The Bracovirus Genome of the Parasitoid Wasp *Cotesia congregata* Is Amplified within 13 Replication Units, Including Sequences Not Packaged in the Particles

Faustine Louis,a Annie Bézier,a Georges Periquet,a Cristina Ferras,b Jean-Michel Drezen,a Catherine Dupuya

Institut de Recherche sur la Biologie de l’Insecte, UMR CRNS 7261, Faculté des Sciences et Techniques, Université de Tours, Tours, France; Laboratory of Chromosome Instability and Dynamics, Institute for Molecular Cell Biology (IBMC), Porto, Portugal

The relationship between parasitoid wasps and polydnaviruses constitutes one of the few known mutualisms between viruses and eukaryotes. Viral particles are injected with the wasp eggs into parasitized larvae, and the viral genes thus introduced are used to manipulate lepidopteran host physiology. The genome packaged in the particles is composed of 35 double-stranded DNA (dsDNA) circles produced in wasp ovaries by amplification of viral sequences from proviral segments integrated in tandem arrays in the wasp genome. These segments and their flanking regions within the genome of the wasp *Cotesia congregata* were recently isolated, allowing extensive mapping of amplified sequences. The bracovirus DNAs packaged in the particles were found to be amplified within more than 12 replication units. Strikingly, the nudiviral cluster, the genes of which encode particle structural components, was also amplified, although not encapsidated. Amplification of bracoviral sequences was shown to involve successive head-to-head and tail-to-tail concatemers, which was not expected given the nudiviral origin of bracoviruses.

Polydnaviruses are unique viruses that have evolved a mutualistic life style with tens of thousands of parasitoid wasp species (1–3). They belong to the Polydnaviridae (PDV), divided into two genera, Ichnovirus (IV) and Bracovirus (BV), associated with parasitoid wasps from several subfamilies of Ichneumonidae and Braconidae, respectively (4). These unconventional viruses have an atypical life cycle that requires two separate species: the parasitoid wasp, in which viral particles are produced in specialized cells of the calyx in the ovaries, and the lepidopteran larvae, in which the viral genes packaged in the particles are expressed (5). One original feature of these atypical viruses is that their genomes are divided into two parts, both of which reside permanently in the wasp genome and are transmitted vertically (6–8). The first part of the genome is composed of proviral segments used to produce multiple double-stranded DNA (dsDNA) circles packaged in the particles and injected into the lepidopteran host (5, 9–14). This packaged genome carries virulence genes causing host immune suppression and developmental arrest that are necessary to ensure successful wasp larva development in the host (15, 16). Recently, *in silico de novo* annotation predicted more packaged genes than previously reported (222 coding DNA sequences or CDS) (17) and numerous gene families. Seven of these gene families encoded proteins containing eukaryotic conserved domains (PTP, VANK, cystatin, RNase T2, BEN, Cys rich, and C-type lectin), one family codes for a P94-like baculovirus protein, and 29 families are specific to BVs (EP1-like, EP2-like, Ser rich, and BV families 1 to 26). However, unlike free viruses, the packaged bracovirus genome does not contain the genes involved in particle production (18), and as a consequence, virions are not produced in parasitized larvae (6–8, 19–22). Indeed, the genes involved in particle production, constituting the second part of the virus genome, reside permanently in the wasp genome (22–24). For example, in the ichneumonid wasp *Hyposoter dydimator*, associated with the ichnovirus *Hyposoter dydimator* ichtnovirus (HdIV), these genes, referred to as ichnovirus structural-protein-encoding regions (IVSPERs), are clustered in specialized genomic regions amplified during virus particle production. They show no relatedness to other proteins (21). In bracoviruses, they belong to the gene set typical of nudiviruses (which are closely related to baculoviruses) and appear to have originated from a nudiviral genome captured by the common ancestor of bracovirus-associated wasps (7, 8, 25). The nudiviral genes encode proteins of the viral transcriptional machinery (nudiviral RNA polymerase subunits) and of the particle structural components and envelope proteins potentially involved in host cell entry (homologous to baculovirus *pif* genes [for *per os* infectivity factors]) (7, 8, 20, 22, 26). Interestingly, half of the nudiviral genes identified are clustered within the genome of the braconid wasp *Cotesia congregata* in a region referred to as the nudiviral cluster, thought to reflect the original organization of the nudivirus genome captured by the ancestor of bracovirus-associated wasps (7, 8, 23). The other nudiviral genes identified are dispersed in different *C. congregata* genomic regions containing wasp genes and mobile elements (7, 8).

Based on analyses of two braconid parasitoid wasps, *C. congregata* and *Chelonus inanitus*, the bracovirus proviral segments were originally thought to be located at a single chromosomal locus in a tandem array referred to as the proviral macrolocus (6, 20, 27). Recent genomic analyses of bracovirus proviral regions of *Glyptapanteles flavicoxis*, *Glyptapanteles indiensis*, and *C. congregata* (GbBV, GbBV, and CcBV, respectively) using bacterial artificial chromosome (BAC) insert sequencing revealed that 68 to 75% of the proviral segments were indeed localized within a 550- to 700-
kb-long macrolocus (12, 17, 28). The organization of the macrolocus was found to be conserved within two regions named PL1 and PL2 (for proviral loci 1 and 2), linked by a wasp gene-containing region. In addition, several proviral loci (seven in C. congregata: PL3 to PL9), containing one to three proviral segments, were not localized in close proximity to the macrolocus (Fig. 1). Some of these loci, however, could be present in the same chromosomal region, since CcBV segments S7, S8 (from isolated loci PL4 and PL9, respectively), and S30 (from the macrolocus) were all detected on the short arm of chromosome 5 in a previous study using in situ hybridization on metaphase chromosomes (6).

Analysis of proviral genomes also confirmed that direct-repeat sequences terminate all bracovirus proviral segments. These repeats, named DRJs (for direct-repeat junctions) are involved in a late step of packaged circle production (18). The process of excision leading to circularization was proposed to occur through juxtaposition of the DRJs of a proviral genome segment, followed by a recombination event involving a site-specific recombinase (27, 29, 30). Accordingly, all the bracovirus viral circles sequenced to date were found to retain a single DRJ corresponding to the recombination of proviral segment extremities (12, 17, 28).

Before their excision, DNAs have to be amplified from proviral segments used as master sequences. In CcBV and Chelonus inanitus BV (GBV), viral DNA amplification does not occur after separate excision of individual segments, as initially proposed (30, 31). Indeed in C. congregata, we showed that two segments, S8 and S21 (on PL9), were amplified together within a large progenitor molecule (30). This large molecule was detected from the onset of viral particle production during wasp pupal development and 1 day before dsDNA circles are produced (30).

From the dispersed organization of the proviral sequences, we expected either that isolated loci were amplified separately or that large regions of wasp genome separating proviral loci were amplified along with viral DNA. In the present study, we finely mapped the regions amplified during virus particle production onto the proviral form and searched for conserved sequences that might play the role of replication origins. We also tested whether the nudiviral cluster encoding the most abundant particle components was amplified during virus particle production. Finally, we analyzed the structure of the amplified molecule corresponding to a replication unit (i.e., circular/linear, head-to-tail/other concatemers) by Southern blotting. For the first time, we provide a complete overview of bracovirus amplification and reveal unexpected insights into the bracovirus replication mechanism.

**MATERIALS AND METHODS**

**Insects.** The gregarious larval endoparasitoid wasps C. congregata (Hymenoptera, Braconidae, Microgastrinae) were reared on their natural host, the tobacco hornworm, Manduca sexta (Lepidoptera, Sphingidae), as previously described (32). Females lay their eggs in fourth-instar larvae of the host, and their progeny emerge 10 to 12 days later to spin their cocoons on the back of the caterpillar. Under our rearing conditions, the wasps emerge between 5 and 6 days after cocoon spinning.

**Extraction of wasp DNA.** The sex of each individual C. congregata wasp was visually checked twice under a binocular dissecting microscope. We selected adults 1 day after emergence as a source of DNA because amplification of bracovirus DNA is very active at this time (22, 30). DNA was extracted from adult males and females using the DNeasy Blood and Tissue kit (Qiagen, France) according to the manufacturer’s instructions.

**PCRs.** Semiquantitative PCRs were performed for all targeted regions using 10 ng of wasp DNA, specific primers, and the most adapted Taq polymerase, depending on the fragment size amplified. Fragments below 4 kb were amplified using Advantage 2 polymerase (Clontech, France), and long-range PCR fragments were generated using TaKara LA Taq (Clontech, France). PCR conditions were determined to perform the amplification within the exponential phase, and the wasp actin gene was used as an internal control to normalize male and female samples. With Advantage 2 polymerase, after a 4-min denaturation step at 95°C, amplifications were carried out for 25 cycles with the following steps: 60 s at 95°C for denaturation, 60 s for annealing (at the temperature corresponding to the lowest melting temperature of the primer pair), and an extension
time at 72°C depending on the fragment size (90 s per kb). With LA Taq, after a 4-min denaturation step at 94°C, amplifications were carried out for 14 cycles with the steps 20 s at 98°C for denaturation, 30 s for annealing, and 20 min at 68°C, and then for 16 cycles with the steps 20 s at 98°C for denaturation and 30 s for annealing, with the extension time increased by 15 s in each cycle. PCR products were resolved on an agarose gel stained with ethidium bromide. Amplified DNA band intensities were then estimated using ImageJ software (http://imagej.nih.gov/ij/). PCR specificity was confirmed by sequencing amplified purified products (Nucleospin extract II; Macherey-Nagel, France) on an ABI 3100-Avant capillary sequencer (BigDye Terminator; Life Technologies, France). Relative quantification was also performed by real-time PCR on PL5 (Fig. 2B and C) using primers designed in the RU5 flanking regions. Relative quantitative PCRs (qPCRs) were carried out on an ABI Prism 7000 Sequence Detection System (Life Technologies, France) in 96-well plates (25 μl per well). The PCR conditions consisted of 40 cycles of 15 s at 94°C and 60 s at 55°C, followed by a dissociation protocol. Serial dilutions of DNA ranging from 0.1 to 10 ng were treated in triplicate in a reaction buffer containing 1× SYBR green mix (Mesa Green qPCR MasterMix; Eurogentec, France) and 300 nM specific primers (Fig. 2B; see Table S1 in the supplemental material). The mean threshold cycle (CT) values were used to estimate the fold change in viral DNA quantities between amplified and nonamplified sequences by the 2^−ΔΔCT method (33).

**Primer design.** Primers were designed based on the sequence of each proviral locus (accession numbers HF586472 to HF586480 for PL1 to PL9). The sequences listed in Table S1 in the supplemental material are longer than the usual primers (25-mers) to compensate for TA bias (70%) and to increase PCR specificity in the context of the numerous duplications characterizing the proviral segments (17).

**Replication unit-mapping approach.** A schematic representation of replication unit mapping by a semiquantitative PCR assay using adult male and female genomic DNAs is shown in Fig. 2. Only females produce virus particles, and males are used as controls for proviral DNA normalization (not amplified). By using appropriate primers, the amplification of a bracoviral sequence is visualized in a PCR assay by an intensity of the band in the female sample that is higher than that obtained with male DNA. Two (theoretical) proviral segments, X and Y, separated in the wasp genome by an intersegmental sequence (IS), are represented. To test whether the IS was part of the replication unit, we used two primers located at the 3′ end of the first segment (Vx3′) and the 5′ end of the next segment (Vy5′). To test whether the region flanking a proviral segment (FS) was part of the replication unit, primers were designed at the extremities of the segments (Vx5′ and Vy3′) and in the wasp flanking regions (Gx-A and Gy-A). Primers were then designed further upstream or downstream of the proviral segment (Wx-NA and Wy-NA) until the region tested was no longer found to be amplified.

**Sequence analysis.** To highlight conserved motifs, the replication units were analyzed using MEME (http://meme.sdsc.edu/meme/intro.html) and WebACT (http://www.webact.org/WebACT/home) software (34, 35). The alignment was performed using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). Secondary-structure motif predictions were based on the consensus and were obtained using mfold software (http://mfold.rna.albany.edu/?q=mfold/).
Southern blotting. Digested DNA (10 μg genomic DNA and 80 ng virus DNA) was electrophoretically separated on a 0.8% agarose gel and transferred onto a nylon membrane (Biotrans ICN, France), according to the manufacturer’s protocol. The filters were incubated for 30 min in hybridization solution (7% SDS, 0.5 M sodium phosphate buffer, pH 7.2, 1 mM EDTA). 32P-labeled probes were prepared by random priming and denatured at 94°C before membrane hybridization at 65°C overnight. The membranes were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS for 20 min at 65°C and twice in 0.2× SSC containing 0.1% SDS for 15 min at 65°C before autoradiography. The EP1 probe corresponds to the HincII 634-bp fragment of EP1 cDNA (32) that is located in segment S8 (positions 28470 to 29104) in proviral locus 9. The immediate downstream probe (IDP) is a 1.2-kb-long HindIII-PstI fragment isolated from a λ phage harboring the EP1 gene (X121) (27). The sequence is located at bp 23979 to 25203 in PL9 in the S8′ flanking sequence and also hybridizes, to a lesser extent, in the S21′ flanking sequence (positions 35386 to 35931).

RESULTS

Amplified sequences from the major proviral locus and isolated regions. Analysis of data obtained in CcBV revealed that the 6 PL1 segments and their ISs were coamplified along with contiguous segments (Fig. 3). Altogether, these results showed that PCR products were obtained with female than with male DNA using a 5′ primer located 317 bp upstream of the first segment (S19; 619-bp PCR product) (Fig. 3 and Table 1). This result indicated that amplification of the molecule begins upstream of the DRJ core sequence and includes a sequence that we named flanking sequence 1.1 (FS1.1). FS1.1 does not extend be-

FIG 3  Mapping of PL1 sequences amplified during particle production by semiquantitative PCR. Shown is a PL1 map with the sequences amplified within RU1 indicated inside the double arrow. Intersegments (IS1.1 to IS1.5) are represented by gray-hatched boxes, and their sizes are indicated. The FSs within RU1 are represented by black-hatched boxes. The positions of the primers used are indicated by black arrows, and the numbers in between primer pairs correspond to amplified fragment sizes. The numbers of fragments obtained were compared using gel electrophoresis after PCR amplification had been performed using the same amounts of DNAs extracted from male and female wasps. The intensities of the bands were measured with ImageJ (the results are represented above the electrophoresis gels). The names of the primers used for each PCR are indicated under the electrophoresis gels.

Intersegmental sequences of PL1 are amplified. Most ISs of PL1 were amplified using specific primer pairs designed near the ends of neighboring segments. For the larger intersegmental sequences IS1.4 (1,548 bp) and IS1.5 (6,346 bp), two and three primer pairs, respectively, amplifying overlapping fragments were used to assess the amplification state of the entire sequence (Fig. 3 and Table 1). In both male and female samples, we observed PCR products of the expected sizes. A larger quantity of PCR products was obtained in females than in males as measured using Image J software, indicating that ISs were amplified during replication, along with contiguous segments (Fig. 3). Altogether, these results indicated that the 6 PL1 segments and their ISs were coamplified within the same molecule.

Short sequences in flanking regions of PL1 are amplified. To determine whether this amplified molecule terminated at the extremities of the first and last segments of PL1, we assessed whether flanking regions were amplified (Fig. 3 and Table 1). A larger quantity of PCR products was obtained with female than with male DNA using a 5′ primer located 317 bp upstream of the first segment (S19; 619-bp PCR product) (Fig. 3 and Table 1). This result indicated that amplification of the molecule begins upstream of the DRJ core sequence and includes a sequence that we named flanking sequence 1.1 (FS1.1). FS1.1 does not extend be-
yond 501 bp, since similar amounts of PCR products were obtained using DNA from females and males with a primer localized at this position (793-bp product) (Fig. 3). The same approach indicated that 369 bp of flanking sequence downstream of the last PL1 segment (S5) was amplified during viral replication (FS1.2). Amplified FSs and ISs are not present in the genome packaged in the bracovirus particles, and thus, we propose to refer to these sequences as amplified not encapsidated sequences (ANES). The molecule containing the 6 PL1 segments and their ANES constitutes a replication unit (RU).

The PL2 region is amplified within three replication units. A similar analysis was performed on the 17 ISs separating the 18 segments of PL2. The results obtained (Table 1) indicated that most ISs were amplified with contiguous segments (summarized in Table 1).
in Fig. 1). However, two ISs (joining S13 to S29 and S3 to S28) were not amplified, showing that bracoviral DNA of the PL2 region was not amplified as a single replication unit but within 3 different molecules, namely, RU2.1, RU2.2, and RU2.3. Analysis of RU2.1, RU2.2, and RU2.3 flanking sequences revealed that the sizes of the amplified flanking sequences (5'-3', respectively) were 4,723 bp to 1,077 bp for RU2.1, 1,113 bp to 1,837 bp for RU2.2, and less than 746 bp to 1,214 bp for RU2.3 (Fig. 1 and Table 1). Altogether, macrolocus proviral segments were amplified within 4 replication units (1 for PL1 and 3 for PL2).

**Isolated PLs are generally amplified within a single replication unit.** Using the same approach, we found that the segments located in PL4, PL8, and PL9 were amplified within a single replication unit, along with FSs (Table 1). PL6 segments were amplified within 2 RUs; the first RU encompassed the two proviral segments S12 and S11 and their IS, and the second contained only segment S14 (Fig. 1 and Table 1). PL3 and PL7 (containing 2 and 1 proviral segments, respectively) could not be tested for FS amplification because there are either no or too few sequence data on the FSs of these segments. However, the IS separating the two segments of PL3 was detected as amplified, indicating the locus corresponds to a single RU. Circle 4, for which proviral sequence is contained in PL7, was sequenced from the DNA extracted from virus particles (9); thus, we know that it is amplified, and moreover, the fact that it contains a single segment strongly suggests it constitute only one replication unit.

In summary, we have precisely mapped the amplified molecules onto the proviral form of the viral genome (Fig. 1), and we showed that the 35 CcBV dsDNA circles identified are produced from segments amplified in 12 replication units. These RUs are variable in length, from 11,244 bp (RU9, PL9) to 217,013 bp (RU2.3, PL2). They carry ANES corresponding to FSs and ISs (11.4 to 6,346 bp long) that represent 3 to 16.6% of each RU (Table 1).

**Sequences from the nudiviral cluster are amplified during virus particle production.** Previous studies have shown that the nudiviral genes involved in bracovirus particle production are expressed in the ovaries (7, 8, 20, 22) but not encapsidated in the particles (9, 11). In ichnoviruses, the IVSPERs correspond functionally to the nudiviral cluster; although not encapsidated, they are amplified during ichnovirus particle production (21). We used a PCR approach to assess whether nudiviral genes could be similarly amplified during bracovirus particle production. We observed that for almost all genes of the nudiviral cluster, the amount of PCR product obtained was larger using DNA extracted from females than using DNA from males (Fig. 4). In order to test whether nudiviral genes of the cluster were amplified within the same molecule, we assessed the amplification of the sequences between the different genes by designing primers at the 3' extremity of one gene and the 5' extremity of the next gene. Almost all of them were amplified, as well as a 695-bp sequence downstream of the pf-3 gene, but not the Pmv gene located at the 3' extremity of the cluster (Fig. 4). The sequence of the region upstream of the 38K gene is not available, making it impossible to map the 5' extremity of the amplified molecule. In contrast to genes present in the cluster, the nudiviral genes isolated in the wasp genome (orf140-like [Fig. 4]; lef-8, odv-c56, and p74 [data not shown]) were not found to be amplified during virus particle production. In conclusion, most of the genes from the nudiviral cluster are specifically amplified during virus particle production within a replication unit more than 14,163 bp long.

**A specific sequence motif is shared by RUs.** The fact that ANES were amplified along with wasp genomic DNA raised the question of their role in the amplification of the bracoviral segments and the nudiviral cluster. We hypothesized that these sequences possess signals that promote the initiation of DNA amplification in the wasp, such as origins of replication. To identify sequences shared by RUs, we analyzed the 53 CcBV ANES sequences with WebACT and MEME software (34, 35).

We found a conserved common TA-rich sequence motif constituted of a C-rich sequence linked to a TA repeat that is variable in size (up to 100 bp) (Fig. 5A). This motif is present in all RUs, including the nudiviral cluster RU (short common sequence
localizations are indicated by asterisks in Fig. 1 and 4). The TA-rich stretches can form hairpins (Fig. 5B) that could be involved in genome replication as replication origins.

The amplified molecules form successive head-to-head and tail-to-tail concatemers. We had proposed initially that proviral segments in tandem array were excised from the chromosome in the calyx cells, forming a large circle, and then amplified by rolling-circle replication (Fig. 6)(30, 36). According to this model, successive concatemers linked head-to-tail are obtained, and head-to-tail junctions on the circle and RU concatemers are predicted, the production of which can be assessed by an inverse PCR using primers designed in opposite orientations at the extremities of the first and last segments of the RU (Fig. 6). We investigated the production of a large master circle and concatemers of RU9 (S21 and S8) by this PCR approach, but we did not obtain the expected PCR fragment (Fig. 6). Using the same DNA sample, the amplification of a specific product was obtained using primers in opposite orientations at the extremities of S21 (Fig. 6). These results strongly suggest that neither a large circle nor rolling-circle concatemers are produced during bracovirus DNA amplification.

To confirm these results, we analyzed the amplified molecule by Southern blotting using specific probes that hybridized in a flanking sequence (IDP) and within the segment S8 (EP1) (30)( Fig. 7A). The hybridization patterns obtained using 4 restriction enzymes to digest male, female, and purified-particle DNAs were compared. Interestingly, the sizes of the fragments did not correspond to those expected from a rolling-circle replication model, with production of head-to-tail concatemers confirming the negative PCR results (Fig. 7B). In contrast, a model of linear replication with production of head-to-head and tail-to-tail concatemers fully predicted the patterns of the most intense bands obtained following hybridization of the two probes (Fig. 7C and D). Such a mechanism was previously described in DNA viruses, such as Poxviridae (37, 38).

DISCUSSION
Recent studies have unraveled the complex organization of BV proviral genomes composed of two distinct components: the “core” genome, which includes nudiviral genes essential for the production of BV particles, and the proviral segments, from which the circular dsDNA molecules packaged in the particles are produced (7, 8, 20, 22). However, our understanding of how proviral segments are amplified in order to provide the large number of circular molecules encapsidated in bracovirus particles is far from complete. It was initially proposed that circles were excised directly from the chromosomal form in the calyx cells (27, 30, 31, 36) and then amplified by rolling-circle replication. However, Southern blot analyses of DNAs from C. congregata females revealed that two sequences from contiguous viral segments were amplified together within the same molecule before individual circles were produced (30). In the parasitoid wasp C. inanitus, a first step of polyploidization of calyx cells was reported, increasing the overall amount of DNA; then, in a second phase, proviral DNA was shown to be selectively amplified by quantitative PCR (36). It was also reported both in C. congregata and C. inanitus that circular viral segments once produced were not further amplified (30, 36). The recent sequencing of genomic regions containing CcBV proviral sequences offered us the opportunity to map DNA molecules amplified during particle production onto the chromosomal regions containing proviral segments (7, 12, 17, 22, 28). Compared to previous analyses performed on a very limited num-
FIG 6 Schematic representation of the macrocircle excision and rolling-circle amplification model (tested on RU9). The first step consists of the excision of a large circle from the wasp chromosome, including S8 and S21 sequences and their ANES (FL1-IS-FL2). This molecule is then used as a template for rolling-circle replication. After a nick on one strand of the circular dsDNA molecule (step 1), the 3’ OH end is released to serve as a primer for DNA synthesis by DNA polymerase (step 2 and step 3). Using the other strand as a template, replication proceeds around the circular DNA molecule, displacing the nicked strand as single-stranded DNA (step 3). The molecule produced is a head-to-tail concatemer, which can later be converted to double-stranded circles (C8 and C21 for RU9). This model predicts the production of a junction between ANES flanking sequences (FL1 and FL2) on the template, which is amplified in the concatemer and can be detected by a specific PCR, thus giving rise to an amplified fragment from 1,492 to 1,567 bp long, depending on the actual size of RU9 FL1 and FL2 (Table 1). No PCR fragment was obtained using primers in opposite orientations at the extremities of S8 and S21 (black arrows), while a product of the expected size (507 bp) was obtained using primers (gray arrows) in opposite orientations at the extremities of S21 (assessing the production of the C21 circle junction). These results indicated that the DNA used can be amplified by PCR but that the large circle and concatemers predicted by the model are most probably not produced (unless a particular structure prevents their amplification by PCR) and that bracovirus packaged-sequence amplification is achieved using a different mechanism.
FIG 7 Southern blot analysis of amplified RU9 molecules in DNA extracted from virus particles (Vi) and female (F) and male (M) wasps undergoing virus replication. The experiment was performed using DNA extracted from purified virus particles (80 ng) and male and female wasps (10 μg) digested with ClaI (C), BglII (Bg), BamHI (Ba), and SalI (S) restriction enzymes and hybridized, as indicated, with IDP localized in RU9 flanking sequences amplified during replication and the ep1 gene probe (EP1) corresponding to a CcBV gene packaged in the particles. (A) Five-hour-exposure autoradiography using IDP and EP1 probes. (B) Map of the amplified RU9 in a model of rolling-circle amplification forming head-to-tail concatamers. (C) Map of RU9 in an alternative model of amplification forming successive head-to-head and tail-to-tail concatamers. The predicted fragments are indicated under the maps, and the corresponding bands are identified in panel A. Note that the fragments that would correspond to RU9 head-to-tail concatamers were not found, whereas all the fragments corresponding to the model of replication implicating head-to-head concatamers were detected and corresponded to the most intense bands of circle C8 uncut (C8 uc) (containing the ep1 gene), whose restriction map is shown on the right in panel A. The less intense bands obtained were those expected from the map of the proviral form (not shown) or corresponded to uncharacterized replication intermediates produced in small amounts or to broken molecules. (D) Linear-replication model (based on poxvirus replication). The linear genome (with covalently closed hairpin termini) replication begins with the introduction of a nick, which exposes a free 3’ OH that serves as a primer for DNA polymerase (step 1). The nascent strand (gray lines) is synthesized from the displaced strand (black lines; step 2), and each forms self-complementary hairpins, which allows leading-strand synthesis to replicate the entire molecule (steps 3 and 4). This process generates a tail-to-tail concatemer (step 5).
ber of segments, we provide here an extensive view of the molecules amplified during particle production.

We determined that the 35 CcBV segments were amplified during particle production in 12 different molecules, each one constituting a different RU containing 1 to 8 segments. Strikingly, ISs and FSs were also amplified within each RU, but they were not encapsidated. However, these ANES do not extend to wasp genomic regions separating proviral loci, and the hypothesis that all bracovirus sequences were amplified within a single molecule was thus clearly excluded (31). ANES are variable in size (114 bp to 6,436 bp) and are mostly constituted of noncoding DNA with only 2 hypothetical genes (ep2-like3 and ep2-like4), located on one large ANES in PL2, that are not expressed during viral-particle production (data not shown). The boundaries of amplified FSs appear to be very precise. Indeed, for several RUs (RU1, RU5, and RU8), the boundaries of amplified regions could be restricted to a region of 40 to 200 bp (Table 1), suggesting that there is a nucleotide start of amplification on the chromosome or that proviral sequences are excised from the chromosome prior to their linear amplification. To our knowledge, the existence of amplified but not encapsidated viral sequences is a unique feature of polydnaviruses. This might be explained by the particular life cycle of the symbiotic viruses, which requires that only the virulence genes involved in antagonist relationships with parasitized hosts are packaged in the particles.

All RUs possess a conserved SCS of 28 bp to 100 bp potentially forming a hairpin that could confer replication origin (ori) functions similar to those described in Saccharomyces sp. or in Droso-sophila for the amplification of choriogen genes (39–41). While the biochemical mechanism and factors involved in replication initiation appear to be highly conserved, the DNA sequences at which these events take place are not (41). Indeed, in baculoviruses, ori sequences (named hrs) are closely related within a genome but show very limited homology between different baculoviruses (42–44). In nudiviruses, direct-repeat sequences have been identified and hypothesized to function as an ori (45–48). The SCs identified in bracoviruses are not similar to sequences from other viruses, but their conservation in both the Glypta panetes and Cote sia lineages, which separated approximately 17 million years ago (49), in sequences that are not packaged and thus are not involved in host-parasite antagonistic interactions suggests they have an important function, which could be initiation of replication.

We also found that the nudiviral cluster, including several genes that are involved in bracovirus particle production but that are not encapsidated, is amplified during viral replication and thus constitutes an additional RU. Strikingly a similar situation—amplification, but not encapsidation—was described for regions involved in ichnovirus particle production called IVSPERs (21, 24). In the ichneumonid wasp H. dydimator, the three IVSPERs are characterized by high coding sequence densities (exon density, 62.2%), making them atypical compared to the rest of the wasp genome (exon density, 21%). There was also a significant difference in the mean lengths of intergenic sequences between these regions (638 bp) and other portions of the wasp genome (1,669 bp). Moreover, the 40 IVSPER genes in these regions each consist of a single exon, while a large majority of wasp genes are predicted to contain multiple exons. The organization of the nudiviral cluster is comparable to that of IVSPERs, with an exon density of 50%, a mean intergenic-sequence length of 857 bp, and 10 genes each consisting of a single exon (7, 8, 21). Due to these similarities, it could be considered a bracovirus structural-protein-encoding region (BVSPER). The amplification of IVSPERs and BVSPERs in ichnoviruses and bracoviruses, respectively, points to a striking convergence of replication strategies between polydnaviruses, despite their different viral origins (21, 24). It is probably significant that in bracoviruses the BVSPERs encode 38K and VP39, the most abundant proteins of CcBV particles (19, 23). Amplification of the bracovirus structural-protein genes appears to mimic the process described in baculoviruses and other viruses in which late genes are expressed after DNA replication (50, 51). Therefore, amplification might not only increase the copy number, but could also provide access to the viral RNA polymerase and facilitate gene expression. Accordingly, a motif previously characterized in baculovirus genes transcribed by baculovirus RNA polymerase has been found in the promoter of vp39 (7). The presence of this small motif, (A/T/G)TAAG, within 300 nucleotides upstream of the translation start site would lack the specificity necessary to selectively express chromosomally integrated genes but would be sufficient to ensure expression from amplified sequences using viral RNA polymerase, as described for baculovirus late genes (52). In accordance with this hypothesis, it has been shown recently in Microplitis demolitor BV (MdBV) that vp39 and p74 genes coding for structural proteins were preferentially transcribed by the nudivirus-like RNA polymerase. The BVSPER amplification might be required to achieve the production of VP39 at the level required to make bracovirus nucleocapsids (26).

The role of the nudiviral machinery in bracovirus particle production prompted us to hypothesize that amplification of viral sequences might involve a rolling-circle mechanism. Indeed, such a mechanism was described for the baculovirus Autographa cali-fornica nucleopolyhedrosis virus (AcMNPV) (53), sharing core genes involved in replication with nudiviruses (the replication mechanism of which is unknown). We identified the presence of fragments corresponding to a succession of head-to-head and tail-to-tail concatamers for RU9. Surprisingly, these types of concatamers are found in the context of linear replication of viral genomes, such as for poxviruses, whose genome is constituted of a closed linear double-stranded DNA that possesses at its extremities TA-rich inverted terminal repeats (ITR) forming a hairpin, in which replication occurs in the cytoplasm (38) (Fig. 7). The production of the head-to-head and tail-to-tail concatamers explains the lack of PCR products expected from a rolling-circle model of replication. This model is also in accord with the lack of nudiviral DNA polymerase detection in transcripts from three braconid species, even when high-throughput sequencing approaches were used (22). Most of the nudiviral genes potentially involved in replication were lacking in braconid wasp ovaries, except a nudiviral helicase and a flap endonuclease we have recently identified by a probabilistic method (22, 54). This led us to propose that bra- covirus DNA amplification might involve the wesp replication machinery (7, 8, 22). In accordance with this hypothesis, massive parallel sequencing performed on M. demolitor ovaries has identified transcripts from many cellular DNA polymerases. In addition, DNA polymerase genes from Maverick transposable elements (TE) that are abundant in parasitoid wasp genomes were also detected (22). The contribution of a third partner for bracovirus DNA amplification is also plausible, since domesticated genes from TE have been found to serve in important cellular processes, such as programmed genome rearrangement in ciliates (55). We expect the detailed characterization of bracovirus repli-
cation units and the identification of successive head-to-head and tail-to-tail concatamers as replication intermediates will constitute important clues to sort out which genes are actually involved in bracovirus DNA amplification.

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