Polydnavirus DNA of the braconid wasp *Chelonus inanitus* is integrated in the wasp's genome and excised only in later pupal and adult stages of the female

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Many endoparasitic wasps inject, along with the egg, polydnavirus into their insect hosts, the virus being a prerequisite for successful parasitoid development. The genome of polydnaviruses consists of multiple circular dsDNA molecules of variable size. We show for a 12 kbp segment of the braconid *Chelonus inanitus* (CiV12) that it is integrated into the wasp genome. This is the first direct demonstration of integration for a bracovirus. PCR data indicated that the integrated form of CiV12 was present in all male and female stages investigated while the excised circular virus DNA only appeared in females after a specific stage in pupal-adult development. The data also indicated that after excision of virus DNA the genomic DNA was rejoined. This has not yet been reported for any polydnavirus.

Sequence analyses in the junction regions revealed the presence of an imperfect consensus sequence of 15 nucleotides in CiV12, in each terminus of the integrated virus DNA and in the rejoined genomic DNA. Within these repeats two sequence types (ATA, TAC) were observed in the various virus clones and in the clones encompassing the rejoined genomic DNA; they corresponded to the sequence type in the right and left junction, respectively. To explain this, we propose a model of virus DNA replication in which the genomic DNA is folded to juxtapose the direct repeat of the left with that of the right junction; recombination at specific sites would then yield the two types of virus and rejoined genomic DNA.

**Introduction**

Polydnaviruses are characterized by having polydisperse dsDNA genomes and occur in parasitic wasps of the families Ichneumonidae and Braconidae (reviewed in Fleming, 1992; Fleming & Krell, 1993; Stoltz, 1993). Accordingly, they are classified as the genera *Ichnovirus* and *Bracovirus* (Francki et al., 1991). Ichnovirus particles consist of lenticular nucleocapsids of usually uniform size and are surrounded by two unit membrane envelopes. Bracovirus particles, on the other hand, consist of nucleocapsids of uniform diameter but of variable length and may contain one or more nucleocapsid within a single unit membrane envelope (reviewed in Stoltz & Vinson, 1979; Fleming & Krell, 1993). Ichnoviruses and bracoviruses replicate only in the nuclei of calyx cells of wasp ovaries from where they are secreted into the oviduct and injected into the host egg or larva (reviewed in Stoltz, 1993). Polydnaviruses are of crucial importance for survival of the parasitoid larva as they play a role in abrogation of the immune response against the parasitoid (reviewed in Lavine & Beckage, 1995) and in host regulation (reviewed in Lawrence & Lanzrein, 1993; Stoltz, 1993).

Genetic studies indicated that both ichnoviruses and bracoviruses are transmitted vertically through germ line tissue (Stoltz et al., 1986; Stoltz, 1990). The mechanism utilized for ichnoviruses appears to be integration of virus DNA into the wasp’s genome. Several segments of the ichnovirus of *Campeolitis sonorensis* were detectable as off-size restriction fragments in male and female somatic tissue (Fleming & Summers, 1986; Fleming & Krell, 1993) and for one virus segment integration into the wasp genome has been unequivocally demonstrated (Fleming & Summers, 1991). In the case of the ichnovirus of *Hyposoter fugitivus* physical mapping and Southern blot data also indicated integration of virus DNA.
into male cellular DNA (Xu & Stoltz, 1991). For bracoviruses the mechanism of transmission of virus DNA (integrated into wasp genomic DNA or episomal) is still unclear.

We recently characterized the bracovirus of *Chelonus inanitus* and observed that its genome consisted of at least 10 different segments (sizes between 7 and 31 kbp) which appear to be singly encapsidated (Albrecht et al., 1994). Here we show for a 12 kbp segment that it is integrated in the wasp genomic DNA and is only excised and circularized in females in the late pupal and adult stages. We also show that after excision of virus DNA the wasp genomic DNA is rejoined. Sequence analyses of the integration/excision regions revealed the existence of two types of excision patterns and we propose a model of how these might be generated.

**Methods**

**Insects.** *C. inanitus* (Bracoidae. Hymenoptera) are solitary egg-larval parasitoids and were reared on one of their natural hosts, *Spodoptera littoralis* (Noctuidae. Lepidoptera). The *C. inanitus* are highly inbred as they are all descendants from one or two females from which the colony was established in 1989. Pupal–adult and ovary development of *C. inanitus* as well as designation of pupal stages have been described and it was shown that ovary development is strictly correlated with the external pigmentation pattern (Albrecht et al., 1994). Adult *S. littoralis* and a semi-artificial diet were kindly given to us by CIBA-Geigy (Basel).

**Calyx fluid collection, DNA isolation and description of clones.** Calyx fluid was collected from excised female reproductive tracts and DNA was isolated as described (Albrecht et al., 1994). Cloning and physical mapping of the 12 kbp segment of the polydnavirus of *C. inanitus* (CiV12) used in this study is also described in Albrecht et al. (1994). For generation of PCR1 clones the PCR product obtained with primers LR/RL (see Fig. 2) and DNA of adult females was polished with T4 polymerase and ligated into a Smal-cut pSP64 vector. Methods were as described in Ausubel et al. (1994).

**Construction of C. inanitus genomic library.** Total nucleic acids were isolated from 100 adult male wasps (the sex of each male was visually rechecked before extraction). Insects were frozen in liquid nitrogen and ground in a mortar with a pestle to a fine powder which was then suspended in 10 ml homogenization solution (150 mM-EDTA, 5 g/l sodium lauryl sarcosinate, 30 g/l saccharose and 2 g/l SDS). After addition of 20 mg proteinase K, the solution was incubated at 37 °C for 5 h. Then the sample was extracted twice with 10 ml buffer-saturated phenol, twice with 10 ml phenol-chloroform-isoamyl alcohol (25:24:1) phases the vial was put on a seesaw for 10 min. Nucleic acids were precipitated with 25 ml of cold (−20 °C) ethanol after addition of 1 ml RNase A at 37 °C for 30 min followed by an incubation with 1 mg/ml proteinase K and 10 mg/ml SDS at 37 °C overnight. Phenol extractions and ethanol precipitations were done as described above. One µg of heat-denatured DNA in 5 × SSC (0.75 M-NaCl, 75 mM-sodium citrate) was dotted onto a positively charged nylon membrane (Boehringer Mannheim) using a dotting manifold (Bio-Rad). DNA was cross-linked to the nylon membrane by irradiation with UV light (160 000 µJ/cm²; UV Stratalinker 2400, Stratagene). The blots were prehybridized for 4 h at 41 °C in 5 × SSPE (1 × = 150 mM-NaCl, 10 mM-NaH2PO4, 1 mM-EDTA pH 7.4), 2 × Denhardt’s solution (1 × = 0.2 g/l Ficoll 400, 0.2 g/l polyvinyl pyrrolidone, 0.2 g/l BSA), 1 g/l SDS, 100 µg/ml denatured herring sperm DNA and 50% formamide. The blots were hybridized at 41 °C overnight in the same buffer, which in addition contained approximately 10⁶ c.p.m. (Cerenkov) per ml of [α-3²P]dCTP-labelled HindIII-digested calyx fluid DNA. Labelling was done with the Random Primed DNA Labeling Kit according to the manufacturer (Boehringer Mannheim). The blots were washed in 2 × SSPE containing 2 g/l SDS at room temperature and in 0.2 × SSPE containing 2 g/l SDS at 65 °C. The nylon membranes were exposed to a Storage PhosphorScreen (Molecular Dynamics) and quantitatively analysed at a PhosphorImager (Molecular Dynamics).

**DNA sequence analysis.** Nucleotide sequences were determined by the dideoxynucleotide method (Sanger et al., 1977) using the Sequenase kit version 2.0 (USB). Sequence data were analysed with the University of Wisconsin Genetics Computer Group software (release 8).

**Results**

The male *C. inanitus* genomic library was screened with the 944 bp and 924 bp EcoRI fragments of clone 1G10 of segment CiV12. The physical map of the genomic clone λA21 is shown.
Fig. 1. Restriction maps of CiV12 and genomic clone µA21. In the CiV12 map the various clones as obtained by partial digestion of virus DNA with HindIII or EcoRI and subsequent ligation with HindIII-cut pSP65 or EcoRI-cut pSP64 are given together with the orientation of the sP6 promoter (arrows). For details of cloning CiV12 see Albrecht et al. (1994). Clones with an asterisk lack the EcoRI site marked with an asterisk (sequence analyses revealed that this results from a point mutation). Bars denote regions which were sequenced and the dotted lines denote the λ arms.

Fig. 2. Design of primers for PCR. The first letter designates the junction (L, left; R, right) and the second letter the direction of elongation (L, R). LL/RR thus amplifies excised circular virus DNA, LR/LL and RL/RR the left and right junction, respectively, and LR/RL the rejoined genomic DNA. Thin lines represent virus DNA and thick lines wasp DNA.

Together with that of the entire CiV12 clone (Fig. 1). It shows that clone λA21 contains a complete collinear copy of CiV12 which is linearized at a site within the 924 bp EcoRI fragment. This indicates that segment CiV12 is integrated in genomic C. innitus DNA. The regions around the left and right junctions were sequenced in CiV12 clone 1G10 and genomic clone λA21 (Fig. 1); the sequence data have been deposited in the EMBL database and a small portion is shown in Fig. 6 and will be discussed later. On the basis of the sequence data we designed primers for PCR which would allow us to distinguish between the integrated and excised/cyclized virus DNA and which would also reveal rejoining of genomic DNA after excision of virus DNA (Fig. 2). Sequences and positions of primers are available in the EMBL database and are indicated as LL, RR, LR and RL. The following primer combinations were used: LL/RR (for circular CiV12 DNA, expected length 265 bp), LR/RL (for rejoined genomic DNA, expected length 267 bp), LL/LR (left junction, expected length 300 bp) and RR/RL (right junction, expected length 232 bp). With these primer combinations male and female pupae at various stages of pupal-adult development, abdomens and head–thoraces of males and females and calyx fluid were analysed; as positive controls the respective clones were used, and negative controls were from PCR reactions carried out without template DNA (Fig. 3). The excised/cyclized form of CiV12 DNA was found in all females after pupal stage 3 and also in calyx fluid, but not in males (Fig. 3a). Accordingly, in females after pupal stage 3 we observed a PCR product with a length of 267 bp with the primer combination LR/RL (Fig. 3b) indicating rejoining of genomic wasp DNA after excision of virus DNA. With thoraces–heads of females a strong band was seen with primers LL/RR (Fig. 3a) and a weak band with primers LR/RL (Fig. 3b). In another independent experiment with head–thoraces of females only a weak band was seen with primers RR/LL and no band with primers LR/RL; in still another experiment weak bands were seen with both primer combinations (data not shown). We do not know whether these bands are due to contamination of the head–thorax fraction with the calyx-containing abdominal fraction during the process of tissue collection. Otherwise these results would suggest that head and/or thorax of females contain a tissue which excises and cyclizes virus DNA. The right (Fig. 3c) and the left (Fig. 3d) junctions were detected in all stages of both male and female pupae, in head–thorax and abdomens of adults and also in calyx fluid. The latter is probably due to the presence of traces of DNA of ovarian tissue as, for the collection of calyx fluid, ovaries had to be punctured at various places with forceps.

We then investigated whether the entire CiV12 segment including flanking wasp genomic sequences could be amplified...
Fig. 3. Amplification of excised circular virus DNA with primers LL/RR (a), of the rejoined genomic DNA with primers LR/RL (b), of the right junction with primers RR/RL (c) and of the left junction with primers LL/LR (d). Ten ng of DNA was used in (a) and (c) and 100 ng in (b) and (d) (these differences compensate for a lower effectiveness of primer LR with genomic DNA). Lane numbers designate stages in pupal-adult development as described in Albrecht et al. (1994). T, thorax-head of adults; A, abdomens of adults; C, calyx fluid; G, clone 1G10 of CiV12 (1.66 pg); L, clone 2A21 (4.58 pg); P, clone pCR1, the cloned PCR product of primers LR/RL (0.36 pg); the quantities of DNA used corresponded to $10^5$ molecules. N, no DNA; M, pBR322 cut with Hpal as a marker.

Fig. 4. Amplification of the entire integrated CiV12 segment with primers LR/RL by means of the Expand Long Template PCR System. Lane 1, 100 ng DNA of female pupae of stage 4 (Fig. 4, lane 1) and of clone 2A21 (Fig. 4, lane 2) yielded the same product of the expected length of slightly more than 12 kb. With pupal DNA the 267 bp PCR product representing the rejoined genomic DNA was also seen (Fig. 4, lane 1). This confirms that the entire CiV12 segment is integrated in genomic wasp DNA as it is in clone 2A21.

To investigate to what extent virus DNA accumulates in the course of pupal–adult development we analysed the same DNA from male and female pupae as in Fig. 3 by Southern dot blot hybridization using HindIII-digested calyx fluid DNA as probe. As a negative control, DNA from non-parasitized S. littoralis was used and as a positive control HindIII-digested calyx fluid DNA was employed (Fig. 5a). The results revealed a strong signal with calyx fluid DNA, no signal with DNA of non-parasitized S. littoralis and a signal of intermediate intensity with male pupae of all stages and female pupae up to stage 3. With DNA of later stages of female pupae stronger signals were detected and computation of the intensities of the dots using a PhosphorImager (Fig. 5b) indicated a rapid increase of virus DNA between pupal stages 3 and 5.

Sequence analyses around the excision region of CiV12 clone 1G10 and the junctions of clone 2A21 revealed the presence of an imperfect consensus sequence of 15 nucleotides in CiV12 and in each terminus of 2A21 (sequences deposited in
Fig. 5. Hybridization of HindIII-digested calyx fluid DNA to 1 µg of DNA of male and female pupae of stages 1–6 (described by Albrecht et al., 1994). (a) Southern dot blot. Neg., negative control, 1 µg of DNA from 5th instar non-parasitized S. littoralis larvae. Pos., positive control, 20 ng of calyx fluid DNA. (b) Quantification of signals on a Phosphorimager. Dots represent females, squares represent males.

Fig. 6. Sequence analyses of the junction region in all 12 CiV12 clones (see Fig. 1), right and left junction in clone λA21 and in 11 pCR1 clones (rejoined genomic DNA). Lower case letters ('a') designate virus DNA, roman capital letters ('A') designate wasp genomic DNA and bold capital letters ('A') designate the excision region. Underlined letters denote divergent nucleotides. The triplets ATA or TAC categorize the sequence variants found in the CiV12 clones (eight TAC, four ATA) and in the pCR1 clones (nine TAC, two ATA). 'Right' and 'left' are as defined in Fig. 1.

data library). To gain more information on the integration/excision site, a region of approximately 250 bp was sequenced in all 12 CiV12 clones (Fig. 1) and also in 11 pCR1 clones (these were obtained by cloning of PCR product LR/LR) representing the rejoined genomic DNA. The parts encompassing the consensus sequences are shown together with the corresponding sequences of the genomic 2A21 clone in Fig. 6. The data revealed an imperfect direct repeat sequence of 15 nucleotides between the CiV12 clones, the pCR1 clones and the left and right junctions of 2A21. We found that at position 5 the right junction and all virus clones had an A residue while the left junction and all clones of rejoined genomic DNA had a T. Between positions 9–11 two sequence types (ATA or TAC) were found in CiV12 and pCR1 clones, corresponding to the right and left junction of λA21, respectively. Of the 12 sequenced CiV12 clones eight were of the TAC type, namely 3B6, 1A2, 1B10, 1G3, 1C1, 1C8, 1G10 and 2A2, and four of the ATA type, namely 1F3, 1B12, 1C9 and 1B9 (for description of clones see Fig. 1). Of the 11 pCR1 clones sequenced nine were of the TAC type and two of the ATA type. One possibility on how these findings might be interpreted is shown in Fig. 7. According to this model the wasp DNA would form a loop in such a way as to juxtapose the terminal repeats for recombination. For formation of the TAC type of CiV12, recombination would take place between position 5, 6, 7 or 8 resulting in a rejoined site of the ATA type (Fig. 7a). For formation of the ATA type, recombination would occur at position 11, 12, 13, 14 or 15 resulting in a rejoined site of the TAC type (Fig. 7b).

Discussion

Our data demonstrate that a 12 kbp segment of the polydnavirus of the braconid wasp C. inanitus is integrated into wasp genomic DNA (Figs 1, 3 and 4). This is the first demonstration of integration for a bracovirus. Integration has so far been demonstrated for two ichnoviruses, those of C. sonorensis (Fleming & Summers 1986, 1991) and Hyposoter fugitivus (Xu & Stoltz, 1991). Thus, chromosomal integration of virus DNA appears to be common to both genera of the family Polydnaviridae. The PCR results indicated that the integrated form of virus DNA, exemplified by CiV12, was present in all male and female stages and tissues investigated (Fig. 3 c, d). The circular (extrachromosomal) form of virus DNA, however, was found in females after pupal stage 3 and in calyx fluid, but not in males (Fig. 3 a). In the ichneumonids C. sonorensis (Fleming & Summers, 1986) and H. fugitivus and the braconid C. melanoscela (Stoltz et al., 1986) hybridization experiments also indicated the presence of small amounts of extrachromosomal virus DNA in male tissues. Our experimental approach was different and it is conceivable that trace amounts of circular virus DNA would have remained undetected under the conditions we used. Nevertheless, H. fugitivus and C. melanoscela crossing experiments involving isogenic virus DNA markers suggested
that the structure of the virus genome is determined by the integrated form of virus DNA rather than by extrachromosomal molecules (Stoltz, 1990). Thus, the integrated virus DNA appears to represent the point of departure of the amplification/repllication of the virus DNA. It is not yet clear how this occurs but our results add substantial new information. The PCR data obtained with primers LR/RL (Fig. 3 b) and sequence analyses of the cloned PCR product (Fig. 6 and sequences deposited in data library) indicated that, after excision of virus DNA, the genomic DNA is rejoined; this has not yet been reported for any polydnavirus. Our data are compatible with a scenario in which the direct repeats of 15 nucleotides at both termini of the integrated copy (Fig. 6) recombine during replication, resulting in excision and circularization of the virus DNA and rejoicing of the genomic DNA (Fig. 7). The model in Fig. 7 proposes recombination sites which would lead to the two observed sequence types (ATA, TAC) in virus and rejoined genomic DNA. The data are also compatible with more complex mechanisms of site-specific recombination, e.g. involving staggered DNA ends being joined with or without additional nucleotide excision. In the only other polydnavirus—wasp system where integration/excision regions have been sequenced, the ichnovirus of C. sonorensis, some similar observations were made. Short imperfect direct repeats of 59 nucleotides were found at both termini of integrated virus DNA and a single copy was found in the virus DNA (Fleming & Summers, 1990, 1991; Fleming & Krell, 1993). Rejoining of genomic DNA was not investigated in this system but the presented sequence data are compatible with the model of excision that we propose for the bracovirus of C. inanitus in Fig. 7. Thus, it is conceivable that the mode of virus DNA replication follows the same principle in both genera of the family Polydnaviridae.

Excision and cyclization of virus DNA was seen to begin in female pupae at stage 3 (Fig. 3 a) and, from this stage on, the quantity of virus DNA increased rapidly (Fig. 5 b). At this stage, eyes, ocelli and mandibles of the pupae are pigmented, the nutritive cords become long in the ovary and maturing oocytes begin to be discernible in the ovarioles (Albrecht et al., 1994). Histological investigations revealed that the calyx cells are discernible at this stage and are characterized by a large nucleus; electron microscopy analyses showed that in the nucleus the first virions appeared at this stage (T. Wyler & B. Lanzrein, unpublished results). In the ichneumonid C. sonorensis circular virus DNA first appeared in a similar stage of pupal—adult development (Webb & Summers, 1992), coincidental with the appearance of virions (Norton & Vinson, 1983). In C. sonorensis it was hypothesized on the basis of thoracic ligations and in vitro applications of the molting hormone 20-hydroxyecdysone that this hormone might regulate virus DNA replication (Webb & Summers, 1992). In C. inanitus, measurements of fluctuations of various ecdysteroids in pupal—adult development revealed that 20-hydroxyecdysone increased rapidly after pupation, reached a peak between stage 1 and 2 and then continuously decreased; ecdysone fluctuated at a lower level until stage 4 and then decreased (C. Grossniklaus, V. Meyer & B. Lanzrein, unpublished results). Histological data indicated that the calyx cells differentiate between stage 1 and 2 (T. Wyler & B. Lanzrein, unpublished results); it is thus conceivable that 20-hydroxyecdysone plays a role in initiating the differentiation of the calyx cells, a process which is most likely a prerequisite for virus replication to occur.

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References


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