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Polydnavirus DNA is integrated in the DNA of its parasitoid wasp host

(insect virus/ichneumonid/*Campoletis sonorensis*/parasitic hymenoptera)

JO-ANN G. W. FLEMING* AND MAX D. SUMMERS*†

*Department of Entomology and the †Center for Advanced Invertebrate Molecular Sciences, Texas A&M University, College Station, TX 77843

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ABSTRACT **The polydnavirus *Campoletis sonorensis* virus (CsV) is present in the oviducts of all adult *C. sonorensis* female wasps and appears to be required for these wasps to parasitize hosts successfully. Physical mapping, Southern blot analysis, and nucleotide sequence analysis demonstrate that the viral DNA B-specific sequences in cloned wasp DNA are colinear with viral genomic segment DNA B from nucleocapsids and are covalently linked to nonviral wasp sequences. Integrated DNA B terminates in 59-nucleotide imperfect direct repeats, but a single repeat exists in the extrachromosomal superhelical viral DNA B. Sequences near each junction form imperfect inverted repeats with sequences near the ends of an internal viral 540-base-pair repeat element gene. CsV appears to be the first documented integrated, nonretroviral DNA virus of insects and probably is vertically transmitted as a provirus.**

Polydnaviruses are distinguished from other viruses by their large genomes composed of multiple superhelical DNAs. These viruses appear to be mutualistically associated with certain wasp species that appear to require the viruses for successful parasitization of host larvae. The polydnavirus *Campoletis sonorensis* virus (CsV) replicates in the calyx epithelia of the oviducts of all adult female *C. sonorensis* wasps (1). During oviposition the wasp injects eggs and virus from the lumen of the oviducts into lepidopteran host larvae where viral genes are expressed (2, 3). Several pronounced immunological, physiological, and developmental changes occur in parasitized hosts concomitant with viral gene expression and appear to be essential for normal endoparasitic development of the wasp in the host (4, 5).

All adult females in several species of ichneumonid or braconid wasps contain polydnaviruses that appear to be species specific (6–10). Genetic studies suggested that the polydnavirus of *Cotesia melanoscela* braconid wasps is vertically transmitted, but physical evidence for viral integration has not been reported (11). In contrast, CsV-specific DNA is detectable as offsize restriction fragments in nonoviduct somatic tissues of both male and female *C. sonorensis* ichneumonid wasps, suggesting that CsV DNA is integrated in the wasp genome (12). However, an alternative interpretation that the CsV offsize restriction fragments represented polymorphic or rearranged viral DNAs could not be excluded due to the incompletely understood, complex patterns of intragenomic cross-hybridization (3, 6, 12–15).

In this study, we examine CsV DNA segment B and related cloned *C. sonorensis* sequences by Southern blot hybridization and nucleotide sequence analysis to determine whether the offsize restriction fragments detected in wasp DNA indicate the integration of polydnaviral DNA or rearranged sequences in the viral genome.‡ Our data show that unrearranged viral DNA B is covalently linked to wasp sequences. Polydnaviruses like CsV likely are maintained in parasitic hymenoptera as integrated proviruses.

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MATERIALS AND METHODS

Insects, Virus, Genomic Libraries, and Plasmid DNA. Methods of rearing an inbred population of *C. sonorensis* wasps and virus and viral DNA purification have been published (2, 7, 12). Total cellular DNA was extracted from male wasps by standard methods because they lack oviducts (16). The predominant form of viral DNA in males is detectable as offsize restriction fragments (12). A *C. sonorensis* genomic library was constructed by ligating *Bam*HI-cleaved EMBL3 λ DNA arms (Promega) with size-selected *Mbo* I partial digestion products of male wasp DNA (16). Methods of plaque purification, λ DNA isolation, plasmid extraction and purification, and generation of overlapping deletions with exonucleases *Exo* III and *Exo* VII or exonuclease BAL-31 have been described (16–20). Extrachromosomal CsV DNA B was cloned in its entirety as independent 6.7-kilobase-pair (kbp) *Bam*HI (pBB6700) or *Eco*RI (pBE6700) fragments by D. A. Theilmann, who provided the clones (21). The *Bam*HI clone (pBB6700) was used routinely.

Southern Blots. Southern blotting, high-stringency hybridization and washing conditions, autoradiography, and nick-repair labeling of gel-purified, vector-free DNAs with [^{32}P]dATP to high specific activity (≥10^8 cpm/μg) were performed as described (12). For lower-stringency condition hybridizations, the percentage of formamide in the hybridization buffer was decreased to 30%, and the washing conditions were altered by lowering the temperature of the 0.1× standard saline citrate/0.1% SDS washes to 37°C.

DNA Sequence Analysis. The nucleotide sequence of both strands was determined with single-stranded DNA by the dideoxynucleotide chain-termination method (22). Sequence data were analyzed with the University of Wisconsin Genetics Computer Group (UWGCG) software for VAX computers (release 60) (23).

RESULTS

Characterization of Wasp DNAs Related to Viral DNA B. Previous Southern blot analyses suggested that a 1.25-kbp *Xho* I fragment of CsV DNA B (6.7 kbp) is detectable in male *C. sonorensis* cellular DNA as offsize restriction fragments (12). Additional Southern blots of male wasp DNA and CsV DNA digested with several other restriction endonucleases and hybridized with radiolabeled cloned CsV DNA B (pBB6700) or subcloned fragments of it indicated that other DNA B sequences are not detectably altered in wasp DNA

Abbreviations: CsV, *Campoletis sonorensis* virus; nt, nucleotide(s); DR_L, direct repeat, left; DR_R, direct repeat, right; IR, inverted repeat; ORF, open reading frame.

‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M80621, M80622, and M80623).

(data not shown). Sequences in a 0.4-kbp *Bgl* II fragment within the 1.25-kbp *Xho* I fragment are consistently associated with DNA B-specific offsize restriction fragments in wasp DNA. Physical mapping of several independent, overlapping clones isolated from a *C. sonorensis* genomic library with cloned DNA B (pBB6700) probes indicated that isolate λCsB2.3 contains a complete copy of DNA B that is colinear with CsV DNA B isolated from nucleocapsids but is linearized at a site within the 0.4-kbp *Bgl* II fragment (Fig. 1).

Identification of Flanking DNA Sequences. Southern blot hybridizations were conducted to identify the DNA flanking the DNA B-specific sequences in λCsB2.3. First, when the entire insert in λCsB2.3 was used to probe blots of the undigested segmented viral genome, the superhelical and relaxed circular forms of the viral DNA segments designated A_3, B, and R hybridized (Fig. 2). An identical pattern was obtained with control cloned DNA B probes. Under the stringency conditions used CsV DNA B normally cross-hybridizes with DNAs A_3 and R (12). Second, blots of digested λCsB2.3 DNA were hybridized with the entire CsV DNA genome. The DNA B-specific sequences gave an intense signal, whereas there was no detectable hybridization with the flanking sequences (data not shown). Third, to reduce probe complexity, the sequences flanking the DNA B-specific DNA in the λCsB2.3 insert were hybridized with blots of CsV genomic DNA under low-stringency conditions. Neither the left nor the right flanking DNA probe hybridized with CsV DNA to levels detectable even after prolonged autoradiographic exposures (Fig. 2). In contrast, the control CsV DNA probe yielded an intense signal in short exposures after hybridization under high-stringency conditions. These results indicate that the DNA B-specific DNA in λCsB2.3 is covalently linked to sequences of nonviral (i.e., wasp) origin.

The left and right flanking wasp DNA probes hybridized strongly under high-stringency conditions with one to three fragments in digested DNA isolated from several hundred male wasps (Fig. 3). Each of the additional, independently isolated DNA B-specific recombinant λ clones hybridized with either the left or right flanking sequence probes (data not shown). The DNA B-specific sequences in male wasp DNA are detectable as single, high molecular weight fragments when enzymes that do not cut within DNA B (e.g., *Sal* I) are used (12). Collectively, these data indicate that unrearranged CsV DNA B is stably integrated at a single locus in nonrepetitive DNA in the *C. sonorensis* wasps studied.

Sequence Analysis of the Junctions of Integrated DNA B. The nucleotide sequences of the 0.4-kbp *Bgl* II fragment of extrachromosomal viral DNA B and the viral and wasp sequences at each junction were compared (Fig. 4). As illustrated schematically in Fig. 5, the mapping and blot data indicated that integrated DNA B is linearized at a site between the *Sma* I and *Pvu* II sites in the 0.4-kbp viral *Bgl* II fragment of the circular extrachromosomal molecule (Figs. 1 and 5). The sequence data confirmed that the first 108 nucleotides (nt) upstream of the *Sma* I site in extrachromosomal DNA B are identical to the comparable viral sequences at the left junction (U_L in Fig. 5), and the sequences 3′ of the *Pvu* II site in the circular molecule are identical to the

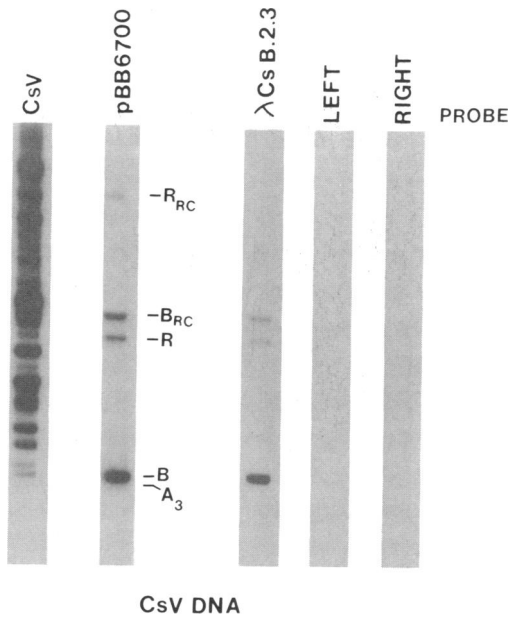

FIG. 2. Southern blot hybridization of λCsB2.3 and flanking sequence probes with CsV DNA. Southern blots of undigested CsV DNA were hybridized with genomic CsV DNA, pBB6700, or the entire insert in λCsB2.3 under stringent conditions. Duplicate lanes of CsV DNA were hybridized under low-stringency conditions with *Sal* I/*Eco*RI (Left) or *Sal* I (Right) fragments that flank the DNA B-specific sequences in λCsB2.3 as illustrated in Fig. 1. Autoradiographs of blots hybridized with the left or right flanking sequence probes were exposed for 7 days with an intensifying screen. Representative overnight exposures without intensifying screens of lanes hybridized with CsV DNA, pBB6700, or the entire λ insert are shown.

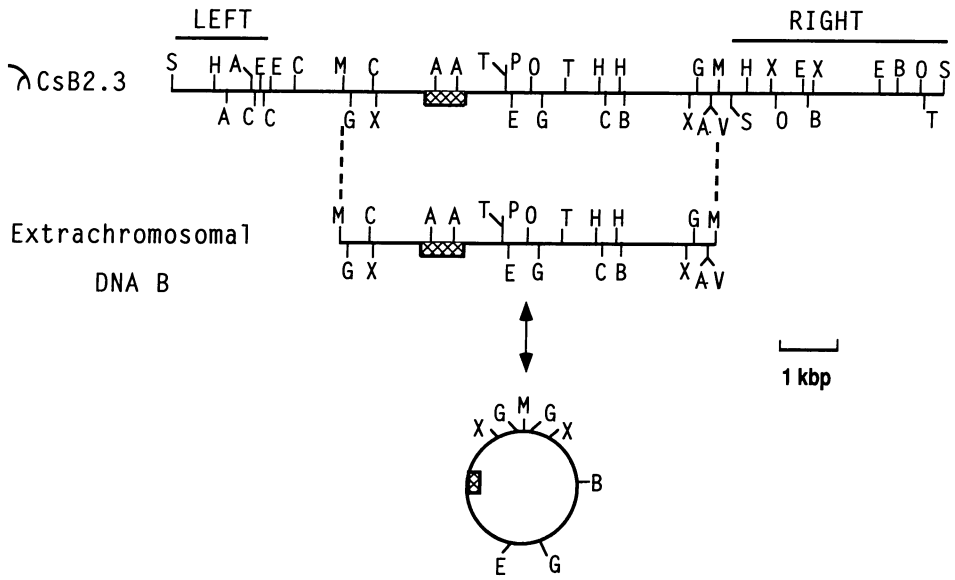

FIG. 1. Physical maps of integrated and extrachromosomal CsV DNA B. The physical map of extrachromosomal viral DNA B (6.7 kbp) in its circular form and linearized at the *Sma* I site is compared with the map of the insert in λCsB2.3 (13.7 kbp). The location of the DNA B-specific sequences in λCsB2.3 is indicated by vertical dashed lines. Cross-hatched box indicates the location of the 540-bp repeat element gene on DNA B. The 1.5-kbp *Sal* I/*Eco*RI and 3.6-kbp *Sal* I fragments labeled Left and Right, respectively, were isolated from the DNA that flanks the DNA B-specific sequences in λCsB2.3 and were used as radiolabeled probes in Southern blot hybridizations as described in the text. B, *Bam*HI; G, *Bgl* II; C, *Cla* I; E, *Eco*RI; A, *Hae* III; H, *Hin*dIII; P, *Pst* I; V, *Pvu* II; S, *Sal* I; M, *Sma* I; T, *Sst* I; O, *Xba* I; X, *Xho* I.

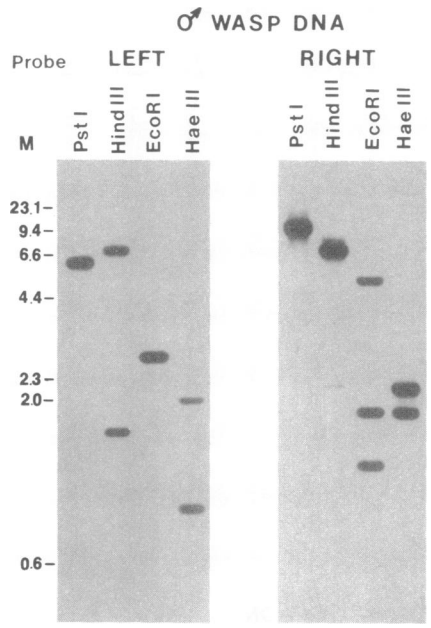

FIG. 3. Southern blots of wasp DNA hybridized with the nonviral flanking sequences. Southern blots of restriction endonuclease-digested *C. sonorensis* male wasp DNA (10 μg per lane) were hybridized under high-stringency conditions with *Sal* I/*Eco*RI (Left) or *Sal* I (Right) fragment DNAs that flank integrated DNA B at the left or right junction, respectively, as illustrated in Fig. 1. Representative overnight exposures without intensifying screens are shown.

comparable viral sequences that are unique to the right junction (U_R in Fig. 5) (Fig. 4).

The relationship of the integrated and extrachromosomal copies of the sequences between the *Sma* I and *Pvu* II sites was more complex. Integrated DNA B terminated in 59-nt imperfect (11 mismatches) direct repeats (DR), which overlap the *Sma* I recognition sequences at each junction (Figs. 4 and 5). The 7 nt immediately 3′ of the left (DR_L) and right (DR_R) terminal repeats differed, but these short regions of dissimilarity were followed by 53-nt sequences that are related (14 mismatches) (Fig. 4). Due to this region of similarity 3′ of the terminal repeats, the junction of the wasp and viral sequences is less clear-cut at the left terminus than at the right end. Extrachromosomal DNA B contained a single identical copy of the 59-nt sequence DR_L that overlaps the *Sma* I site (Figs. 4 and 5). However, in extrachromosomal DNA B the sequences immediately 3′ of DR_L were identical to the sequences found immediately 3′ of DR_R at the right junction rather than the sequences that are downstream of the integrated copy of DR_L (Fig. 4). This suggests that the terminal repeats recombine during virus replication.

The sequences within and immediately adjacent to the terminal direct repeats were structurally complex. A 21-bp sequence DR_{3A} overlapped the 3′ end of the right direct repeat DR_R by 18 nt and differed by 4 nt from a 21-nt viral sequence (DR_{3B}) located 25 bases 3′ of the *Pvu* II site in the U_R sequences at the right junction (Fig. 4). DR_L was less related to DR_{3B} than DR_R because of the mismatches in the two terminal direct repeats. In the left junction, the sequence CAGCTCT was directly repeated 15 nt 3′ of DR_L, although 5 nt separate the two copies (Fig. 4). In the right junction, short inverted repeats (IR_V) (underlined) (TACCAGCTCTGGTA) overlap the first copy of this 7-nt sequence, which is located 15 nt 3′ of DR_R and which is followed 7 nt downstream by a partial second copy (CAGCT) (Fig. 4). Short imperfect IRs were found in the wasp DNA close to the left (IR_{WL}) or right (IR_{WR}) junctions (Fig. 4). No repeats suggestive of target duplications were noted.

The longest open reading frames (ORFs) in the extrachromosomal viral DNA 0.4-kbp *Bgl* II fragment or the left junction start at an ATG 31 nt 5′ of the *Sma* I sites in the viral sequences (Fig. 4). In the left junction, a stop codon occurs ≈90 bp downstream of the *Sma* I site in the flanking wasp sequences. In the extrachromosomal DNA, the ORF extends through the *Bgl* II fragment, terminating ≈75 nt downstream of the 3′-most *Bgl* II site (data not shown). The longest ORF in the right junction begins at an ATG 34 nt 5′ of the *Sma* I site (Fig. 4) and continues through the *Bgl* II fragment-specific DNA to a stop codon in the adjacent viral DNA (data not shown). Sequences resembling transcriptional signals are present in the upstream wasp DNA in the right junction but were not found in upstream viral sequences at the left junction or in extrachromosomal DNA B (Fig. 4). A 5-amino acid ORF occurs between the proximal putative TATA box and the AUG of the long ORF in the right junction (Fig. 4).

Sequence Comparison of Polydnaviral DNAs. Members of the CsV 540-bp repeat element gene family occur on most of the CsV genomic segments, but their function is not known (15). The genes are composed of ≈540-bp sequence elements, which occur singly or in tandem arrays and which are ≈60% similar (15). A 13-nt sequence overlapping the directly repeated CAGCTCT sequence at the left junction (5′-TGACAGCAGCTCT-3′) and a 13-nt sequence close to the 5′ end of the single 540-bp repeat element on DNA B (5′-AGAGCGGCTGTCA-3′) are imperfect IRs IR1 (Figs. 4 and 5). A 30-nt sequence near the *Bgl* II site at the right junction (5′-TTGCTCGGAACAGATGACGTGGTGCAGATG-3′) and a 26-nt sequence close to the 3′ end of the 540-bp repeat element on DNA B (5′-CATCTGGACGACG . . . . CCGTACCGAGCAA-3′) form imperfect IRs IR2 (Figs. 4 and 5). (Mismatches are underlined, and periods are inserted for optimal alignment.) The IR1 and IR2 sequences are not closely related to one another or the 540-bp repeat elements of other CsV genomic segments (15). The viral and wasp sequences in Fig. 4 were not significantly related to other sequences in the GenBank data base (release 60).

DISCUSSION

The hybridization and DNA sequence data demonstrate that the CsV DNA B-specific offsize restriction fragments previously detected in *C. sonorensis* somatic tissue DNA result from the covalent linkage of complete, unrearranged viral genomic segment B to wasp DNA. Several additional CsV genomic segments that we have examined also are reproducibly detectable as offsize restriction fragments in wasp DNA, consistent with the data for DNA B (ref. 12; J.G.W.F., unpublished data). Although *in situ* hybridization or genetic analyses are necessary to demonstrate that the viral DNA is integrated in the chromosomes rather than in mitochondrial DNA, the large size of the CsV genome (estimated aggregate genome size, 210–260 kbp) suggests that the viral genomic segments likely are integrated in wasp chromosomal DNA (7). CsV thus appears to be the first documented integrated, nonretroviral DNA virus of insects.

All polydnaviruses have segmented DNA genomes, but polydnaviruses from ichneumonid and braconid wasps are dramatically different in terms of morphology, methods of nucleocapsid release from cells, and possibly packaging of the multiple genomic DNAs (4). Whether the two viral subgroups follow the same replication strategy is not known. Our data on CsV and the recently detected viral offsize restriction fragments in *Hyposoter fugitivus* DNA suggest that polydnaviruses of ichneumonid wasps generally may be integrated and vertically transmitted as proviruses (24). Whether polydnaviruses of braconid species also are inte-

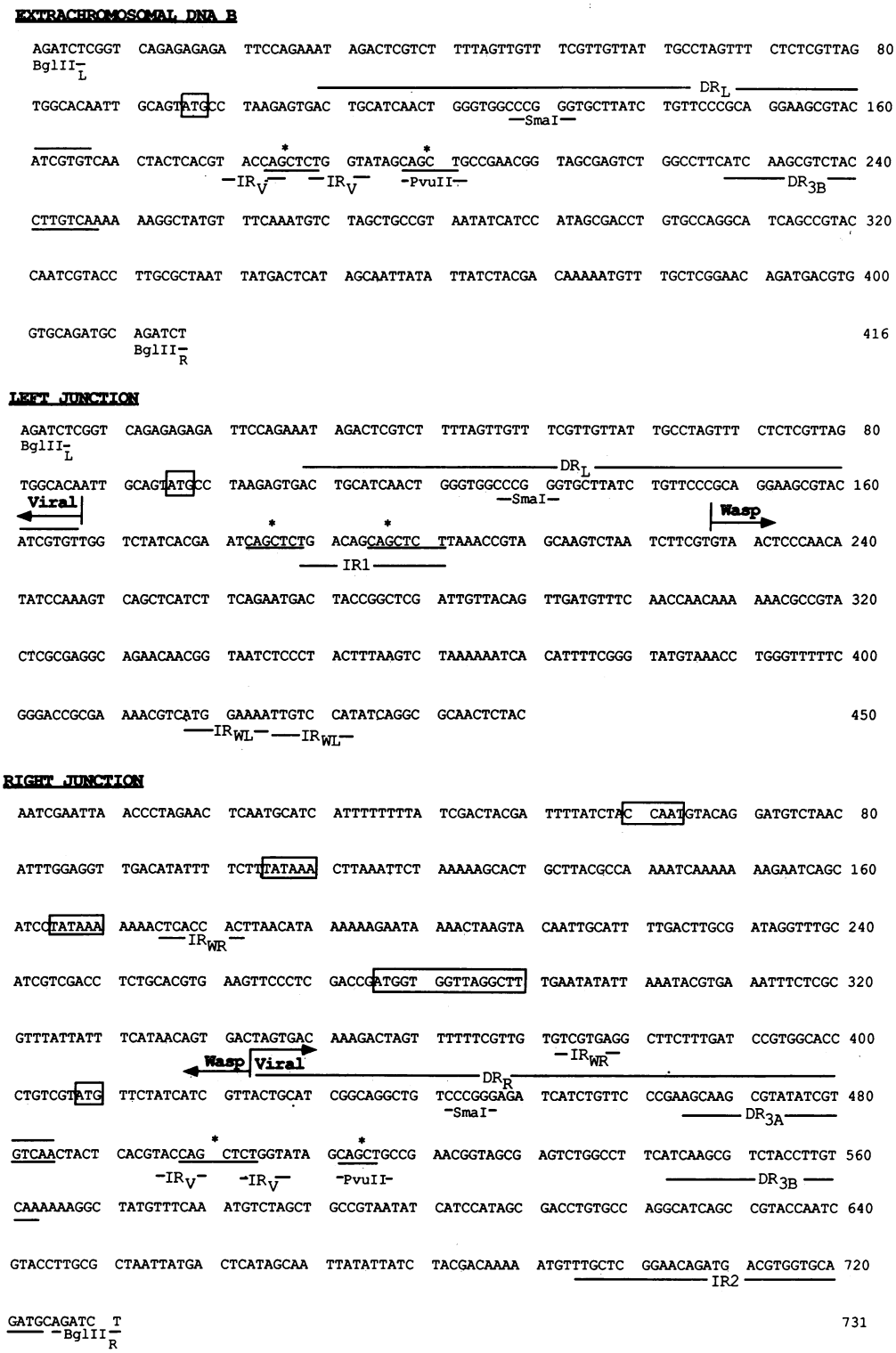

FIG. 4. Nucleotide sequences of the viral and wasp sequences at the junctions of integrated DNA B or the extrachromosomal viral homologue. Nucleotide sequences of the 0.4-kbp *Bgl* II fragment of extrachromosomal DNA B, the comparable viral sequences at the left or right junctions of integrated DNA B, and the flanking wasp DNA are shown in standard 5′ → 3′ orientation. The terminal direct repeats DR_L or DR_R are indicated by lines above the sequences. Other sequence pairs forming direct (DR) or inverted (IR) repeats are underlined and are identified by similar subscripts. Sequences related to the 540-bp repeat element gene (IR1 and IR2) are noted. A 7-nt sequence motif discussed in the text is indicated by an asterisk. Possible CCAAT and TATA sequences, the ATGs of the longest ORFs detected, and a 5-amino acid ORF located upstream of the ATG of the long ORF in the right junction are boxed.

grated is not clear from conflicting published data. Only episomal viral DNAs were detected in male *C. melanoscela* braconid wasps, and viral DNA probes did not hybridize to the chromosomal DNA (11). In a genetic analysis of the same species, however, two polymorphic forms of the virus segregated among wasp progeny in ratios consistent with possible chromosomal inheritance of the virus (24).

The replication strategy of polydnaviruses is not known. Unlike plant multipartite mitochondrial genomes, no evidence for a large, "master" circular DNA from which the

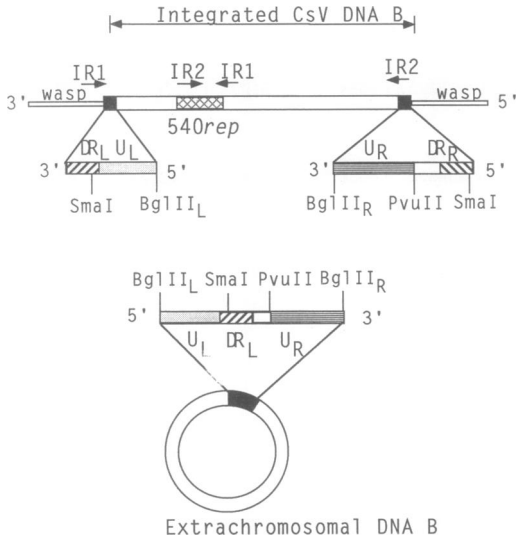

FIG. 5. Schematic diagram of integrated and extrachromosomal DNA B. Extrachromsomal superhelical DNA B with an enlarged schematic map of the 0.4-kbp *Bgl* II fragment showing the locations and orientations of selected sequences (U_L, U_R, or DR_L) defined in the text is illustrated at the bottom. The diagram of λCsB2.3 at the top illustrates the relative positions and orientations of these sequences and DR_R in integrated DNA B (wide bars), which is flanked by wasp sequences (narrow bars). The extrachromosomal DNA is pictured in standard 5′ → 3′ orientation. The unusual 3′ ← 5′ orientation shown for the integrated copy and flanking sequences in the schematic at the top reflects the linearization of the circular DNA B molecule, resulting in the apparent "reversal" of the orientation when reading along the same strand. The location of the 540-bp repeat element gene (cross-hatched box) in integrated DNA B is shown together with the positions of the sequence pairs forming the imperfect inverted repeats IR1 or IR2.

several, smaller circular DNAs are derived was found in blot analyses (12). The terminal direct repeats flanking the integrated form versus the single copy of the repeat in the extrachromosomal homologue not only argue against integrated DNA B being an artifact of illegitimate recombination but also suggest that the integrated copy may act as template and that the terminal direct repeats recombine during replication. Models involving DNA excision and strategies that do not involve excision can be hypothesized. The similarity of the sequences immediately 3′ of each terminal direct repeat may facilitate recombination and also suggests that these sequences may be part of longer, ancient repeats that subsequently have diverged during the coevolution of the wasp and virus. Other structures in the viral and wasp DNA near the junctions may be involved in replication. IR1 and IR2, for example, may facilitate intramolecular recombination since base pairing between the junction-associated sequences and the related sequences in the centrally located 540-bp repeat element gene could result in a looping of the DNA by which the terminal direct repeats are juxtaposed. Phage Mu has evolved an analogous mechanism involving DNA–protein interactions of the terminal sequences, the Mu A protein, and an internal operator sequence for the synapsis of its termini (25). Alternatively, IR1, IR2, the CAGCTCT motifs or other sequence elements may bind viral- and/or wasp-encoded proteins involved in virus replication. Our continuing analysis of other integrated CsV genomic segments should indicate what organizational features or sequence elements are conserved at junctions and hence are likely to be functionally significant.

Note. The 0.4-kbp *Bgl* II fragment from the independent *Eco*RI clone of extrachromosomal DNA B has been sequenced (B. Webb and M.D.S., unpublished data). The *Eco*RI clone contains a copy of DR_R, whereas we found a copy of DR_L in the *Bam*HI clone. The sequences from the two cloned DNAs were otherwise identical, suggesting that the terminal repeats recombine during replication.

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