Tranosema rostrale ichnovirus repeat element genes display distinct transcriptional patterns in caterpillar and wasp hosts

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The endoparasitic wasp Tranosema rostrale transmits an ichnovirus to its lepidopteran host, Choristoneura fumiferana, during parasitization. As shown for other ichnoviruses, the segmented dsDNA genome of the T. rostrale ichnovirus (TrIV) features several multi-gene families, including the repeat element (rep) family, whose products display no known similarity to non-ichnovirus proteins, except for a homologue encoded by the genome of the Helicoverpa armigera granulovirus; their functions remain unknown. This study applied linear regression of efficiency analysis to real-time PCR quantification of transcript abundance for all 17 TrIV rep open reading frames (ORFs) in parasitized and virus-injected C. fumiferana larvae, as well as in T. rostrale ovaries and head–thorax complexes. Although transcripts were detected for most rep ORFs in infected caterpillars, two of them clearly outnumbered the others in whole larvae, with a tendency for levels to drop over time after infection. The genome segments bearing the three most highly expressed rep genes in parasitized caterpillars were present in higher proportions than other rep-bearing genome segments in TrIV DNA, suggesting a possible role for gene dosage in the regulation of transcription level. TrIV rep genes also showed important differences in the relative abundance of their transcripts in specific tissues (cuticular epithelium, the fat body, haemocytes and the midgut), implying tissue-specific roles for individual members of this gene family. Significantly, no rep transcripts were detected in T. rostrale head–thorax complexes, whereas some were abundant in ovaries. There, the transcription pattern was completely different from that observed in infected caterpillars, suggesting that some rep genes have wasp-specific functions.

INTRODUCTION

Hymenopteran endoparasitoids deposit their eggs within the haemocoele of arthropods, most of which are insects (Eggleton & Belshaw, 1993). To protect their eggs from detection by the host immune system and to provide an appropriate developmental and physiological milieu for survival of their immature progeny within the host, female wasps typically inject their eggs along with various materials capable of disguising the egg surface and/or altering host physiology. For example, some members of the families Ichneumonidae and Braconidae transmit, to their caterpillar hosts, a virus that is essential for survival of the immature wasp within the parasitized insect (reviewed by Stoltz, 1993). These viruses, known as polydnaviruses (PDVs), feature a segmented, circular dsDNA genome, with individual genome segments varying in size and genetic content. A copy of the viral genome is present as a provirus within the wasp’s chromosomes, thus providing a mechanism for the vertical transmission of PDVs within parasitoid populations. Virus replication is restricted to the calyx region of the wasp ovary, from which virions are released into the lumen of lateral oviducts. There, they form the particulate fraction of the calyx fluid (CF). During oviposition, a female wasp injects one or more eggs, along with CF and other secreted proteins and venom, into the lepidopteran host. Although no virus replication occurs in the parasitized caterpillar, expression of PDV genes causes developmental and immune dysfunctions that protect the egg and wasp larvae from encapsulation by host haemocytes and/or lead to retardation or arrest of host metamorphosis, thus providing more time for the wasp larva to complete its development in advance of host pupation (reviewed by Kroemer & Webb, 2004; Stoltz, 1993).

PDV genes are divided into three broad categories based on whether they are expressed in the carrier wasp (class I), the infected caterpillar (class II) or both (class III) (Theilmann...
& Summers, 1988). Because of their potentially major significance in the success of parasitism, class II genes have been studied more extensively than those of the other two groups. A number of these genes encode proteins displaying motifs or structural and sequence features observed in previously characterized eukaryotic proteins. Based on these similarities, it has been possible to generate and test hypotheses about their probable functions. Such an approach has led to proposed functions for various PDV genes (e.g. the vankyrins) (Falabella et al., 2007; Kroemer & Webb, 2005; Thoetkiattikul et al., 2005).

Other PDV genes, however, display no known similarity to other eukaryotic or viral (non-PDV) genes, rendering their functional analysis more difficult. Such is the case for the repeat element (rep) gene family, the largest gene family identified to date in the genus Ichnovirus (PDVs associated with ichneumonid wasps). These genes consist of imperfectly conserved repeats of ~540 bp, arranged either singly or in direct tandem arrays (Theilmann & Summers, 1987). Members of the rep gene family encode non-secreted proteins that are conserved among several ichnovirus species (Tanaka et al., 2007; Volkoff et al., 2002; Webb et al., 2006). Expression of rep genes has been detected in both parasitoids and their parasitized hosts (Galibert et al., 2006; Theilmann & Summers, 1988). The Transoemma rostrale ichnovirus (TrIV) genome contains at least 17 different open reading frames (ORFs) identified as belonging to the rep gene family; they are located on ten different genome segments (Tanaka et al., 2007). In an earlier study, two TrIV rep genes (TrFrepl and TrFrep2) were shown to be expressed from TrIV genome segment F (Volkoff et al., 2002); this genome segment has been renamed F1 and the two rep genes it contains are now referred to as F1-1 and F1-2 (Tanaka et al., 2007). As a first step towards elucidating the function(s) of these gene products, we initiated a study of the temporal and tissue-specific transcription of all known and putative TrIV rep genes. A similar study of ten rep genes from the ichnovirus of Hyposoter didymator (HdIV) has revealed important differences in gene-specific transcript abundance but minor differences in host and tissue specificity (Galibert et al., 2006). Using a recently developed and powerful quantitative real-time PCR (qPCR) approach (Rutledge & Stewart, 2008a, b), the present study examined transcriptional patterns in the host Choristoneura fumiferana, either naturally parasitized by T. rostrale or injected with 0.5 female equivalents (FE) of T. rostrale CF, as described previously (Doucet & Casson, 1996a, b). For each sampling point [1, 3 and 5 days post-parasitization (p.p.) or post-injection (p.i.)], total RNA was extracted and pooled from three to five whole C. fumiferana larvae, using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions (Béliveau et al., 2000). RNA was also extracted from the fat body, cuticular epithelium, midgut and haemocytes obtained from a pool of three to five larvae 48 h p.i. In addition, total RNA was extracted and pooled from five ovary pairs dissected from post-emergence 5–10-day-old T. rostrale females, using a QiAmpshredder and RNeasy Mini Kit (Qiagen), according to the manufacturer’s instructions. The head–thorax complexes of the same five females were also subjected to total RNA extraction using TRIzol reagent.

TrIV DNA was extracted from the CF of 16 T. rostrale female wasps as described previously (Stoltz et al., 1986). The DNA was first ethanol precipitated and then resuspended in 100 μl Tris/EDTA buffer (pH 7.6).

**Methods**

**RNA and DNA extraction.** Within 24 h of the moult to the last (sixth) instar, C. fumiferana larvae were either parasitized once by T. rostrale or injected with 0.5 female equivalents (FE) of T. rostrale CF, as described previously (Doucet & Casson, 1996a, b). For each sampling point [1, 3 and 5 days post-parasitization (p.p.) or post-injection (p.i.)], total RNA was extracted and pooled from three to five whole C. fumiferana larvae, using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions (Béliveau et al., 2000). RNA was also extracted from the fat body, cuticular epithelium, midgut and haemocytes obtained from a pool of three to five larvae 48 h p.i. In addition, total RNA was extracted and pooled from five ovary pairs dissected from post-emergence 5–10-day-old T. rostrale females, using a QiAmpshredder and RNeasy Mini Kit (Qiagen), according to the manufacturer’s instructions. The head–thorax complexes of the same five females were also subjected to total RNA extraction using TRIzol reagent.

To assess the proportion of each rep gene-bearing genome segment within a TrIV DNA extract, the same qPCR approach was applied directly to 0.01 ng TrIV DNA, using one of the primer pairs designed for transcript quantification for each genome segment (see Supplementary Table S1).

**Reverse transcription and qPCR.** To remove DNA contaminants from RNA extracts, 500 ng total RNA was treated with 2 U amplification-grade DNase I (Invitrogen) for 15 min at 25 °C. We ran controls with no reverse transcriptase for the four most highly transcribed ORFs and detected no significant amplification, indicating the absence of genomic DNA contamination in the extracts. RNA (500 ng) from parasitized and CF-injected C. fumiferana larvae, as well as from T. rostrale head–thorax complexes, and 200 ng RNA from ovarian tissue was reverse transcribed using 0.5 μg of an oligo(dT) primer and 200 U Superscript II RNase H − reverse transcriptase (Invitrogen). The reaction was carried out in 1 x PCR buffer, with 0.5 mM each dNTP and 40 U RNguard RNase inhibitor (Amersham Biosciences), at 42 °C for 50 min.

For qPCR analysis, four primers were initially designed for each rep gene, using diverse regions among aligned rep nucleotide sequences. These four primer pairs were used to assess primer performance and quantitative precision. Initial amplification tests were conducted on reverse-transcribed RNA obtained from parasitized C. fumiferana larvae. A single primer pair was then selected for each rep gene (see Supplementary Table S1, available in JGV Online), based on high amplification efficiency and lack of non-specific amplification products, and used for analysis of the remaining samples.

PCR amplifications were carried out on aliquots of individual reverse transcription reactions containing cDNA in amounts equivalent to 2.5 ng RNA, except for ovarian samples, which contained amounts of cDNA equivalent to 1 ng RNA. Four replicate amplification reactions containing 500 nM each primer were conducted for each sample, using an MX3000P spectrophuorometric thermal cycler (Stratagene) and a Quantitect SYBR Green PCR kit (Qiagen), initiated with a 15 min incubation at 95 °C, followed by a cycling regime (30 cycles) of 95 °C for 10 s and 65 °C for 2 min. Each run was completed with a melting-curve analysis to confirm the specificity of amplification and the absence of primer dimers. Amplification efficiency was determined for each amplification reaction using linear regression of efficiency (LRE) analysis, and the number of target molecules was calculated using i genomic DNA as a quantitative standard (Rutledge & Stewart, 2008a, b). LRE is a powerful methodology recently developed for modelling real-time qPCR amplification. It provides absolute target amounts without the need to produce standard curves and can generate absolute accuracies of less than ±25 %, whilst displaying single-molecule sensitivity.

To assess the proportion of each rep gene-bearing genome segment within a TrIV DNA extract, the same qPCR approach was applied directly to 0.01 ng TrIV DNA, using one of the primer pairs designed for transcript quantification for each genome segment (see Supplementary Table S1).
To evaluate the accuracy of the measurements made here using LRE analysis, we applied a limiting-dilution assay (LDA; Wang & Spadaro, 1998) approach to three of our samples, and compared the estimates obtained with each method. Briefly, based on values determined by LRE, samples were diluted so that each of 20 replicate aliquots would contain approximately one copy of cDNA or genomic DNA. As dictated by Poisson distribution, a large proportion of aliquots will not contain a target molecule and will fail to produce an amplification profile. The mean number of molecules per aliquot ($N_{\text{mean}}$) can be calculated using the equation:

$$N_{\text{mean}} = -\ln \left( \frac{\text{nil}}{\text{total}} \right)$$

where nil is the number of amplification reactions failing to produce an amplification profile and total is the total number of reactions [see Rutledge & Stewart (2008b) for additional details about LDA]. Multiplication of $N_{\text{mean}}$ by the dilution factor provides the LDA estimate.

Bioinformatics. To explore the possibility that sequences recently deposited in GenBank may be homologous to ichnoviral rep genes, all TrIV rep proteins were submitted to a BLASTP analysis. Alignments of amino acid sequences were performed with CLUSTAL_X (Thompson et al., 1997) using default settings.

RESULTS AND DISCUSSION

Critical assessment of the LRE methodology

To assess the reliability of the qPCR estimates made in this study using the LRE approach, target quantities in three of our samples were determined using both LRE and LDA analysis. The LDA method generated estimates that were congruent with those obtained by LRE analysis, for one DNA and two RNA samples (Table 1), confirming the accuracy of the LRE methodology. In addition, amplification efficiencies were high and uniform across all 17 rep genes, in all treatment groups, and across all ten TrIV genome segments, with maximal amplification efficiencies (see Rutledge & Stewart 2008a, b) of 101.3 $\pm$ 1.7% and 101.5 $\pm$ 1.5% (mean $\pm$ SD) for transcript and genome segment abundance, respectively. Thus, in assessing transcript levels for large multi-gene families such as those found in PDVs, or for measuring the relative abundance of many PDV genome segments, application of the LRE approach to qPCR determinations provides unprecedented accuracy and substantially improves analytical throughput over methods requiring the production of standard curves for each DNA examined.

Table 1. Critical assessment of the accuracy of LRE-based qPCR determinations (Rutledge & Stewart, 2008a, b) by comparison with estimates obtained by application of the LDA method (Wang & Spadaro, 1998).

<table>
<thead>
<tr>
<th>Sample id</th>
<th>LRE value</th>
<th>Dilution factor</th>
<th>Nil</th>
<th>$N_{\text{mean}}$</th>
<th>LDA value</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1.1, 3 days p.p.</td>
<td>40 297</td>
<td>40 000</td>
<td>7</td>
<td>1.05</td>
<td>41 993</td>
</tr>
<tr>
<td>F1.1, 3 days p.i.</td>
<td>137 822</td>
<td>140 000</td>
<td>8</td>
<td>0.92</td>
<td>128 281</td>
</tr>
<tr>
<td>C166 DNA</td>
<td>19 338</td>
<td>20 000</td>
<td>6</td>
<td>1.20</td>
<td>24 079</td>
</tr>
</tbody>
</table>

Transcript abundance in parasitized larvae

TrIV rep genes displayed important differences in gene-specific, time-dependent and tissue-specific levels of transcripts in naturally parasitized last-stadium C. fumiferana larvae. In whole caterpillars, transcripts were detected for almost all genes examined, but transcript abundance was generally low [<550 transcripts (ng total RNA)$^{-1}$] except for F1-1 (~16 000 at 3 days p.p.) and, to a lesser extent, F1-2 (~2200 at 1 day p.; Fig. 1). Whether these differences in transcript abundance among rep genes are indicative of their relative importance in the subjugation of C. fumiferana hosts is not clear, but the strong predominance of F1-1 transcripts suggests that the product of this gene plays a vital role in the success of parasitism.

Levels of rep gene transcripts were not stable during the course of parasitism and tended to decrease by $>$50% between the first (day 1) and last (day 5) sampling points p.p., although three genes (most notably F1-1) displayed higher levels of transcripts at 3 days p.p. than at the other two sampling times (Fig. 1). A temporal pattern of expression similar to that observed here for F1-1 has been reported previously for another TrIV gene, TrVI, in parasitized C. fumiferana larvae (Béliveau et al., 2000). Differences in temporal patterns of expression among viral genes, in a given host, have been observed for other PDVs, including examples where maximal transcript levels were seen several days after oviposition (e.g. Ibrahim et al., 2007). Although such differences suggest that individual PDV gene products may target specific phases of parasitism, the observed transcriptional patterns may be dictated, at least in part, by the stability of the viral genome segments from which transcripts are generated, a variable that could differ considerably according to whether or not the developing wasp larva feeds on infected tissues supporting viral gene expression (Beck et al., 2007).

In our investigation of tissue-specific transcription at 2 days p.p., the overall gene-specific pattern of transcript abundance (Fig. 2) was similar to that observed in whole larvae (Fig. 1), but with some notable exceptions. For example, in the four tissues examined, F1-2 displayed lower proportions of transcripts relative to F1-1 than in whole larvae, whilst the opposite trend was observed for F3-2. This suggests that the tissues supporting high levels of F1-2 transcription were not sampled in the present study.
Fig. 1. Transcript levels of 17 TrIV rep genes in naturally parasitized C. fumiferana sixth-instar larvae, as determined by qRT-PCR using total RNA extracted from whole caterpillars at 1, 3 and 5 days p.p. Larvae were parasitized within 24 h of the moult to the sixth (last) stadium. Actual transcript numbers are provided above each bar for values ≤ 50 transcripts (ng total RNA)^{-1}. Each value presented here is the mean ± SD of four technical replicates carried out on an RNA extract obtained from a pool of three to five parasitized larvae.

Fig. 2. Transcript levels of 17 TrIV rep genes in naturally parasitized sixth-instar larvae, as determined by qRT-PCR using total RNA extracted from four different tissues: FB, fat body; CE, cuticular epithelium; HL, haemocytes; MG, midgut. The larvae were parasitized within 24 h of the moult to the sixth (last) stadium, and RNA was extracted from individual tissues 2 days p.p. Each value is the mean ± SD of four technical replicates carried out on an RNA extract obtained from a pool of three to five parasitized larvae.
whereas some of the sampled tissues were enriched for F3-2 transcripts. More significantly, TrIV rep genes exhibited important differences in their tissue specificity: whereas F1-1 transcripts were most abundant in C. fumiferana cuticular epithelium and the fat body, corroborating previous assessments made by Northern blot analysis (Volkoff et al., 2002), the transcripts of several other genes were at higher levels in haemocytes (B2-2, C7-2 and F3-2) or the midgut (C4-2, D5-2, D6-1 and F1-2) than in the other three tissues (Fig. 2). These results are in contrast to those obtained by Galibert et al. (2006), who found that the fat body and cuticular epithelium of parasitized Spodoptera littoralis hosts had the highest levels of HdIV rep transcripts for all ten rep ORFs examined, followed by nervous tissue, which was not investigated in the present study. It remains to be seen whether the observed trend in HdIV rep gene expression was influenced by the choice of rep ORFs that were studied, as we now know that the HdIV genome contains many additional rep genes (A. N. Volkoff, personal communication). Thus, this apparent difference between the two biological systems could be due to a gene-sampling bias.

Tissue-specific differences in PDV gene transcript abundance in parasitized hosts have also been observed for ichnovirus ank genes (Kroemer & Webb, 2005) and bracovirus PTP genes (Gundersen-Rindal & Pedroni, 2006; Provost et al., 2004). Such tissue-specific expression suggests that the diversity of genes within a given PDV gene family may be associated with the existence of tissue-specific roles for different family members in the caterpillar hosts, or that some of these related gene products, whilst having the same function, are more effective in one tissue than in another. Irrespective of its functional significance, tissue-specific variation in transcript levels implies that there exist tissue-specific host factors modulating the transcription of specific rep genes.

Transcript abundance in CF-injected larvae

The TrIV rep transcript levels observed in CF-injected larvae (Fig. 3) displayed gene-specific and time-dependent differences similar to those observed for parasitized whole larvae (Fig. 1), with the exception that absolute transcript levels were generally much higher than those observed at equivalent sampling times in parasitized larvae, particularly 1 day after treatment (>85 times higher in the case of F1-1), indicating that the virus dose contained in 0.5 FE of CF is much higher than that injected by a female wasp during natural parasitization. As a point of comparison, the dose of virus injected by the wasp Microplitis demolitor into its

![Fig. 3. Transcript levels of 17 TrIV rep genes in sixth-instar larvae injected with 0.5 FE of T. rostrate calyx fluid, as determined by qRT-PCR using total RNA extracted from whole caterpillars at 1, 3 and 5 days p.i. Larvae were injected within 24 h of the moult to the sixth (last) stadium. Actual transcript numbers are provided above each bar for values <2000 transcripts (ng total RNA)^-1. Each value is the mean ± SD of four technical replicates carried out on an RNA extract obtained from a pool of three to five injected larvae.](http://vir.sgmjournals.org)
host has been estimated to be between 0.04 and 0.005 FE of CF per ovipositional event (Beck et al., 2007).

Another difference between patterns found for parasitized and CF-injected larvae was the rise seen in F1-1 transcript abundance on day 3 p.p., an increase that was not observed in injected caterpillars, although absolute levels of F1-1 transcripts were higher in the latter than in the former group, at all three sampling points. With few exceptions, transcript levels decreased substantially from day 1 to days 3 and 5 p.i., suggesting that the unusually high inoculum injected in larvae may have triggered faster clearance or breakdown of some viral DNA in the host than in parasitized caterpillars. The present qPCR findings for F1-1 (TrF
rep
1) are in agreement with an earlier assessment made by Northern blot analysis, which showed F1-1 to be transcribed at much higher levels in CF-injected larvae than in parasitized caterpillars (Volkoff et al., 2002).

Transcript abundance in wasp ovary and head–thorax complexes

The pattern of TrIV rep gene transcription in T. rostrale ovaries was markedly different from that seen in naturally parasitized or CF-injected C. fumiferana larvae. Whereas F1-1 and F1-2 were the most highly expressed rep genes in infected caterpillars (Figs 1, 2 and 3), transcripts generated from these two genes displayed low abundance in wasp ovaries compared with other genes such as C166-1, the transcript levels of which were by far the highest (Fig. 4). Interestingly, the C3-1 gene, whose transcription was barely detectable in infected caterpillars (Figs 1, 2 and 3), was the second most highly transcribed gene in wasp ovaries. In addition, the transcript levels of C3-2, C7-2, D5-2 and F3-2, which were modest in infected C. fumiferana larvae (Figs 1, 2 and 3), varied between ~5000 and ~10 000 transcripts (ng total RNA)⁻¹ in wasp ovaries (Fig. 4). In comparison, all TrIV rep genes had undetectable or very low transcript levels in wasp head–thorax complexes (Fig. 4).

Using Northern blot analysis, Theilmann & Summers (1988) provided the first report on the transcription of Campoletis sonorensis ichnovirus (CsIV) rep genes in Cam. sonorensis female reproductive tissues. These authors observed that some rep genes were transcribed exclusively in the parasitized host, whilst others produced transcripts only in wasp ovaries or in both hosts. The quantitative transcriptional data provided here for 17 TrIV rep genes in both parasitized hosts and wasp ovaries are in agreement with this earlier finding. The distinct transcriptional patterns of rep genes in T. rostrale ovaries (Fig. 4) and parasitized or CF-injected larvae (Figs 1, 2 and 3) suggest that individual rep genes may play either wasp- or caterpillar-specific roles. In contrast, HdIV rep1 was the most highly expressed rep gene in both infected caterpillar hosts and wasp ovaries (Galibert et al., 2006), suggesting that the host-specific expression reported here may not apply to all ichnoviruses. With respect to TrIV,

![Fig. 4. Transcript levels of 17 TrIV rep genes in T. rostrale ovaries and head–thorax complexes, as determined by qRT-PCR. Total RNA was extracted from five ovary pairs dissected from post-emergence 5–10-day-old T. rostrale females and from the head–thorax complexes of the same females. Each value is the mean ± SD of four technical replicates carried out on each RNA extract. Actual transcript numbers are provided above each bar for values <500 transcripts (ng total RNA)⁻¹.](image-url)
observation that some rep genes may be expressed only in the wasp (e.g. C3-1) raises the questions as to (i) why such genes are found in a packaged virus meant to be delivered to the lepidopteran host and (ii) whether there are additional, unpackaged rep genes in the T. rostrale genome, expressed only in wasp ovaries. Of course, the possibility exists that some of the TrIV rep genes that were found to be weakly expressed in parasitized C. fumiferana larvae would be expressed strongly in other lepidopteran hosts (e.g. Choristoneura rosaceana; Cusson et al., 1998) or tissues not sampled yet, including genes that were found here to be expressed only in the ovary. Additionally, it is not quite clear whether rep genes that are expressed in the wasp ovary are transcribed from episomal or chromosomal DNA, or both. Interestingly, none of the rep genes that were found to be expressed in T. rostrale ovaries was transcribed at significant levels in the other wasp tissues examined (Fig. 4), thus suggesting an ovary-specific role for those that are transcribed in that tissue. Given that rep gene products are not predicted to be secreted, rep proteins expressed in wasp ovaries are not expected to be released in the lumen of the oviduct for subsequent injection into the caterpillar during parasitization. For this reason, their expression in the ovary suggests that they could play a role in virus replication, a hypothesis that could be tested by following developmental changes in ovarian rep transcript abundance in pupae, the stage at which virus replication begins (Marti et al., 2003; Webb & Summers, 1992).

**Gene dosage**

In earlier work examining the relationship between the abundance of PDV gene transcripts and the proportion of the genome segments bearing these genes within the packaged viral genome, no clear correlation between the two variables was observed (Beck et al., 2007; Galibert et al., 2006). Here, the three most highly expressed TrIV rep genes in parasitized caterpillars, F1-1, F1-2 and C166 (Fig. 1), were found to be borne by the two most abundant TrIV genome segments (Fig. 5), suggesting that gene dosage, in this particular instance, may have some impact on transcript abundance. Yet, when all TrIV rep genes were considered, we observed no significant correlation between transcript levels and the proportion of the originating genome segments. Clearly, factors other than, or in addition to, gene dosage affect transcript levels, including possible differences in promoter strength, the presence or absence of host factors that may affect the transcription of individual rep genes and/or differences in mRNA stability. For example, there were important differences in the abundance of F1-1 and F1-2 transcripts, which are generated from genes present on the same genome segment. Integration of genome segment F1 into the lepidopteran host genomic DNA could also be a factor resulting in the enhancement of F1-1 and F1-2 transcription. Although the integration of genome segment F1 has not been demonstrated in the parasitized host, it clearly occurs in infected C. fumiferana CF-124T cells in culture (Doucet et al., 2007). Such an integration event would permit sustained expression of the integrated genes when titres of episomal DNA decrease. The question of whether other rep-containing genome segments undergo integration into C. fumiferana genomic DNA remains to be examined.

**Comparison of TrIV rep proteins and identification of non-PDV rep homologues**

A **CLUSTAL_X** alignment of all 17 deduced TrIV rep proteins revealed regions that are well conserved across all members of this family, including five cysteine residues that are present in all proteins except C7-2; the latter lacks the second and third cysteines, and its N terminus is substantially truncated relative to the other TrIV rep proteins (Fig. 6). The most conserved region is observed in the vicinity of the fifth cysteine residue (Fig. 6), comprising a segment of ~18 aa that also appears well conserved among rep proteins from other ichnoviruses, including *Hyposoter fugitivus* ichnovirus (HfIV) and HdIV (Fig. 7). A **BLASTP** search using all TrIV rep proteins as query sequences revealed the existence of two putative rep homologues in *Helicoverpa armigera* granulovirus (HearGV), the genome of which has recently been sequenced and annotated (Harrison & Popham, 2008). One of these two proteins, hear76, has only 70 aa and displays a modest level of similarity to ichnoviral rep proteins (e.g. **BLASTP** expect value of 0.36 for similarity to TrIV F3-1); however, the other predicted protein, hear75, has 171 aa, contains four of the five conserved cysteine residues referred to above and shows significant similarity to many ichnoviral rep proteins, most notably within the aforementioned highly conserved region (Fig. 7). **BLASTP** expect values for similarity between hear75 and ichnoviral rep proteins varied between 6 \( \times \) 10\(^{-09} \) and 3 \( \times \) 10\(^{-05} \) for
HfIV D3-2 and TrIV F3-1, respectively. No rep homologues have been detected in the other baculovirus genomes sequenced to date; thus, their presence in HearGV may well be the result of lateral gene transfer from an ichnovirus genome (Harrison & Popham, 2008).

As observed in earlier analyses of rep proteins, no conserved domains were detected in any of the 17 TrIV representatives of this family, with the exception of F1-2, in which a PIWI-like domain was detected in the region between residues 60 and 150, although with a low (0.001) expect value. The same protein was also found to display a modest level of similarity to a bacterial transposase (NCBI protein database accession no. ABM04822) within its C terminus, an interesting observation given that TrIV genome segment F1 has been shown to integrate spontaneously into the genome of C. fumiferana cells in culture, through an unidentified mechanism (Doucet et al., 2007).

Although the new bioinformatics analyses performed here provided few new insights into the function(s) of rep genes, the presence of rep homologues in the recently sequenced genome of a granulovirus could eventually provide an indirect means of assessing their role through the

**Fig. 6.** CLUSTAL_X alignment of all known and predicted TrIV rep family proteins. Arrows indicate the positions of conserved cysteine residues. Asterisks (*), double dots (••) and single dots (•) above letters in the alignments denote identical residues, and conserved and semi-conserved substitutions, respectively. NCBI protein database accession nos: C7-1, BAF45598; C7-2, BAF45599; D5-1, BAF45610; D5-2, BAF45611; C289-1, BAF45769; C3-2, BAF45588; F3-2, BAF45622; F3-3, BAF45627; F3-1, BAF45769; F1-1, BAF45589; C166-1, BAF45590; B2-2, BAF45626; B2-1, BAF45627; C3-1, BAF45588; D6-1, BAF45614; F1-1, AAN32723; F1-2, ACJ72220; C166-1, BAF45767.
production of a HearGV rep knockout, followed by an assessment of the effect of this genetic alteration on viral replication or other aspects of the infection cycle. Deployment of this strategy, however, would require the prior development of an efficient in vitro system for HearGV.

In summary, the present study suggests that the very high level of diversification seen within the ichnoviral rep gene family may have evolved in response to the necessity to fine-tune the function(s) and/or effectiveness of rep proteins for expression in different hosts and tissues. Given that rep genes encode proteins that are not secreted and that some of them are expressed at relatively high levels in wasp ovaries without any overt pathological consequence, the possibility exists that their function has more to do with cell homeostasis (in ichnovirus- or granulovirus-infected lepidopteran cells or in ovarian wasp cells supporting virus replication) than virulence. Some PDV-encoded proteins are secreted and display deleterious effects on other cells (e.g. Béliveau et al., 2003); because PDVs do not replicate in the lepidopteran host, sustained viral gene expression for the duration of immature parasitoid development is predicted to require a mechanism preventing infected cells from being negatively affected by secreted PDV proteins and/or suppressing breakdown of viral DNA and transcripts by host cells. Some CsIV ank gene products appear to have such a function, given that they have been shown to delay lysis of baculovirus-infected cells (Fath-Goodin et al., 2006). We are currently examining the effect of TrIV rep gene expression on C. fumiferana host-cell gene expression, with the aim of identifying the pathway(s) targeted by rep proteins.

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