Characterization of two genes of the polydnavirus of *Chelonus inanitus* and their stage-specific expression in the host *Spodoptera littoralis*

Andrea Johner and Beatrice Lanzrein

Institute of Cell Biology, University of Berne, Baltzerstrasse 4, CH-3012 Bern, Switzerland

*Chelonus inanitus* (Braconidae, Hymenoptera) is a solitary egg-larval parasitoid of *Spodoptera littoralis*. Along with the egg the female wasp injects polydnaviruses, which are prerequisites for successful parasitoid development. The polydnavirus genome is segmented and consists of double-stranded circular DNA. Proviral DNA is integrated in the wasp’s genome; virus replication is restricted to the wasp’s ovary and does not occur in the parasitized host. The polydnavirus of *C. inanitus* (CiV) protects the parasitoid larva from encapsulation by the host’s immune system and causes a developmental arrest of the host in the prepupal stage. Here we report on the first two cloned CiV genes, which are named CiV14g1 and CiV14g2 because of their localization on segment CiV14. The cDNA of CiV14g1 has a size of 2036 bp; the gene contains seven exons interrupted by six introns of similar size and encodes a putative polypeptide of 548 amino acids. The cDNA of CiV14g2 has a size of 618 bp; the gene consists of three exons and encodes a putative peptide of 77 amino acids. Transcript quantities of both genes are very low up to the penultimate larval instar of the host. In the last instar, at the stage of pupal cell formation, CiV14g1 expression increases about 5-fold and CiV14g2 expression about a 1000-fold. These are the first data to show strong upregulation of polydnavirus genes towards the end of parasitization. These two genes might be involved in the reduction of host ecdysteroids observed at this stage.

Introduction

Endoparasitic wasps of the families Ichneumonidae and Braconidae develop inside other insects. For successful parasitoid parasitoids have to avoid or suppress the immune response of the host (reviewed in Schmidt *et al.*, 2001) and many also manipulate the development of the host (reviewed in Lawrence & Lanzrein, 1993). For both effects polydnaviruses, which are injected into the host at oviposition, play an important role. The polydnavirus genome is segmented and consists of double-stranded circular DNA; proviral DNA is integrated into the wasp genome and transmitted vertically through the germ line. Viral DNA replication and virion formation take place in special cells of the ovary, the calyx cells; in the parasitized host viral DNA does not replicate (reviewed in Stoltz *et al.*, 1995; Webb, 1998). Polydnaviral gene expression was found in the wasp as well as in the parasitized host larvae whereby the majority of characterized genes are transcribed shortly after parasitization and appear to be associated with suppression of the host’s immune system (reviewed in Webb, 1998).

We are working with the polydnavirus of the braconid wasp *Chelonus inanitus* (CiV). *C. inanitus* is a solitary egg-larval parasitoid of *Spodoptera littoralis*. The egg stage is believed not to be immune competent (Salt, 1968) and thus the immediate early protection of the parasitoid egg at the host’s egg stage may not be important. Later on, however, once the host has become a larva, inhibition of encapsulation of the parasitoid larva by polydnaviruses appears to be essential (Pfister-Wilhelm & Lanzrein, 1996; Stettler *et al.*, 1998). Parasitization by *C. inanitus* has a great influence on the development of *S. littoralis* as metamorphosis is induced precociously in the fifth instar followed by a developmental arrest in the prepupal stage (Grossniklaus-Bürgin *et al.*, 1994). The parasitoid larva in the presence of polydnavirus/venom has been shown to be
responsible for the precocious onset of metamorphosis and to cause a premature decline in juvenile hormone (Pfister-Wilhelm & Lanzrein, 1996; Steiner et al., 1999). Polydnaviruses, synergized by venom, were shown to be responsible for the developmental arrest in the prepupal stage (Soller & Lanzrein, 1996) and to cause an inhibition of the prothoracic gland and a reduction in haemolymph ecdysteroids at the stage of pupal cell formation (Grossniklaus-Bürgin et al., 1998). The CiV genome is composed of at least 10 different DNA segments with sizes between 7 and 31 kbp (Albrecht et al., 1994). Four segments (CiV12, CiV14, CiV14.5 and CiV16.8) have been sequenced completely and for CiV12 and CiV14 the integration/excision site in the wasp genome has been characterized (Gruber et al., 1996; Wyder et al., 2002). Northern dot blots with total CiV DNA or segments CiV12, CiV14 and CiV16.8 as probe revealed the presence of viral transcripts throughout the time-course of parasitization; but levels were very low and mainly increased in the last larval instar, in particular in the case of CiV12 and CiV14 (Johner et al., 1999).

Here we present data for the first CiV genes to be cloned and characterized. As both are situated on segment CiV14 they are named CiV14g1 and CiV14g2. The cDNA of CiV14g1 has a size of 2036 bp and encodes a predicted protein of 548 amino acids; the cDNA of CiV14g2 has a size of 618 bp and encodes a predicted protein of 77 amino acids. Transcript quantities of both genes are low up to the penultimate instar and increase in the host’s last instar at the stage of pupal cell formation.

Methods

- **Insects and X-ray irradiation.** C. inanitus (Braconidae, Hymenoptera) is a solitary egg-larval parasitoid and was reared on its natural host S. littoralis (Noctuidae, Lepidoptera). Details of the biology and rearing of the parasitoid and its host are described by Grossniklaus-Bürgin et al. (1994). To study the effect of polydnavirus/venom in the absence of a developing parasitoid larva, eggs were parasitized with X-ray-irradiated C. inanitus females as described (Soller & Lanzrein, 1996). Larvae developing from eggs parasitized with X-ray-irradiated wasps are designated ‘X-ray-parasitized’. Nonparasitized and X-ray-parasitized larvae pass through six larval instars but the latter become developmentally arrested in the prepupal stage, as do larvae developing from eggs injected with calyx fluid/venom (Soller & Lanzrein, 1996). The last instar of X-ray-parasitized larvae was subdivided into feeding early, feeding late, cell formation early and cell formation late as described by Grossniklaus-Bürgin et al. (1998). Parasitized larvae enter metamorphosis precociously in the fifth instar and the parasitoid larva emerges from the precocious prepupa and consumes the host remains (Grossniklaus-Bürgin et al., 1994).

- **Total RNA isolation.** RNA from first to fourth instar S. littoralis was isolated as described by Johner et al. (1999), but with minor modifications. The purification of the homogenate by the QIAshredder was replaced by a digestion with proteinase K. Briefly, DEPC H2O and proteinase K (10 mg/ml) were added to the homogenate in the ratio of 1:65:0.35:1 (by vol.), incubated for 1.5 h at 45 °C and centrifuged for 10 min at 11000 g. The supernatant was then mixed with an equal volume of lysis buffer RLT (Qiagen) and an equal volume of ice-cold ethanol and applied onto RNaseasy mini spin columns (Qiagen). For isolation of RNA from fifth and sixth instar larvae the RNeasy Midi Kit (Qiagen) was used. Larvae were homogenized with a Polytron PT10-35/PTA 10s (Kinematica) in lysis buffer RLT (0.45 ml per 100 mg tissue) containing 1% (v/v) β-mercaptoethanol. The proteinase K digestion of the homogenates was done as described above and the subsequent steps were performed according to the RNeasy Midi Kit protocol, including the DNase digestion on the column. After elution a second DNA digestion was carried out as described by Johner et al. (1999).

- **cDNA library construction.** RNA from either 10-day-old feeding fifth instar parasitized larvae (= L5p library) or X-ray-parasitized 12-day-old sixth instar larvae in the early pupal cell formation stage (= XL6 library) was isolated as described above. For poly(A)+ mRNA isolation the PolyATtract mRNA Isolation System (Promega) protocol for small scale mRNA isolation was used. cDNA libraries were constructed following the SMART PCR cDNA Library Construction Kit (Clontech) protocol. cDNAs were ligated into vector λgt11 and packaged into E. coli Y1090– with the Gigapack III Gold Packaging Extract (Stratagene), following the manufacturer’s instructions. The unamplified L5p library contained 1·35×10⁶ clones with a recombination efficiency of 44% and the unamplified XL6 library contained 0·62×10⁶ clones with a recombination efficiency of 80%. The two libraries were then amplified according to the Clontech protocol and yielded titres of 5·5×10⁸ p.f.u./ml (L5p) and 3·9×10⁹ p.f.u./ml (XL6).

- **Screening probes.** On the basis of ORF predictions on CiV14 (Wisconsin Package Version 10.1, Genetic Computer Group) two pairs of primers were designed, namely CiV14U3A/CiV14L3A and CiV14U4A/CiV14L4A, resulting in products of 193 bp and 157 bp, respectively (Fig. 1a and accession no. AJ276877). The 5000 bp HindIII fragment of CiV14 was purified by gel extraction (QIAEX II Gel Extraction Kit, Qiagen) and used as a template. The PCRs were carried out in a volume of 50 µl with 10⁵ molecules of template, 0·3 µM of each primer, 2·6 U Expand High Fidelity Enzyme-Mix and 1 × PCR DIG Probe Synthesis Mix (containing 70 µM DIG-11-dUTP). The cycling conditions were: 5 min at 95 °C; 35 cycles of 95 °C, 1 min; 54 °C, 1 min; 68 °C, 3 min; and a final elongation step at 68 °C for 10 min. PCR products were analysed on a 1% agarose gel and the amount of DNA containing DIG-11-dUTP was determined by comparison with a DIG-labelled control DNA (Roche). In addition DIG-High Prime- (Boehringer Mannheim) labelled CiV DNA was used as a probe (Johner et al., 1999). Subsequently, the following screening probes were made: a CiV14g1-specific probe with primers CiV14g1U2/CiV14g1L2 (Fig. 1a and accession no. AJ276877) with a length of 160 bp and two CiV14g2-specific probes with primers CiV14g2U2/CiV14g2L2 and CiV14g2U3/CiV14g2L3 (Fig. 1b and accession no. AJ276877) with a length of 252 bp and 313 bp, respectively.

- **Library screening.** For the first round of screening 0·5×10⁶ phages per library were incubated with 0·5 ml E. coli Y1090r– overnight and plated onto LB plates. Colonies were transferred to positively charged nylon membranes (Roche). Subsequent denaturation, neutralization and washing were according to Sambrook et al. (1989). The DNA was covalently cross-linked to the nylon membrane with a UV Stratalinker 2400 (Stratagene; 254 nm, 160 000 M/cm²) and membranes were prehybridized and hybridized as described by Johner et al. (1999). Detection of positive clones was by a catalysed colour reaction with NBT/BCIP (Roche). In addition DIG-probes (Boehringer Mannheim) labelled CiV DNA was used as a probe (Johner et al., 1999). Subsequently, the following screening probes were made: a CiV14g1-specific probe with primers CiV14g1U2/CiV14g1L2 (Fig. 1a and accession no. AJ276877) with a length of 160 bp and two CiV14g2-specific probes with primers CiV14g2U2/CiV14g2L2 and CiV14g2U3/CiV14g2L3 (Fig. 1b and accession no. AJ276877) with a length of 252 bp and 313 bp, respectively.
cDNA. As no NotI restriction site was detected in the inserts by analysis of the digested DNA on a 0.8% agarose gel, cDNA inserts were finally cut-out with a NotI digestion, purified by gel extraction (QIAEX II Gel Extraction Kit, Qiagen) and cloned into pBluescript II KS (+) vectors. The insert of clone Å6/7.3 was amplified by PCR using Åqt11 forward and reverse primers (Galas et al., 1980) with the Taq PCR core kit (Qiagen) in a volume of 50 µl, consisting of 12.5 pmol of each primer, 200 µM of each dNTP, 2.5 U Taq polymerase and the DNA template (1 µl of phage lysate). Cycling was as follows: 95 °C, 6 min; 30 cycles of 95 °C, 1 min; 48 °C, 1 min; 74 °C, 3 min; and a final extension step at 74 °C for 10 min. After analysing the product on a 1.2% agarose gel the DNA fragment with a size of about 800 bp was cloned into the vector pCR 2.1-TOPO according to the TOPO TA Cloning Kit (Invitrogen) protocol. Plasmid DNA was isolated using Wizard Plus SV Miniprep (Promega) as specified by the manufacturer, followed by ethanol precipitation. Sequencing was done with the Thermo Sequenase Sequencing Kit (Amersham) with IRD800 labelled primers. PCR conditions were: 94 °C, 5 min; 30 cycles of 94 °C, 30 sec; 55 °C, 45 sec; 68 °C, 2 min; and a final elongation step at 68 °C for 10 min. Automatic sequencing was carried out on a Gene ReadIR 4200 (Licor).

**Northern blot analysis.** Total RNA was denatured in 50% (v/v) DMSO, 4% (v/v) deionized glyoxal and 10 mM sodium phosphate, pH 8.5, for 20 min at 50 °C and separated on an agarose gel (1.2 g agarose/100 ml 10 mM sodium phosphate). As a marker, RNA Molecular Mass Marker I (0.3–6.9 kb) (Roche) was used. Transfer to positively charged nylon membranes (Roche) was by capillary forces in 20 x SSC (1 x SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) as described by Sambrook et al. (1989). UV-cross-linking was as described above and membranes were prehybridized for at least 5 h in 6 x SSC, 5 x Denhardt's solution (1 x Denhardt's solution = 0.2 g/l Ficoll 400, 0.2 g/l polyvinylpyrrolidone, 0.2 g/l BSA), 1 g/l SDS and 100 µg/ml denatured herring sperm DNA at 60 °C. The blots were hybridized at 60 °C for 2 days in the same buffer with an [α-32P]dCTP-labelled DNA probe (approximately 106 c.p.m./ml hybridization solution). Labelling was done with the Ready To Go Kit according to the manufacturer (Amersham) whereby 15–50 ng DNA served as template. Finally, the blots were washed 3 x 20 min in 1 x SSC containing 0.1 g/l SDS at 60 °C, exposed to a PhosphorScreen and analysed on a PhosphorImager (Molecular Dynamics).

**Southern blot analysis.** Calyx fluid collection from female reproductive tracts of C. ananassus and DNA isolation was as described (Albrecht et al., 1994). Undigested and HindIII-digested calyx DNA and HindIII-digested done 2A, which contains the entire CiV14 segment, were separated on a 0.8% agarose gel by field inversion gel electrophoresis (FIGE). Electrophoresis as well as blotting, probe labelling and high stringency washes were as described by Wyder et al. (2002).

**3' RACE (rapid amplification of cDNA ends).** To identify the 3′ end of CiV14g1, a 3'RACE was done. Poly(A)+ mRNA was isolated from X-ray-parasitized sixth instar S. littoralis larvae in the early cell formation stage and reverse transcribed with the Superscript First-Strand Synthesis System for RT–PCR (Life Technologies), with the supplied oligo(dT) primer replaced by a modified primer (5′ GACTCGAGTCGACATCG 3′) and DNA isolation was as described previously. The cDNA was precipitated with isopropanol and the dried DNA pellet was dissolved in 10 mM Tris–HCl, pH 8.0. The PCR reaction was performed with the Taq PCR Core Kit (Qiagen) in presence of 50 pmol primer (5′ GACTCGAGTCGACATCG 3′) and 50 pmol of the CiV14g1-specific primer CiV14g1.R (Fig. 1a and accession no. AJ278677). Cycling conditions were: 94 °C, 5 min; 40 cycles of 94 °C, 1 min; 65 °C, 1 min; 74 °C, 2 min; and final elongation at 74 °C for 10 min. This led to a product of about 650 bp which was then gel extracted (QIAEX II Gel Extraction Kit, Qiagen) and cloned into the vector pCR 2.1-TOPO as described above.

**Real-time PCR.** Total RNAs from parasitized, X-ray-parasitized or nonparasitized S. littoralis were reverse transcribed with the Superscript First-Strand Synthesis System for RT–PCR (Life Technologies), with
Fig. 2. For legend see facing page.
1–5 µg template was used with oligo(dT) as primer. For the subsequent real-time PCR primers were designed with Primer Express prerelease version 1.0 (PE Applied Biosystems). The primer pairs for CiV14g1 and CiV14g2 were CiV14g1U1/CiV14g1L1 and CiV14g2U1/CiV14g2L1 (Fig. 1a, b and accession no. AJ278677); both produce an amplicon of 51 bp. As an endogenous standard a primer pair specific for S. littoralis β-actin, namely actin forward and actin reverse (accession no. Z46873), was used. The PCR was performed with the SYBR Green I Reaction System (Eurogentec); briefly, an appropriate amount of cDNA was amplified in a volume of 30 µl containing 200 µM each dNTP, 3.5 mM MgCl₂, 0.75 U Hot GoldStar enzyme, 200 nM of each primer and 0.9 µl of a 1/2000 dilution of Sybr Green I stock. Cycling conditions were: 95 °C, 10 min; followed by 40 cycles of 95 °C, 15 s; and 60 °C, 1 min. Measurement was done in 96-well Optical Reaction Plates (Applied Biosystems) with the GeneAmp 5700 Sequence Detection System (PE Applied Biosystems). The quality of primers was tested by analysis of the melting curves and by analysis of the PCR products on a 3 % MetaPhor agarose gel.

Results
Screening of host cDNA libraries with viral probes and analysis of positive clones

Having found that CiV, and in particular CiV14, transcripts increase in the last larval instar (Johner et al., 1999) cDNA libraries were made with RNA from parasitized larvae in the fifth larval instar during the feeding stage (= L5p library) and with RNA from X-ray-parasitized larvae in the sixth instar during the early pupal cell formation stage (= XL6 library). They were screened with either total CiV DNA or CiV14-specific probes. With the two CiV14-specific PCR probes (U3AL3A, U4AL4A; Fig. 1a) two clones (λ6/4.1, λ6/5.1) could be identified in the XL6 library. Sequencing revealed that both clones contained the same cDNA insert, length 1555 bp, which was named CiV14g1. Due to the lack of a stop codon and a poly(A) tail and the fact that the size was much less than indicated by Northern blots (see Fig. 4a), both cDNAs appeared to be incomplete. Thus a 3’ RACE analysis was made, which yielded the missing sequence information. Apparently, the formation of incomplete cDNAs was due to a short poly(A) stretch within the gene. The cDNA of CiV14g1 and the genomic DNA of CiV14 are shown in Fig. 1(a); CiV14g1 has a size of 2036 bp and is composed of seven exons and six introns, the latter having sizes between 73 and 88 bp. A putative TATA box is located 99 nucleotides upstream of the 5’ untranslated region (UTR). In the 3’UTR two potential unconventional polyadenylation signals (Beaudoin et al., 2000) were seen. One clone, λ6/6.5, was identified from the XL6 library using total viral DNA as a probe. After sequencing it could be localized on CiV14 and was named CiV14g2. With CiV14g2-specific PCR probes (Fig. 1b) a second clone, λ6/7.3, was identified in the XL6 library and was seen to be 92 nucleotides longer in the 5’UTR than λ6/6.5. Fig. 1(b) shows that CiV14g2 cDNA has a size of 618 bp and is composed of three exons and two introns, the latter having lengths of 302 and 1536 nucleotides, respectively. Putative TATA and a CAAT box are present 57 and 74 nucleotides upstream of the 5’UTR. In the 3’UTR a potential polyadenylation signal was seen.

The nucleotide sequences together with the predicted amino acid sequences of CiV14g1 and CiV14g2 are shown in Fig. 2. The open reading frame (ORF) of CiV14g1 comprises 548 amino acids encoding a protein with a predicted molecular mass of 63.5 kDa (Fig. 2a). A potential O-glycosylation site at position 402 and a potential N-glycosylation site in the N-terminal domain of the deduced protein are predicted by NetOGlyc 2.0 (Hansen et al., 1998) and Prosite (Hofmann et al., 1999). The predicted protein seems to have no N-terminal signal peptide and is proposed to be localized in the nucleus (PSORT; Nakai & Horton, 1999). Database searches using BLAST (Altschul et al., 1997) revealed some similarity to sequences of Chironomus pallidivittus and Chironomus tentans (accession numbers AJ311054–AJ311057) which are proposed to act as recombinases (M. Rosén, personal communication). The sequences matched over a length of 339 amino acids with 23% identity and 43% similarity. The search for conserved domains using Blocks (Henikoff & Henikoff, 1994) indicated significant similarity to a transposase of the mutator family, the sequence being situated inside the above-mentioned region.

The ORF of CiV14g2 consists of 77 amino acids and encodes a predicted protein of 8.5 kDa with one potential N-glycosylation site (Fig. 2b). Neither N-terminal signal peptides nor transmembrane helices were detected and the protein is suggested to have a nuclear localization (PSORT). No significant sequence similarity to known proteins or nucleotides was found by BLAST searches. However, CiV14 has significant similarity to CiV12 over large stretches (Wyder et al., 2002) and we recently cloned a gene on CiV12 which is 83% identical to CiV14g2 (D. Kojic, unpublished).

A comparison of CiV14g1 and CiV14g2 cDNAs with genes predicted on the genomic sequence using Fgenes (Solovyev & Salamov, 1999) with either Drosophila or Human settings is shown in Fig. 3. In the case of CiV14g1, 100% of the coding nucleotides were predicted with Human settings compared to 56% with Drosophila settings. Human settings gave 21% of false positives as compared to 7% with Drosophila settings. CiV14g2 was not predicted with Human settings, but with Drosophila 56% of the coding nucleotides were correctly predicted.
A. Johner and B. Lanzrein

Fig. 3. Comparison of predicted genes with the identified cDNAs. Numbers indicate the start and end of exons on the genomic sequence. Correctly predicted nucleotides are underlined. Percentage values designate correctly predicted nucleotides of the corresponding ORF.

<table>
<thead>
<tr>
<th>exon nr.</th>
<th>location</th>
<th>length</th>
<th>Prediction &quot;Drosophila&quot;</th>
<th>exon nr.</th>
<th>location</th>
<th>length</th>
<th>Prediction &quot;Human&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIV14g1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>10328-10671</td>
<td>344</td>
<td>I</td>
<td>10113-10183</td>
<td>71</td>
<td>I</td>
<td>9873-10183</td>
</tr>
<tr>
<td>II</td>
<td>10755-10817</td>
<td>63</td>
<td>II</td>
<td>10413-10671</td>
<td>259</td>
<td>II</td>
<td>10413-10671</td>
</tr>
<tr>
<td>III</td>
<td>10891-11094</td>
<td>204</td>
<td>III</td>
<td>10755-10817</td>
<td>63</td>
<td>III</td>
<td>10755-10817</td>
</tr>
<tr>
<td>IV</td>
<td>11172-11505</td>
<td>334</td>
<td>IV</td>
<td>10891-11094</td>
<td>204</td>
<td>IV</td>
<td>10891-11094</td>
</tr>
<tr>
<td>V</td>
<td>11582-11726</td>
<td>145</td>
<td>V</td>
<td>11379-11505</td>
<td>127</td>
<td>V</td>
<td>11372-11505</td>
</tr>
<tr>
<td>VI</td>
<td>11809-11891</td>
<td>83</td>
<td>VI</td>
<td>11582-11726</td>
<td>145</td>
<td>VI</td>
<td>11582-11726</td>
</tr>
<tr>
<td>VII</td>
<td>11980-12824</td>
<td>863</td>
<td>VII</td>
<td>11809-11891</td>
<td>83</td>
<td>VII</td>
<td>11809-11891</td>
</tr>
<tr>
<td>ORF</td>
<td>10456-12581</td>
<td></td>
<td>ORF</td>
<td>10456-12581</td>
<td></td>
<td>ORF</td>
<td>100 %</td>
</tr>
<tr>
<td>CIV14g2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5607-5737</td>
<td>121</td>
<td>I</td>
<td>5726-5737</td>
<td>12</td>
<td>I</td>
<td>5726-5737</td>
</tr>
<tr>
<td>II</td>
<td>6040-6159</td>
<td>120</td>
<td>II</td>
<td>6040-6169</td>
<td>120</td>
<td>II</td>
<td>6040-6169</td>
</tr>
<tr>
<td>III</td>
<td>7696-8062</td>
<td>367</td>
<td>III</td>
<td>7042-7190</td>
<td>149</td>
<td>III</td>
<td>7042-7190</td>
</tr>
<tr>
<td>IV</td>
<td>8792-8801</td>
<td>10</td>
<td>IV</td>
<td>8792-8801</td>
<td>10</td>
<td>IV</td>
<td>8792-8801</td>
</tr>
<tr>
<td>ORF</td>
<td>5726-7797</td>
<td></td>
<td>ORF</td>
<td>5726-7797</td>
<td></td>
<td>ORF</td>
<td>56 %</td>
</tr>
</tbody>
</table>

Fig. 4. (a) Hybridization of radiolabelled CiV14g1 cDNA to total RNA (20 µg) of parasitized fifth instar feeding larvae (= L5p), X-ray-parasitized sixth instar larva in the early cell formation stage (= L6 X-ray-p) and nonparasitized sixth instar feeding larvae (= L6 np) of S. littoralis. (b) As a control the blot was stripped and rehybridized with radiolabelled S. littoralis β-actin DNA. RNA size marker is shown on the left. Exposure time was 4 days.

predicted. Fgenesh also predicted a third gene on CiV14, but its existence is uncertain as real-time PCRs with parasitized and X-ray-parasitized larvae were inconclusive (data not shown). It remains possible that this gene exists but is either transcribed at a very low level in parasitized and X-ray-parasitized larvae or only in the wasp.

**Northern blot analysis**

On a Northern blot, CiV14g1 cDNA hybridized to total RNA of X-ray-parasitized sixth instar larvae in the early cell formation stage and to a lesser extent to parasitized fifth instar larvae in the feeding stage (Fig. 4a). The size of the mRNA is approximately 2-2 kb. An additional weak band of approximately 1-7 kb was seen in X-ray-parasitized larvae but its significance is unclear. No signal was seen in the control with nonparasitized sixth instar larvae. A fragment of S. littoralis β-actin hybridized to all RNAs tested (Fig. 4b). In order to obtain an overview of transcription of CiV14g2 in the course of X-ray-parasitism, Northern blots were performed with total RNA from each larval stage. The cDNA hybridized only and very strongly to an approximately 0-8 kb mRNA from X-ray-parasitized sixth instar larvae during the early cell formation stage (Fig. 5a). In parasitized fifth instar feeding larvae no signal was detectable (data not shown). The control with a fragment of S. littoralis β-actin showed a clearly visible signal for all tested samples (Fig. 5b).

**Southern blot analysis**

To investigate whether CiV14g1 and CiV14g2 are present only on segment CiV14, Southern blots with undigested and HindIII-digested viral DNA were performed using CiV14g1 and CiV14g2 as probes (Fig. 6). CiV14g1 cDNA (Fig. 6a) and CiV14g2 cDNA (Fig. 6b) both hybridized specifically to CiV14 and also to a larger viral segment. The gels used for the blots...
of Fig. 6 were run in the absence of ethidium bromide and we know that under these conditions CiV DNA occurs as covalently closed relaxed circles only (Wyder et al., 2002). Thus we can exclude the possibility that the second band represents a superhelical form of CiV14. This suggests that the two genes and possibly the entire CiV14 segment are also nested in a larger segment. CiV14g1 hybridized to the same 5 kbp HindIII fragment of either calyx DNA or CiV14 DNA (Fig. 6a) and CiV14g2 hybridized to the same 7.5 kbp HindIII fragment of either calyx DNA or CiV14 DNA (Fig. 6b).

**Expression of CiV14g1 and CiV14g2 in the course of host development**

To determine to what extent expression of CiV14g1 and CiV14g2 changes during parasitism and X-ray-parasitism, real-time PCR experiments were performed. As in Northern blots with CiV14g2 as a probe a strong signal was seen only with X-ray-parasitized sixth instar larvae of the early cell formation stage (Fig. 5a) this stage was used as a reference and these values were defined as 100% in real-time PCR. S. littoralis β-actin was chosen as an endogenous standard to normalize the data. However, measurements of actin transcripts (Fig. 7a) in the course of development of parasitized and X-ray-parasitized larvae revealed that values increased slightly up to the last instar and then dropped at the stage of pupal cell formation. These data and the Northern blot (Fig. 5b) indicate that actin RNA is reduced at the onset of metamorphosis. Actin values in nonparasitized sixth instar larvae in the feeding stage were similar to the corresponding X-ray-parasitized larvae; no transcripts were detectable in the non-template control (NTC). In view of the variability of the actin transcripts, values obtained with CiV14g1 (Fig. 7b) and CiV14g2 (Fig. 7c) are presented in two ways, not normalized and normalized with actin. The CiV14g1 transcript is most abundant in X-ray-parasitized sixth instar larvae during the early cell formation stage; in the earlier stages of parasitized and X-ray-parasitized hosts transcript quantities were much lower (only 5–20% of those in X-ray-parasitized sixth instar larvae during the early cell formation stage). In nonparasitized sixth instar larvae and in the NTC no CiV14g1 transcripts were detectable (Fig. 7b). Also, CiV14g2 transcripts were found to be most abundant in X-ray-parasitized sixth instar larvae during the early cell formation stage; in the earlier stages of parasitized and X-ray-parasitized hosts transcript quantities were only 0.01–0.2% of those at early pupal cell formation. In nonparasitized sixth instar larvae and in the NTC no CiV14g2 transcripts were seen (Fig. 7c). Thus, the onset of upregulation of CiV14g1 and mainly CiV14g2 transcription seems to be at the end of the feeding stage in the last larval instar.

---

**Fig. 5.** (a) Hybridization of radiolabelled CiV14g2 cDNA to total RNA (20 µg) of X-ray-parasitized larvae from the first to the sixth instar in the early cell formation stage (= L1 X-ray-p to L6 X-ray-p) and nonparasitized sixth instar feeding larvae (= L6 np) of S. littoralis. (b) As a control the blot was stripped and rehybridized with radiolabelled S. littoralis β-actin DNA. RNA size marker is shown on the left. Exposure time was 3 days.

**Fig. 6.** Undigested and HindIII-digested calyx DNA (1 µg each) and HindIII-digested CiV14 DNA (100 ng) were separated by FIGE. Hybridization was performed with radiolabelled CiV14g1 cDNA (a) and radiolabelled CiV14g2 cDNA (b). A linear size marker is shown on the left. Exposure time was 2–5 days.
Fig. 7. Relative quantities of actin (a), CiV14g1 (b) and CiV14g2 (c) transcripts in the course of parasitism in X-ray-parasitized and parasitized larvae (insets in b, c). Transcript quantities were measured by real-time PCR and data are means ± SD of five or six measurements, with the exception of X-ray-parasitized sixth instar larvae in the late cell formation stage (three measurements only). The values for X-ray-parasitized sixth instar larvae in the early cell formation stage were defined as 100%. XL1–XL6, first to sixth instar X-ray-parasitized larvae; L1p–L5p, first to fifth instar parasitized larvae; npL6, nonparasitized sixth instar larvae; NTC, non-template control. The sixth larval instar was subdivided into feeding early (= fe), feeding late (= fl), cell formation early (= cfe) and cell formation late (= cfl) stage. (a) Transcripts of *S. littoralis* β-actin to be used as an endogenous standard were measured in parasitized and X-ray-parasitized host larvae from the first to the last larval instar and in nonparasitized sixth instar larvae feeding early. (b, c) Relative quantities of CiV14g1 transcripts and CiV14g2 transcripts, not normalized and normalized with actin, in X-ray-parasitized and parasitized (insets) larvae.
Discussion

Characteristics of CiV14g1 and CiV14g2

We describe the first two cloned cDNAs, CiV14g1 and CiV14g2 (Figs 1 and 2), of the polydnavirus of the egg-larval parasitoid C. inanitus. With a size of 2036 bp and an ORF encoding 548 amino acids CiV14g1 is among the largest polydnavirus genes described and its structure of seven exons interrupted by six introns of similar size (Fig. 1a) is new for a polydnavirus gene. The cDNA of CiV14g2 contains three exons and two introns and has a size of 618 bp, containing an ORF encoding 177 amino acids (Figs 1b and 2b) with no similarity to known proteins but similarity to a recently cloned polydnavirus gene. The cDNA of CiV14g1 was at least partially predicted using Fgenesh (Fig. 3). As Northern blots with total viral RNA and total CiV DNA as a probe never gave clear signals, gene predictions can help to find genes which are transcribed at low levels or only for a short period of time. The information available up to now, along with dot blot analyses with RNA of last instar hosts and subclones of CiV14 as probes, suggests that CiV14g1 and CiV14g2 are the major if not the only genes on CiV14 transcribed in parasitized and X-ray-parasitized larvae.

CiV14g1 and CiV14g2 are both localized on CiV14 and a larger segment (Fig. 6). As additional probes of CiV14 also hybridized to the same larger segment (Wyder et al., 2002), it appears that the entire CiV14 is also present nested in another larger segment. In three braconid larval parasitoids identified, viral cDNAs hybridized to a single segment only (Harwood et al., 1994; Yamanaka et al., 1996; Strand et al., 1997; Trudeau et al., 2000) and in one case to several segments (Varricchio et al., 1999). In the ichneumonid Campeolits sonorensis some cDNAs hybridized to one segment only and others, encoding the abundantly expressed Cys-motif genes, hybridized to several segments which were nested. It was thus proposed that nested segments are likely to encode abundantly expressed genes (Webb & Cui, 1998).

Expression of CiV14g1 and CiV14g2 in the course of host development and comparison with larval parasitoids

CiV14g1 and CiV14g2 were both found in the XL6 library, which was made from mRNA of X-ray-parasitized sixth instar larvae in the early pupal cell formation stage. Measurement of transcript quantities of CiV14g1 and CiV14g2 in parasitized and X-ray-parasitized larvae from the first to the last instar showed that expression of both genes was highest in X-ray-parasitized sixth instar larvae at the early pupal cell formation stage (Figs 7b, c). In parasitized larvae, the latest stage investigated (L5p) was late feeding in the fifth (last) instar, because later on the parasitoid has grown enormously and occupies a large portion of the host’s body (Grossniklaus-Bürgin et al., 1994). The developmental stage L5p corresponds to that of late feeding sixth instar X-ray-parasitized larvae and quantities of transcripts are very similar in L5p and XL6fl (Figs 7b, c). Thus, only the initial increase in CiV14g2 transcripts can be seen in parasitized larvae (Fig. 7c). Expression of this gene is strongly upregulated in X-ray-parasitized sixth instar larvae at the stage from late feeding to early pupal cell formation (Figs 5 and 7c). This is precisely the time-point when prothoracic gland activity and ecdysteroid titres are reduced in X-ray-parasitized larvae as compared to nonparasitized larvae (Grossniklaus-Bürgin et al., 1998; Lanzrein et al., 2001). Also, transcription of CiV14g1 is upregulated at the stage of pupal cell formation but to a lesser extent than CiV14g2 (Figs 7b, c) and total quantities of transcripts appear also to be lower for CiV14g1 than CiV14g2 as deduced from the Northern blots (Figs 4 and 5). This suggests that both genes might be involved in suppressing host ecdysteroids at this stage.

Our data are the first to show strong upregulation of polydnavirus genes towards the end of parasitization. All other data on the temporal appearance of polydnavirus transcripts stem from larval parasitoids where evasion or immediate suppression of the host’s immune system is required to prevent encapsulation of the parasitoid egg. In hosts of several braconids, viral transcripts with sizes between 0.4 and 2.0 kb have been identified and they appeared as early as 30 min after parasitization and reached highest levels 1–24 h after parasitization (Harwood & Beckage, 1994; Asgari et al., 1996; Yamanaka et al., 1996) or injection of calyx fluid/venom (Strand et al., 1997; Trudeau et al., 2000). Most of these transcripts were found in host haemocytes, but other tissues have also been reported to contain viral transcripts, e.g. fat body, midgut, Malpighian tubules, nervous tissue and the prothoracic gland (Strand et al., 1992; Harwood & Beckage, 1994; Harwood et al., 1994; Asgari et al., 1996; Varricchio et al., 1999). In the egg-larval parasitoid C. inanitus the site of polydnavirus transcription has not yet been analysed in detail, but Northern dot blots with CiV14 as a probe indicate transcription in the haemolymph of parasitized fifth instar larvae (Johner et al., 1999). As CiV14g1 and CiV14g2 are the major genes transcribed from CiV14 (see above) and as Northern dot blots with subclones of CiV14, which encompass CiV14g1 or CiV14g2, gave clear signals with haemolymph (data not shown), it appears that both genes are transcribed in haemocytes. Their expression in other tissues is currently being investigated.

Among the ichneumonids investigated up to now most information is available for C. sonorensis and its host Heliothis virescens. In this host, viral genes are expressed rapidly and persistently throughout endoparasitoid development with little significant variation in mRNA levels from 4 h to 8 days after parasitization; for the Cys-motif genes a role in suppression of the host immune reaction has been documented (Bissard et al., 1987; Theilmann & Summers, 1988; Cui & Webb, 1996; Webb & Cui, 1998; reviewed in Webb, 1998). In Choristoneura fumiferana parasitized by Transeusa rostrata a
650 bp transcript was detected from 1 day after parasitization onwards with a maximum at 3–4 days post-parasitization; highest quantities of transcripts were found in the cuticle and lower amounts in the fat body, midgut and haemolymph (Béliveau et al., 2000). In haemocytes of S. littoralis parasitized by Hypsoper didymus three polydnavirus-specific mRNAs were seen from 2 h onwards (Volkoff et al., 1999).

On Northern blots with 20 μg total RNA from parasitized S. littoralis and CiV DNA as probe we could never see signals (data not shown) whereas in hosts of several larval parasitoids strong signals were seen under similar conditions (Asgari et al., 1996; Varricchio et al., 1999; Volkoff et al., 1999; Béliveau et al., 2000). It thus appears that the level of viral gene expression is generally lower in this egg-larval parasitoid as compared to larval parasitoids. Interestingly, the quantity of viral DNA injected per egg is also much lower, approximately 0·1 ng (Lanzrein et al., 2001) as compared to 2·4 and 6·5 ng for the two larval parasitoids investigated (Theilmann & Summers, 1986; Strand et al., 1992). Additional analyses with other egg-larval parasitoids would be necessary to find out whether the intensity and pattern of expression of polydnavirus genes is generally different between egg-larval and larval parasitoids or whether the observations reported here are specific for the C. inanitus–S. littoralis system.

We would like to thank Novartis AG, Basle, for providing us with adult S. littoralis and the diet for rearing the larvae. We also thank Dr Isabel Roditi for helpful discussions. Financial support from the Swiss National Science Foundation (grant 31-52399-97 to B.L.) and from the Roche Research Foundation to A.J. is gratefully acknowledged.

References


Stage-specific polydnavirus expression


Received 27 September 2001; Accepted 10 January 2002