability of the caterpillar for the growth of wasp larvae. The principal manifestation of this developmental perturbation is seen in the inability of the caterpillar to initiate or complete metamorphosis, a pathology that is usually induced by wasp-borne factors transmitted to the host during oviposition. These factors are believed to disrupt the hormonal events that trigger the larva–pupa transformation (Lawrence & Lanzrein, 1993; Cusson et al., 2000).

Among the factors known to play a role in the induction of developmental arrest, polydnaviruses (PDVs) have received the most attention. These viruses are obligate symbionts of many parasitic wasps belonging to the families Ichneumonidae and Braconidae. Their genomes are segmented and consist of a species-specific number of superhelical dsDNA circles generated from linear copies integrated in the wasp genome. Replication takes place in the calyx cells of the wasp ovary and virus particles are released in the lumen of the oviduct, where they form the particulate fraction of the ‘calyx fluid’ (CxF). Typically, a small quantity of this fluid is injected into the caterpillar host during oviposition. Although no replication takes place in the caterpillar, expression of viral genes is instrumental in altering host physiology to the benefit of the developing wasp larva. Immune suppression and developmental disruption in the caterpillar host are the two best-documented effects of PDV infection. However, viral gene expression and virus replication in the wasp are apparently asymptomatic (reviewed in Stoltz, 1993; Webb, 1998).

Several studies have examined the transcription of polydnaviral genes in lepidopteran hosts and, in some cases, the expressed genes and their protein products have been...
isolated and characterized (Webb, 1998). So far, most of these investigations have pointed to an involvement of the identified genes, transcripts or protein products in immune suppression (as opposed to developmental regulation), either on the basis that polydnavirus genes were expressed predominantly in host haemocytes (Asgari et al., 1996; Hayakawa et al., 1994; Strand, 1994; Strand et al., 1992, 1997; Yamanaka et al., 1996) or that the recombinant protein products had the ability to alter haemocyte behaviour and to inhibit encapsulation (Asgari et al., 1997; Cui et al., 1997; Li & Webb, 1994; Soldevila & Webb, 1996; Soldevila et al., 1997). However, a recent study examining the temporal pattern of PDV transcription in Spodoptera littoralis larvae parasitized by the braconid wasp Chelonus inanitus has shown that the levels of viral transcripts increase in the final instar, coincident with the induction of developmental arrest, an observation that suggests a role for some polydnaviral genes in the disruption of S. littoralis metamorphosis (Johner et al., 1999).

The ichneumonid wasp Transnova rostrale transmits a PDV (TrPDV) to its host, the eastern spruce budworm (Choristoneura fumiferana), in which it delays or prevents initiation of metamorphosis (Cusson et al., 1998a; Doucet & Cusson, 1996a). This developmental arrest results from a TrPDV-induced depression in moulding hormone (20-hydroxyecdysone) titres and is also associated with an inhibition of the activity of juvenile hormone esterase (JHE), a juvenile hormone (JH) degradative enzyme. However, neither the titre nor the activity of juvenile hormone esterase (JHE), a juvenile hormone induced depression in moulting hormone (20-hydroxyrostrale host–parasitoid system may be particularly well suited to the identification of polydnaviral genes involved in the disruption of host metamorphosis.

Here, we present data on the cloning and sequencing of a TrPDV gene, the patterns of transcription and protein accumulation of which, combined with the observed pathologies, suggest that the protein it encodes could play a role in the disruption of C. fumiferana metamorphosis.

Methods

**Biological material, parasitization and injections.** C. fumiferana larvae and T. rostrale wasps were obtained as described previously (Doucet & Cusson, 1996a, b; Cusson et al., 1998a). For parasitization, a T. rostrale female (< 2 weeks old) was introduced in a Petri dish containing a 1-day-old 6th-instar C. fumiferana larva and monitored until oviposition was observed. Injections of 0.5 female equivalents (FE) of CxF and saline were carried out as described previously (Doucet & Cusson, 1996a, b). Whole larvae and dissected tissues were snap-frozen in liquid nitrogen 0–17 °C, 1, 2, 3, 4, 5, 7 and 9 days after treatment and stored at −80 °C until processed.

**DNA and RNA isolation.** TrPDV DNA was extracted as described previously (Stoltz et al., 1986) from the CxF of 16 wasps. The DNA was ethanol-precipitated and resuspended in 100 µl TE, pH 7–6. Total RNA was extracted from C. fumiferana last-instar larvae by using TRIZOL reagent (Life Technologies) according to the manufacturer's instructions. The PolyATract mRNA isolation system (Promega) was employed to purify poly(A)+ mRNA from CxF-injected last-instar larvae by using total RNA extracted and pooled from caterpillars collected at each time-point post-injection (0–17–9 days, see above; 75 µg total RNA per time-point).

**Construction and screening of viral genomic libraries.** Genomic libraries were constructed by transformation of Escherichia coli (Hanahan, 1983) with purified TrPDV DNA that had been digested with either HindIII or Sphl and cloned in the pTZ18R vector (Amersham Pharmacia Biotech). Approximately 7500 clones from each library were plated onto LB Petri dishes containing 50 µg/ml ampicillin; colonies were lifted with Biotrans nylon membranes (ICN Pharmaceuticals) (Sambrook et al., 1989). The probe was prepared by reverse transcription of 1 µg poly(A)+ mRNA (from CxF-injected larvae) by using an oligo(dT)$_{18}$ primer and 200 U M-MLV reverse transcriptase (Life Technologies). The reaction was carried out at 37 °C for 1 h. The second strand was synthesized with 20 U E. coli DNA polymerase I (Life Technologies) at 16 °C for 2 h. Total cDNA was ethanol-precipitated and labelled (Oligo labelling kit; Amersham Pharmacia Biotech) by incorporation of [α-32P]dCTP (3000 Ci/mmol) (NEN Life Science Products). Hybridization was carried out in 6 x SSC, 5 x Denhardt’s reagent, 0.5% SDS and 200 µg/ml denatured salmon sperm DNA for 16 h at 65 °C. Blots were washed twice at 65 °C with 2 x SSC, twice with 2 x SSC, 0.1% SDS and, lastly, twice with 0.1 x SSC. The blots were then autoradiographed at −80 °C for 7 days. Positive clones were selected and subjected to restriction enzyme mapping. On the basis of the latter analysis, one clone (114) was chosen for sequencing.

**RT–PCR for rapid cloning of cDNA.** One µg total RNA extracted from CxF-injected larvae (2 days post-injection) was reverse-transcribed as described above. The reverse-transcription mixture (5 µl) was then submitted to PCR amplification with a SacI primer containing a sequence identical to the gene from clone 114 (from nt +193 to +214, Fig. 1c), 5 TAGGTTGAGCTCCATGACAACTCCTAGAATAGACCA 3, and an XhoI-oligo(dT)$_{18}$ primer, 5 GA$_{18}$ACTAGTCCTGAG(T)$_{18}$ 3' (Stratagene), in the presence of Vent DNA Polymerase (New England Biolabs). The reaction was carried out according to the following protocol: five cycles of 94 °C, 30 s; 43 °C, 30 s; 72 °C, 1 min; followed by 30 cycles of 94 °C, 30 s; 49 °C, 30 s; 72 °C, 1 min; and a final extension step at 72 °C for 5 min. The PCR product was digested with SacI and XhoI and cloned in the corresponding site of the pLITMUS 29 vector (New England Biolabs) for sequence analysis. To establish the position of the putative transcription start site, we carried out a primer-extension analysis (Sambrook et al., 1989) on total RNA from CxF-injected larvae (2 days post-injection) using a primer, 5' CGGCTCTTATTTCTTGGAAAGAGTGTAGTGA 3', complementary to nt +125 to +159 of the genomic sequence (Fig. 1c).

**Southern blot analysis.** One µl TrPDV DNA was separated on a 0.8% agarose gel by electrophoresis and transferred onto a Hybond-N nylon membrane (Amersham Pharmacia Biotech) by using a vacuum blotting system (LKB 2016 VacuGene apparatus, Amersham Pharmacia Biotech). A 284 bp Poul DNA fragment encompassing the first exon of the gene on clone 114 (from nt +239 to +254; Fig. 1a, c) and undigested total TrPDV DNA were used as probes. Labelling and hybridization were carried out as described for colony hybridization. The blots were autoradiographed at −80 °C for 1–4 h.

**Northern blot analysis.** Total RNA was denatured by using the glyoxal–DMSO method (Sambrook et al., 1989). Ten µg total RNA from
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**Fig. 1.**

(a) Partial restriction map of a TrPDV genomic fragment. Restriction sites are indicated as follows: B, *Bam*HI; H, *Hin*III; P, *Pvu*II; Sp, *Sph*I; Ss, *Ssp*I.

(b) The boxes are a schematic representation of the genomic clones obtained from the *Hin*III (shaded) and *Sph*I (open) libraries. In the *Sph*I clone, the hatched box represents the region that was sequenced (referred to as clone 114). Underneath, the bar represents the location of the 284 bp *Pvu*II fragment used as a probe.

(c) Nucleotide (plain) and deduced amino acid (bold) sequences of clone 114. In the nucleotide sequence, † is the putative transcription start site; in the amino acid sequence, † is the putative initiating methionine. A putative TATA box at †57 and a putative polyadenylation signal at †974 are underlined. The putative signal peptide cleavage site is indicated by an arrow between positions A21 and Y22 of the amino acid sequence.

Whole *C. fumiferana* larvae or 5 μg tissue-specific total RNA was separated on a 1–25% agarose gel by electrophoresis and transferred onto a Hybond-N nylon membrane as described for the Southern blot procedure. One probe was made by labelling the PCR product obtained by amplification of DNA from the *Sph*I and *Hin*III genomic libraries. The amplification was carried out by using the T7 promoter primer and the universal primer present at each side of the multiple cloning site of the pTZ18R vector in the presence of Elongase (Life Technologies) in 1× PCR buffer containing 1–9 mM MgSO₄, as described by the manufacturer for amplification of large fragments. The reaction consisted of 30 cycles of the following regime: 94 °C, 30 s; 55 °C, 30 s; 68 °C, 12 min; followed by a final extension step at 68 °C for 5 min. The other two probes were the same 284 bp *Pvu*II DNA fragment used for Southern blot analysis and the *C. fumiferana* JHE cDNA (Feng et al., 1999). All probes were labelled as described for colony hybridization. Hybridization was carried out in 0.5× SSPE, 0.5% formamide, 5× Denhardt’s reagent, 5% dextran sulphate, 0.5% SDS and 200 μg/ml denatured salmon sperm DNA for 16 h at 42 °C. The washes were performed at 42 °C, once in 5× SSPE, once in 1× SSPE, 0.1% SDS and, lastly, once in 0.1× SSPE, 0.1% SDS. The blots were autoradiographed at −80 °C for 0–24 h. The membranes were then stripped and rehybridized with the *Coprinus cinereus* pCc1 rDNA clone (Wu et al., 1983) to monitor the amount of RNA in each lane.

**Bacterial expression of cloned cDNA and production of polyclonal antibodies.** In order to clone the aforementioned cDNA in the bacterial expression vector pET-28b (Novagen), a PCR was carried out with the pLITMUS 29 cDNA clone and the *Sac*I primer used in the RT–PCR procedure described above and a second primer complementary to nt +849 to +835 (Fig. 1c: 5′ GTCTCTCTCGGCGGATAC 3′), designed to remove the stop codon by creating a new XhoI site, thus allowing the C-terminal fusion of the recombinant protein with the polyhistidine tag of the vector. The reaction was carried out as described above. The PCR product and the vector were digested with *Sac*I and XhoI.
and the coding portion of the cDNA was cloned directly in-frame in the vector. The fusion protein was produced in E. coli BL21 (DE3) by IPTG induction and was purified from the bacterial lysate on a Ni–NTA agarose column (Qiagen) under denaturing conditions according to the manufacturer’s instructions (Novagen). The purified His-tagged protein was separated on a 7.7% SDS–PAGE gel in a Tris–Tricine buffer (Khalkhali-Ellis, 1995), stained with a solution of 0.8% Coomassie blue in distilled water and rinsed in distilled water before the His-tagged protein was cut from the gel. Acrylamide pieces containing approximately 100 µg protein were put in 500 µl PBS and submitted to sonication until the acrylamide was thoroughly homogenized. Female New Zealand white rabbits (2.5–3 kg) were immunized with intramuscular injection with 100 µg of the purified protein in Freund’s complete adjuvant and a 100 µg boost was given 6 weeks later (Harlow & Lane, 1988). The antiserum was purified with Affi-Gel blue gel (Bio-Rad).

Western blot analysis. Proteins were extracted from parasitized C. fumiferana larvae (48 h post-parasitization), either from whole insects or individual tissues, in SDS loading buffer (Khalkhali-Ellis, 1995) adjusted to a final 1× buffer concentration. The samples were homogenized, boiled for 5 min and centrifuged. The protein preparations were separated by 7.7% SDS–PAGE, after which they were electrophoresed onto Immobilon-P membrane (Millipore). Immunodetection was carried out according to the membrane manufacturer’s instructions. A 1:250 dilution of the purified antiserum was used as primary antibody, followed by an incubation with a 1:30000 dilution of a goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Sigma). The protein of interest was visualized by using the Alkaline Phosphatase Conjugate Substrate kit (Bio-Rad).

Results

Screening of viral genomic libraries, mapping and nucleotide sequence analysis

In an effort to identify TrPDV genes expressed in TrPDV-infected C. fumiferana larvae, we screened two viral genomic libraries with a probe prepared by labelling a cDNA preparation generated from CxF-injected last-instar larvae. Eighty-six per cent of the positive clones were found to originate from the same viral genomic fragment (Fig. 1a). Positive clones from the HindIII and SphI libraries were 3.5 and 4.2 kb long, respectively (Fig. 1b). The localization of a coding sequence in the cloned genomic fragment was accomplished by Southern transfer of HindIII and SphI clones digested with PvuII and hybridized with the same total cDNA probe used for colony hybridization (data not shown). Clone 114 from the SphI library was selected for sequence analysis and found to encompass the entire gene sequence. The partial sequence revealed the presence of a gene, termed TrV1, composed of two putative exons and one putative intron (Fig. 1c). We identified a putative TATA box 254 bp upstream of the first ATG codon as well as a polyadenylation signal 144 bp downstream of the termination codon (Fig. 1c).

We searched for similarity to the nucleotide sequence of the TrV1 gene in the GenBank database by using the Blast search software (Altschul et al., 1997). The only significant similarity detected involved another TrPDV sequence (T. rostrale virus clone 57; unpublished GenBank accession no. AF052836), which showed 78% identity to the 3’ non-coding portion of the TrV1 gene (from nt +971 to +1292, Fig. 1c). Clone 57 was sequenced in the context of a separate study and was used to monitor the presence of TrPDV in C. fumiferana larvae (M. Laforge, G. Bellemare and M. Cusson, unpublished data). Thus, the similarity observed here merely confirms the presence of homologous sequences within PDV genomes (Theilmann & Summers, 1988; Cui & Webb, 1997; Webb, 1998).

Cloning of the corresponding cDNA and amino acid sequence analysis

In order to obtain the cDNA corresponding to the TrV1 gene, total RNA extracted from CxF-injected larvae (2 days post-injection) was subjected to RT–PCR. An oligo(dT) primer with an XhoI site in its 5’ end was used for the amplification in combination with a primer containing a SacI site at its 5’ end, flanked by a nucleotide sequence identical to nt +193 to +214 of the genomic clone (Fig. 1c). Analysis of the RT–PCR products by agarose gel electrophoresis revealed a single detectable band of ~550 bp (data not shown). Sequence analysis of the cDNA clone confirmed its identity as the cDNA corresponding to the TrV1 gene and supported our prediction regarding the exon–intron arrangement of the gene. The putative transcription start site (nt +1, Fig. 1c) was identified by primer-extension analysis (Fig. 2). The ORF present in the TrV1 cDNA encodes a protein of 103 amino acids (Figs 1c and 3) with a predicted molecular mass of 11.6 kDa (Strider software: March, 1988) and no potential glycosylation site (PROSITE analysis: Hofmann et al., 1999; Bucher & Bairoch, 1994). The first 21 amino acids were recognized as a putative signal peptide, with a region rich in hydrophobic amino acids and with a potential cleavage site between A21 and Y22 (Figs 1c and 3) (Nielsen et al., 1997).

In a Blast search (Altschul et al., 1997) conducted to identify GenBank sequences related to the TrV1 amino acid sequence,
the only significant alignment observed was with the N-terminal region of the VHv1.4 cysteine-rich protein encoded by the PDV of C. sonorensis (CsIV) (Cui & Webb, 1996). The two proteins display 47% similarity over the first 46 amino acids, a region that includes the putative signal peptides (Fig. 3). The remaining portion of the TrV1 protein shows very little similarity to the VHv1.4 protein and displays no Cys motif.

Southern analysis

In Southern analysis, hybridization of undigested viral DNA with the TrV1-specific probe revealed two major bands (Fig. 4a, lane 1). These bands were identified as the open circular (g) and superhelical (G) forms of segment G by comparison with a similar blot in which the probe was made circular (g) and superhelical (G) forms of segment G by digestion with the TrV1-specific DNA fragment. Lanes: B, BamHI digest; H, HindIII digest; Ss, SspI digest; M, DNA ladder.

In an effort to highlight all TrPDV transcription products, the entire DNA from the genomic libraries was labelled and used as a probe (Fig. 5a), while the transcription pattern of TrV1 was analysed by using the gene-specific PvuII probe (Fig. 5b, c). We observed only one band with either probe, apparently representing a single transcript of ~650 bp, which was detectable during the entire sampling period (0–17–9 days post-treatment) in both CxF-injected (Fig. 5a, b) and the TrV1-specific probe revealed bands (Fig. 4b) with mobilities that matched those predicted from the sequence results (see Fig. 1). Altogether, these results suggest that the TrPDV genome contains at least one copy of the TrV1 gene on segment G and that the same or a similar sequence is also present on a few other, less-abundant segments, including segment A.

Transcription patterns of TrPDV genes in C. fumiferana larvae

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Fig. 5. Time-course analysis of TrPDV gene transcription in last-instar C. fumiferana larvae by Northern hybridization. Lane numbers indicate sampling time, from 0-17 to 9 days post-treatment. (a)–(b) Total RNA from CxF- and saline-injected larvae probed with labelled DNA from the SphI and HindIII genomic libraries (a) or the 284 bp PvuII fragment specific to TrV1 (b). (c) Total RNA from parasitized and untreated larvae probed with the 284 bp PvuII fragment specific to TrV1. The picture shown under each blot represents the same membrane that was rehybridized with the C. cinereus rDNA pCc1 clone as a control for the amount of RNA present on the blot. Note that day-9 control insects (saline-injected and untreated) represent a small proportion of the population, as pupation normally takes place on the 7th or 8th day of the final instar.

Fig. 6. (a) Northern hybridization performed on total RNA obtained from various tissues of C. fumiferana larvae parasitized by T. rostrale. HL, haemolymph; M, midgut; FB, fat body; and C, cuticle. Sampling was conducted 24, 48 and 72 h after parasitization. The Northern blot was hybridized successively with the 284 bp PvuII fragment specific to TrV1 (panel 1) and the C. cinereus rDNA pCc1 clone (panel 2) as a control for the amount of RNA present on the blot. (b) Relative abundance of TrV1 mRNA in various tissues of C. fumiferana larvae parasitized by T. rostrale. Signals obtained on the blot shown in (a) were quantified with Image Gauge version 3.0 (Fuji Photo Film) and the values obtained for the 284 bp PvuII probe were corrected for the amount of RNA present on the blot, as measured by pCc1 hybridization.

Bacterial expression of the TrV1 protein for antibody production and protein detection in parasitized larvae

The coding portion of TrV1 was cloned into the bacterial expression vector pET-28b in a fashion that resulted in the addition of a histidine tag at the C terminus of the protein, which would increase its molecular mass to 16.7 kDa. After IPTG induction for 3 h, the TrV1–His protein was found in the insoluble fraction of the bacterial lysate (Fig. 7a, lane +1); it was then purified by Ni–NTA agarose affinity chromatography (Fig. 7a, lane P).

The polyclonal antiserum raised against the purified TrV1–His protein was tested on the bacterial recombinant protein (Fig. 7b, lane P) and on proteins extracted from parasitized (Fig. 7b, lane +) and untreated (Fig. 7b, lane −) larvae. As expected, a signal was detected for the recombinant protein and for the extract obtained from parasitized larvae, but not for that obtained from controls (Fig. 7b). To examine the distribution of the TrV1 protein in the insect, different tissues from parasitized larvae were tested. The signal obtained in the haemolymph (Fig. 7b, lane HL) originated from the plasma fraction (Fig. 7b, lane PL), as it was absent from haemocytes (Fig. 7b, lane HC). The fat body and the cuticle, in which high levels of TrV1 mRNA were detected (Fig. 6), also contained detectable levels of the protein (Fig. 7b, lanes FB and C, respectively). The protein extracted from larvae had a
greater mobility than the recombinant protein, as might be expected given the absence of the His tag and the likely cleavage of the signal peptide in the native protein (predicted molecular mass of 9.3 kDa after cleavage of the signal peptide).

**Transcription pattern of JHE in CxF-injected C. fumiferana larvae**

In a previous study, we showed that parasitization by *T. rostrale*, or injection of its CxF, results in a significant depression of JHE activity in host *C. fumiferana* larvae (Cusson et al., 2000). Work on other host–parasitoid systems suggests that this PDV-associated dysfunction may be caused by translational inhibition of the JHE transcript (Dong et al., 1996; Shelby & Webb, 1997). To determine whether a similar mechanism may be responsible for TrPDV-induced JHE inhibition, we carried out an additional Northern hybridization with the RNA extracted from CxF- and saline-injected larvae employed in a previous experiment (see Fig. 5b) and a *C. fumiferana* JHE (CfJHE)-specific probe. No suppression of CfJHE transcription was observed in CxF-injected insects relative to saline-injected controls (Fig. 8a, b). The pattern of CfJHE transcription observed here in control larvae is similar to that reported earlier by Feng et al. (1999) for the same species. On day 9, the very low level of JHE transcription observed in saline-injected controls is typical of caterpillars that have reached the prepupal stage and is likely the result of transcriptional inhibition by 20-hydroxyecdysone (Feng et al., 1999), the titre of which are depressed in CxF-injected *C. fumiferana* larvae (Cusson et al., 2000).

**Discussion**

In the present paper, we describe the cloning of a cDNA corresponding to a TrPDV transcript that is very abundant in last-instar *C. fumiferana* larvae parasitized by *T. rostrale* at the beginning of the stadium. In Northern analysis, with the entire
viral DNA used as a probe, this transcript was the only one that could be detected during infection (Fig. 5a). Although we strongly suspect the presence of additional, less-abundant viral mRNAs (not yet analysed), the high intensity of the 650 bp signal observed on Northern blots, combined with the absence of any other detectable band, suggests that the TrV1 protein may play an important role in the observed virus-induced pathologies.

Transcription of ichnovirus genes in lepidopteran hosts has been examined in only two other species. With total viral DNA as a probe, Northern analysis of CsIV transcription in *Helothis virescens* led to the detection of at least ten transcripts (Blissard *et al.*, 1986), whereas a similar approach applied to the virus of *Hyposoma didymator* (HdV) in *Spodoptera littoralis* revealed only one band (Volkoff *et al.*, 1999). However, infection of S9 cells *in vitro* with HdV led to the detection of several other, less-abundant transcripts (Volkoff *et al.*, 1999), suggesting that these additional mRNAs are also present in parasitized larvae but escape detection because of the higher relative abundance of host transcripts (compared with S9 cells) and the limited specificity of the probe. The latter results support the hypothesis that TrV1 is unlikely to be the only TrPDV gene expressed in *C. fumiferana*.

Temporal patterns of TrV1 transcription differed between naturally parasitized and CxF-injected caterpillars, with peak transcription levels seen a few days later in the former group (Fig. 5). This difference could stem from the fact that a manual injection is likely to result in a more rapid and more uniform distribution of the virions within the treated animal than is presumably the case following natural parasitization, whereby the virions are believed to diffuse slowly away from the egg, to which they are bound initially (Cusson *et al.*, 1998b). Alternatively, other wasp fluids injected into the host during parasitization and absent from the CxF (e.g. venom) may have regulatory effects on TrV1 transcription. Lastly, the amount of virus contained in a 0.5 FE dose of CxF is likely to be higher than that injected by a female wasp during oviposition, since significantly greater inhibition of JHE activity was observed in CxF-injected 0.5 FE *C. fumiferana* larvae than in parasitized individuals (Cusson *et al.*, 2000), therefore providing a possible explanation for the very high level of TrV1 transcription observed 24 h after injection.

With only 103 amino acids, the TrV1 protein is one of the smallest PDV-encoded proteins reported so far (see Webb, 1998). It shows no relatedness to any other known protein except for the VHv1.4 protein encoded by CsIV. However, most of the similarity is found in the signal peptide region of the polypeptide (Fig. 3). Therefore, the mature protein has little in common with the larger VHv1.4 gene product, which is believed to be involved in immune suppression (Cui *et al.*, 1997).

Unlike most PDV genes for which expression has been documented for other host–parasitoid systems, haemocytes do not appear to be the primary site of TrV1 transcription (see Introduction for relevant references). Instead, the tissues that are usually in contact with the egg following oviposition, the fat body and the cuticle, show the greatest abundance of TrV1 transcripts (Fig. 6). This observation is in agreement with an earlier study, in which we documented the entry of TrPDV particles in tissues that are in direct contact with the chorionic hair-like projections that coat *T. rostrale* eggs and to which the virions adhere (although we did not see virions entering the cuticle itself; Cusson *et al.*, 1998b). The fact that the level of transcription was somewhat higher in the cuticle than in the fat body was surprising, since the egg is usually laid underneath the cuticle (i.e. in the fat body) and not within it. However, the tissue referred to here as ‘cuticle’ could not always be dissected clean of all adhering fat body and muscle. It is, therefore, possible that the signal observed in the ‘cuticle’ originated, at least in part, from the contaminating tissues. To resolve this question, it will be necessary to use approaches such as *in situ* hybridization and immunohistochemistry. In the present study, immunolocalization of the TrV1 protein by Western analysis of individual tissues suggests that this protein is secreted into the haemolymph; although it was detectable in the two tissues identified as the primary sites of transcription (cuticle and fat body), it was most abundant in the plasma fraction of the haemolymph (Fig. 7b).

Inhibition of JHE activity by TrPDV (Cusson *et al.*, 2000) does not appear to result from virus-induced suppression of JHE transcription, as indicated by the levels of CjJHE transcripts, which were at least as high in CxF-injected larvae as they were in saline-injected controls (Fig. 8). These results are in agreement with data reported for other PDVs, which suggest that PDV gene products likely act at the post-transcriptional (i.e., translational) level (Dong *et al.*, 1996; Shelby & Webb, 1997, 1999; Shelby *et al.*, 1998). Although we did not measure the actual levels of the JHE protein in the plasma of *C. fumiferana* larvae (e.g. by immunodetection), other workers have shown that inhibition of JHE activity by PDVs is a result of depressed JHE titres, as opposed to interference with the activation of the secreted enzyme. Whether the TrV1 protein is involved in the inhibition of CjJHE translation remains to be determined.

In conclusion, given (i) that the TrV1 transcript is clearly the most abundant TrPDV transcript during the last instar of *C. fumiferana*, (ii) that haemocytes are not the primary site of TrV1 transcription and protein accumulation, (iii) that the mature TrV1 protein shows little relatedness to other PDV proteins believed to be involved in immune dysfunction and (iv) that during the period when TrV1 transcription is seen, disruption of host metamorphosis is the principal pathology observed, with no obvious effect on the cellular immune response (Doucet & Cusson, 1996b), it seems possible that the TrV1 protein plays a role in blocking the initiation of metamorphosis, either alone or in concert with other low-abundance viral proteins. To test this hypothesis, we are currently examining the existence of other TrPDV transcripts
that may have escaped detection using the methods described here and we are producing the TrV1 protein in a baculovirus expression system, with the view to using it in both in vivo and in vitro bioassays. The observation that the TrV1 protein is secreted into the caterpillar haemolymph suggests that it acts on tissues that may differ from those involved in TrV1 expression and that an approach involving injection of the recombinant protein into whole caterpillars may be successful for documenting pathological effects.

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References


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