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Homologous Sequences in the *Campoletis sonorensis* Polydnavirus Genome Are Implicated in Replication and Nesting of the W Segment Family†

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Polydnaviruses (PDVs) are double-stranded DNA viruses with segmented genomes that replicate only in the oviducts of some species of parasitic wasps and are required for the successful parasitization of lepidopteran insects. PDV DNA segments are integrated in the genomes of their associated wasp hosts, and some are nested; i.e., smaller segments are produced from and largely colinear with larger segments. To determine the internal structure of nested viral segments, the first complete nucleotide sequence of a PDV genome segment and its integration locus was determined. By restriction mapping, Southern blot, and sequence analyses, we demonstrated that the *Campoletis sonorensis* PDV segment W is integrated into wasp genomic DNA. DNA sequence analysis revealed that proviral segment W terminates in two 1,185-bp direct long terminal repeats (LTRs) in the wasp chromosome, while only one LTR copy is present in the extrachromosomal (viral) W. The results suggest that terminal direct repeats are a general feature of PDV DNA segment integration but that the homology and size of the repeats can vary extensively. Segment W contains 12 imperfect direct repeats of six different types between 89 bp and 1.9 kbp with 65 to 90% homology. The orientation and structure of the repeats suggest that W itself may have arisen through sequence duplication and subsequent divergence. Mapping, hybridization, and sequence analyses of cloned R and M demonstrated that these segments are nested within segment W and that internal imperfect direct repeats of one type are implicated in the homologous intramolecular recombination events that generate segments R and M. Interestingly, segment nesting differentially increases the copy number of genes encoded by segment W, suggesting that the unusual genomic organization of PDVs may be directly linked to the unique functions of this virus in its obligate mutualistic association with parasitic wasps.

Polydnaviruses (PDVs) are obligate symbiotic viruses of some parasitic wasps in the families *Braconidae* and *Ichneumonidae*. PDVs are characterized by their unique genome structure consisting of multiple segments of closed circular double-stranded DNA (37). PDVs have essential roles in the life cycle of endoparasitic wasps that require the viruses to be efficiently transmitted (36). PDVs appear to be transmitted vertically through the germ line in an integrated form as proviruses (16, 35, 36, 38). Two viral genera, *Bracovirus* and *Ichnovirus*, have been defined on the basis of their differences in morphology and host range (37). PDVs replicate asymptomatically only in the nuclei of calyx cells of wasp ovaries and are injected into lepidopteran larvae during oviposition. Although PDV replication has not been detected in parasitized insects (42), viral genes are expressed either transiently (1) or persistently (5). Viral gene products suppress the immune systems and arrest development of their lepidopteran hosts, and they are required for the successful development of the parasitic wasp (22, 41).

PDV genome organization is complex and poorly understood. The *Campoletis sonorensis* polydnavirus (CsPDV), the *Ichnovirus* type species, is composed of over 28 DNA segments with a genome size that may exceed 250 kbp (2, 21). An exact measure of the genome size is complicated by comigrating and nested DNA segments. Viral segments are also present in nonequimolar ratios, with certain segments present in much greater amounts than others (14, 21). The CsPDV genome contains many segments that cross-hybridize due to the presence of repetitive sequences on most segments (43) and to segment nesting (46).

Molecular hybridization experiments detect off-size restriction fragments in male *C. sonorensis* cellular DNA for CsPDV genome segments B, O^1, H, and W, suggesting that viral DNAs are integrated as linear DNA in the wasp genome (15, 16). Analyses of segment B demonstrated that it is linear and covalently linked to nonviral wasp DNA (16). For all viral DNA segments analyzed to date, there exists a cognate locus within the parasitoid's genome (15, 16, 49, 50). These data indicate that integration of viral DNA segments in the parasitoid's genome is a common feature of PDVs. To date, only the integration locus of CsPDV segment B has been analyzed at the DNA sequence level (16).

Early studies suggested that the CsPDV genome is composed of largely unique sequences (21). More recently, significant hybridization between viral segments has been observed (5, 43), raising the possibility that the viral genome consists of DNA segment families. However, the observed cross-hybridization between segments could be due to related genes on different segments rather than segment nesting, or to some combination of segment nesting and gene-specific cross-hybridization. The cross hybridization of genome segment W to segments R, M, and C^2 under stringent conditions suggests that these segments have large blocks of sequence in common.

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However, the possibility that W is a template segment for R, M, and C^2 has not been investigated. In studies using a related ichnovirus, Xu and Stoltz used wasp genomic Southern analyses to show that *Hyposoter fugitivus* PDV (HfPDV) segment L is nested within the larger segment U (50). We have revisited the issue of segment nesting in the CsPDV in relation to viral gene function. The results of genomic Southern analyses suggest that the viral genome segment V and the cross-hybridizing segments (T, L^2, K, and C) also are nested (46). However, the absence of sequence analyses of the nested segments makes it difficult to assess the extent of segment nesting in PDVs and difficult to study the mechanisms by which the nested segments are generated.

In this study, we have examined the integration of CsPDV segment W in the wasp genome and the nesting of segments W, R, and M. DNA sequence analyses of proviral DNA integration sites and of nested segment recombination junctions suggest a mechanism through which nested viral DNA segments are produced. The long terminal direct repeats present at the W integration site and one of the internal direct repeats within segment W appear to serve as regions for the homologous recombination events that produce nested segments during viral replication.

MATERIALS AND METHODS

Insects and viruses. The wasp *C. sonorensis* was maintained on its host *Heliothis virescens* larvae, and virus and viral DNA were isolated from the female wasp ovaries as described previously (30).

Cloning and mapping of viral segments. Viral DNA was separated on 0.7% agarose gel in TAE buffer, and bands of superhelical viral DNA segments R and M were isolated (2, 5). DNA segments were purified by using Geneclean (Bio 101), digested with a restriction enzyme (*Bam*HI, *Pst*I, or *Xba*I), and cloned into compatibly digested plasmid pZero-1 (Invitrogen). Since some DNA bands contain comigrating but different viral DNA segments, the resulting clones were screened for hybridization to labeled W. Hybridizing clones were mapped with restriction enzymes (27), and subclones were generated in pZero-1 for detailed mapping and sequencing.

Wasp genomic DNA isolation and genomic library. Wasp genomic DNA was extracted from pooled male wasps by using a modified procedure (46). A *C. sonorensis* genomic library was constructed by ligating *Bam*HI-digested λGEM-11 (Promega) arms to size-selected, *Sau*3AI-digested wasp genomic DNA (27). Genomic library screening, plaque purification, and phage DNA isolation were done as described previously (27). More than 75,000 plaques were screened with labeled total viral DNA to isolate lambda clones containing the proviral segment W. Phage inserts were released by *Sac*I digestion (which exists in the λGEM-11 arms) and subsequently subcloned in pBluescript for restriction analyses and sequencing.

Southern blots. DNA was labeled with [^{32}P]dATP by random priming by using the Prime-a-Gene labeling system (Promega). For genomic Southern blots, 10 μg of wasp DNA digested with restriction enzymes was separated in a 1% agarose gel, and the DNA was transferred to a nylon membrane. The membranes were prehybridized in hybridization buffer (50% formamide, 5× Denhardt's solution, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% sodium dodecyl sulfate) for 2 h at 42°C (8). Blots were hybridized overnight, washed under conditions of high stringency (65°C, 0.1× SSC, 1% sodium dodecyl sulfate), and exposed to X-ray film at −80°C.

DNA sequence analysis. For sequencing of segment W and its integration sites, subclones were first generated in pBluescript KS(−) (Stratagene). Segments R and M and their recombination junctions were subcloned in pZero-1. The nucleotide sequences of segment W and recombination sites were determined by the dideoxy termination method (28), using a Sequenase 2.0 kit (US Biochemical). Universal primers (T7, T3, and M13) were used for most sequencing reactions, and sequence-specific primers were synthesized when necessary. Both strands of the DNA were sequenced. DNA sequences were analyzed with the GCG program (University of Wisconsin Genetics Computer Group; release 7.5) designed for the VAX computer (10). Sequence comparison and search for homologous sequences were performed with FASTA and BESTFIT (26).

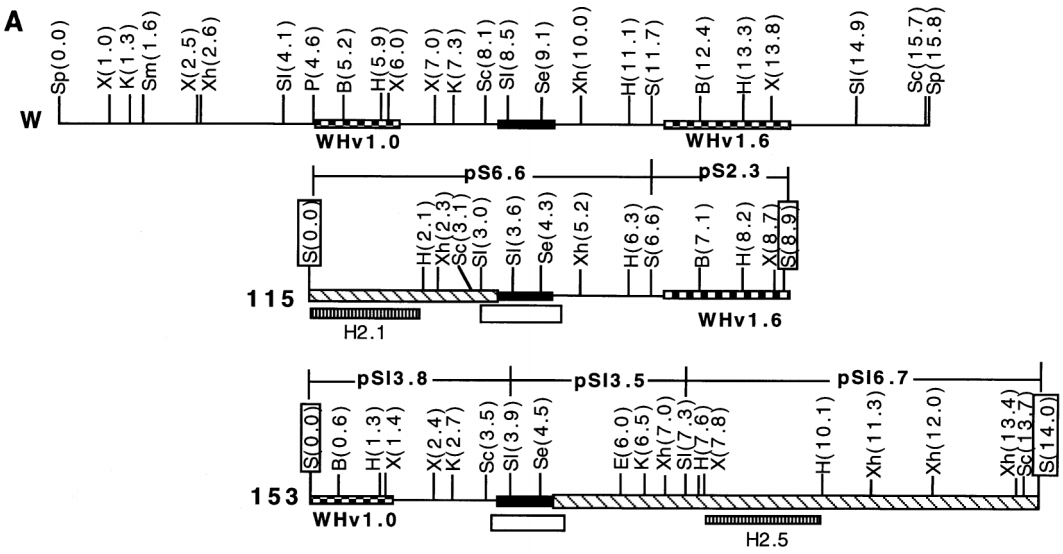
Nucleotide sequence accession numbers. The complete sequence of segment W and sequences at the left and right junctions of integrated W have been deposited in GenBank under accession no. AF004378, AF004557, and AF004558.

RESULTS

Isolation of integrated viral DNA segment W. To isolate the integrated segment W, a *C. sonorensis* genomic library was constructed in λGEM-11 and screened with labeled CsPDV DNA to identify phage plaques containing integrated viral DNA segments. Hybridizing plaques (total of 407) were seeded on 150-mm-diameter plates in a grid format and rescreened with labeled viral segment W. DNA was isolated from three hybridizing plaques and digested with *Sac*I, and the blot was probed with labeled viral segment W (data not shown). The hybridizing bands were isolated, purified from agarose, and subcloned into pBluescript for mapping and sequencing. Due to the large size of viral segment W (15.8 kbp), no clones were found to contain the full-length segment W. The restriction map of one hybridizing clone was identical to part of the extrachromosomal segment W (data not shown). Maps of the other two clones matched only portions of the extrachromosomal segment W, while other regions of these clones did not match the extrachromosomal segment, suggesting that these clones contained wasp DNA sequences flanking the W integration locus. These clones (115 and 153) were analyzed in more detail (Fig. 1A). A 3.5-kbp *Sal*I fragment [Sl(3.5)] (see legend to Fig. 1A for key to naming of restriction sites) from clone 153 that partially overlapped with segment W was subcloned for Southern blot and sequence analyses. When the two *Sac*I clones and the *Sal*I subclone were digested, separated, and hybridized to labeled segment W, regions whose restriction sites matched to cloned segment W hybridized strongly, whereas regions with physical maps that diverged from segment W did not hybridize (Fig. 1B). Therefore, we analyzed the putative junction regions, i.e., the 2.9-kbp *Xho*I fragment [Xh(2.9)] on clone 115 and Sl(3.5) on clone 153, in more detail (Fig. 1A).

To determine if clones 115 and 153 represented the left and the right junctions of integrated W, Southern hybridizations were performed with putative wasp DNA fragments flanking W. When *Sal*I- and *Xho*I-digested male wasp DNA was probed with the labeled 2.1- and 2.5-kbp *Hin*dIII fragments (Fig. 1A) of the putative left and right flanking regions, hybridizing bands were seen under high-stringency conditions after 5-h exposure (data not shown). In contrast, CsPDV genomic DNA did not hybridize even after prolonged exposures. These results indicate that regions in these subclones whose restriction maps do not match segment W represent wasp DNA sequences.

Sequence analysis of the junctions of integrated W. The junction regions of clones 115 and 153 were sequenced and compared with the complete sequence of extrachromosomal segment W. Part of the left and right junction sequences and sequence of the 1.85-kbp Sc(8.07)-Xh(9.92) fragment of W are shown in Fig. 2. We identified an identical 1,185-bp sequence present at each terminal junction and in a single copy in the extrachromosomal molecule (Fig. 2). This observation is consistent with previous findings that integrated PDV genome segments terminate in direct repeats (16, 18, 50), but the high degree of homology and length of the long terminal repeats (LTRs) were unexpected. The integrated W terminates in an identical four-nucleotide palindromic motif of GATC. Searches for repeat sequences larger than 8 bp within the 1.18-kbp LTR and wasp sequences flanking the junctions revealed 45 short direct and inverted repeats and six short palindromes. Multiple short open reading frames are also located within the LTR, but gene expression has not been detected from this region of segment W (4).



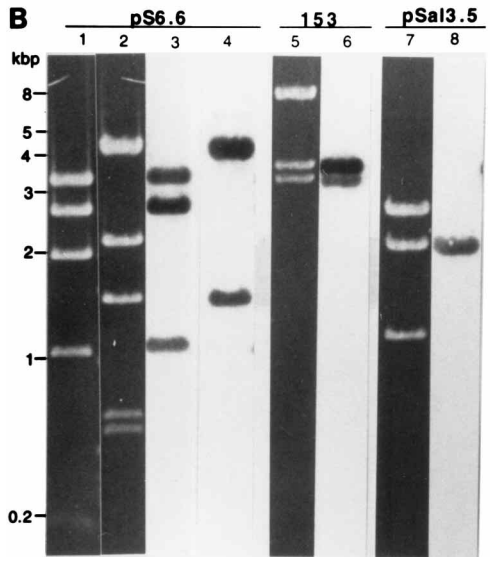


FIG. 1. Identification of the junctions of integrated segment W by restriction mapping and Southern blotting. (A) Comparison of restriction maps of viral segment W (linearized at the *Sph*I site) and two clones (115 and 153) isolated from male wasp genomic DNA library. The numbers in parentheses indicate kilobase pairs. The WHv1.0 and -1.6 genes are shown as checkered boxes; the LTRs are shown as solid boxes. In clones 115 and 153, the hatched boxes represent wasp host sequences, and the subclones (pS6.6, pS2.3, pS13.8, pS13.5, and pS16.7) are shown above the map. The open boxes are the junction regions that have been sequenced (Fig. 2). The *Hin*dIII fragments [H(2.1) and H(2.5)] used to probe wasp and viral genomic Southern blots are also shown below the maps. The boxed restriction sites *Sac*I are from the phage arms that flank the insert. Abbreviations: B, *Bam*HI; H, *Hin*dIII; E, *Eco*RV; K, *Kpn*I; S, *Sac*I; Sc, *Sac*II; Se, *Spe*I; Sl, *Sal*I; Sp, *Sph*I; X, *Xba*I; Xh, *Xho*I. (B) Southern blots of clones pS6.6, clone 153, and pSa13.5. Clones containing putative left and right junctions of integrated W were digested with restriction enzymes and probed with labeled W. The ethidium bromide-stained gel (lane 1, 2, 5, and 7) and its Southern blot (lane 3, 4, 6, and 8) are compared in parallel. Lanes 1 and 3, *Xho*I and *Hin*dIII; lanes 2 and 4, *Xho*I and *Sal*I; lanes 5 and 6, *Sal*I; lanes 7 and 8, *Sal*I and *Eco*RV.

Complete sequence of segment W. To complete the sequencing of segment W, over 60 subclones were generated in pBluescript. Regions of the WHY1.0 and -1.6 genes have been published (3, 11). The complete sequence of segment W, 15,812 bp, has many unusual features. Dot matrix plot analysis revealed many homologous regions over 50 bp (64 to 91% identical) that belong to six different repeat types. These repeats are named according to their specific features; they are schematically shown in Fig. 3A, and their lengths and homologies are shown in Table 1. Similar to the LTRs of the integrated W, all of these repeats are in direct orientation. The recombination repeats, ranging from 311 to 383 bp, are highly homologous (75 to 90% identical) (Fig. 3B). Recombination repeats flank the WHv1.0 gene and are also present within the LTRs. The cysteine-rich gene (Cys-gene) repeats include the WHv1.0 and WHv1.6 genes, are the longest repeats (1,227 to 1,891 bp), and are highly homologous (73 to 81%). The Cys-LTR repeats include the 3′ regions of the two cysteine-rich genes and a region in the LTR. They are about 75% identical and range from 526 to 624 bp. The *rep* repeats are the least homologous (~65%) and are located where three putative genes expressed only in the female wasp ovaries (CsW1 to -3) map (4). Portions (~400 bp) of the *rep* repeats show homology (56 to 60%) with the *rep* gene family that is present on most viral segments. Under reduced stringency, a region on W downstream of the WHv1.6 gene hybridized to the *rep* gene probe HC1185 from segment O^1 (4), while two other regions were not detected under these hybridization conditions. More interestingly, two long open reading frames encoding 237 and 206 amino acids are found within two *rep* repeats where CsW1 and CsW2 genes are mapped (4). They are in the opposite orientation to the cysteine-rich genes and show over 72% similarity in amino acid sequence. Two kinds of unique short repeats are also listed in Table 1.

Evidence for nesting of segments R, M, and C^2. We have adopted criteria described by Xu and Stoltz (50) to identify nested viral segments by high-stringency viral and wasp genomic Southern hybridizations (46). In this case, segments W, R, M, and C^2 cross-hybridize under high stringency (3, 4). If these segments were located at different chromosomal loci, hybridization of digested wasp genomic DNA with labeled W would generate a more complex hybridization pattern than if the segments were nested and integrated at a single genomic locus.

Digestion of male wasp genomic DNA with enzymes that cut only once in extrachromosomal W (*Sph*I, *Sac*I, and *Sma*I) generated two off-size fragments (Fig. 4, lane 1 to 3). As expected from the sizes of the fragments produced by *Sph*I di-

Extrachromosomal W

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GGAAACTTCCAGCATTCAAG AAAGTCTAAGCGTCTGGCAA GACAAACCTTTTTCATGCAT GAAATCGATATACCCAAACT CCCAGATAAACACGTACAAT  100
                                  <Viral sequence from left junction | LTR>
CTCTTGAAATCGGAGAACCT AAGTTCTAGGTCACCCAAAT TTGTAGGCTGGATTTAACGA CACTCTTCCGATCCGATAAA TGATTTTATATCGGAATAGA  200
CATCGTACATTTAGTTACAT GCGAGTGACGCATGGTATCG TCAACCATCGTGAAAATCTA ATTCCCCCTTCATAAGCTGC ACATCGGCTTGCTTTATGAA  300
CGTCGACTAAATTAATGTTA TATTGTTTACTTTCAGTGCC GAGGTTCCAACAAGCCCTGC TGCGGACACGACAACTAGAA GATGGTACGTTAGGACATAA  400
GTATTGGACTTGTTTTGAAT TCGGACGGGGGATCTGTACA CCACTCCATGAAATTTTTAA TTTAGATTTCTTTGCTAAGT TGATCATTAAACTGAACAGC  500
ACCAATTACGCGGAACTGCA TAACAAATATTGGCAGTCTG TTCACACAGTCTAGTTAGAC GAACGATTTGTTACTAACTT AAGAGGAATATGCATTAACT  600
TATGTAAAGTAAAATGTATA CATTAACGTTTTACATTTTG CTGGTACTGCTAGTCATTGA GATGTATGTTTGGCTTACGA GGAGTTCCCGAAATTGCTGT  700
CGTCGATTCAATTCTTACTA CATCTTAAGAAAAAAGTTCA AAGGTTTTATTGAACCGTAA GTTTAATAAGAGCTTTTAAC TCTTCAAGTTAATTTTAGAT  800
TATGTTTAGTAAATACGCTT TTGAATTGTCAAACATGGCC AAAGTGATGCACCATAATTA TATTTTCTGAATAAATTGCA CATGATAATATGATGTTATT  900
CGTTCTTTATTGTAAAACCC CCGATACCTTGGCACTCAAC ATTCAACTAGTGCGTTATGA AGAAAGGTTCACCCGGACCT GATTCTGCTGGATGAATCTA 1000
CAGTATGACACTGTGCTTGA TGTCCTGTGGTTACCCCGGC TTACATTTCTTAGTTACTAA GTACTCAACAGAAAGAAAAC CTATTTCAATGTTTAAAGTT 1100
CTAGCACGAAGAACAGCAGC AGGACGCGGGTAGGTGCTGT AACGGTGTAACGAATGGCTG CTATGCATTCTATGCTACAA TCCATCGCTTAGTCTGATGT 1200
ATGCGGTGCTTAGTGACTTC ACACGCGTGGTACAGAGCCA ATGGCAAAATGTCTGTCAAA GTTGCTCTTGGCGTTGCGCG CGGGCTAGGCATTCCATTTG 1300
                                                <LTR | Viral sequence from right junction>
GGTAAGGGTAGGACATAAAG GATGACATCGAACGTTGTGC TCAGCGCTCTGATCGGTCAC ATGGTACAAACAACTATGGC GTGACCATGCATTCATACAC 1400
ATCTGGTATGCATATGGTAG AAATGTTTCTCACTTAGTGA GTTTCGTTATCCTGCACTCT TTCTATCATTCTATAGTCGT CAGGTTATGGGAAAAAAAAA 1500
CTCAACATACTTGAAACATG ACACTTTGTTAATTGTACGA CATCAGTAGGTTACATGAGT TGCATTATTCCTATATGCAA ATTGACGCCGGCGTTGCTTT 1600
AAATCCGCTGTCATTCGGTT GTCTCCGGAAATTTGATTGT CCTTCCCAGGAACCGCAAGT AACCTTTGGTGGTTTCCTCG TCGAAGAATGCATTCCCATG 1700
GGGCGTAAGGGAATTATTCA AAAAATTATTCAGCCAATGA CTAACGTGTTGCCAGCAAAA ATGGTGAAAATGTCCATGTT CGCACGGTTCCGCAGGAGGC 1800
TTGCTAAACGTTCGAGCGCT TCGGTTGCCATTCTTCTC                                                                  1838
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Left junction

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GTCGACGCGGTTTTCAAACA TGTAAATTATAGAGAGAAAG GGAGAAACGCTATGAGAACC TTATTCATTTTAATCATCAA AGTTTTTTGGAGGGATATCG 100
AGGGGGTTAATTAAGGTTGG AAAAGCCTAGTTGGGTACGG ACTTTGGATACATCTGAGGG TTTCCTGGCTTCGGAGTTAA TTATGCATTCGTAAGTGTTA 200
TCAGGGCATTAAGGGTTTAT ATACACCTAGAGGTGCCAAA AATGGGATCTCATGAGTTCT CAAAAGCATGTAATATGCCT CAACAAATTCGGATTACCTG 300
TATTATAAGTTCATGATATC CCCTTTTGCAATAACGATGT AAATGGATACCATTCCGTAG TAGGGCAATATTTCAATAGC TGGAAGCTACAGACACACTA 400
                                                <Host | Viral>
ATTAACGTTATGTCGCTAAC GAGCCACAAATTACGAGGCA TGTACACAACGTTTGTTTGT TTTAGATCCGATAAATGATT TTATATCGGAATAGACATCG 500
```

Right junction

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                                                                <Viral| Host>
CGCGCGGGCTAGGCATTCCA TTTGGGTAAGGGTAGGACAT AAAGGATGACATCGAACGTT GTGCTCAGCGCTCTGATCGA ACCAGGAAGCTGAACGAACG 100
CGAGACACACGAGGGCGTCA AGAAAAATTTGCCTCCACAT GATTTTTGTCTTTGTCTTAT TTGTTTATGGTAATTTCTGT CTATTTTTATTCAAAACAAA 200
TATTCAAACGAAATTTTCAA AAAAGTTGAAAAATTGATTC AAATGTTCATTTATTTTTGT TTGTATTTACATTTTTATCT GAATGTTTTTTTTTTATATT 300
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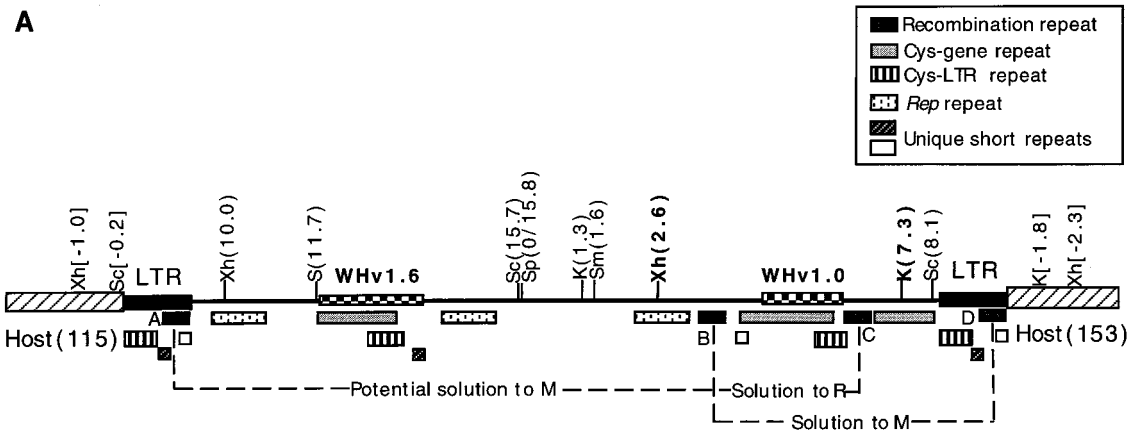
FIG. 2. Nucleotide sequences of regions of extrachromosomal segment W, left and right junctions. The region on viral genome segment W is between Sc(8.07) and Xh(9.92) (3). LTR, viral sequences, and wasp sequences are indicated. The two four nucleotide motifs are in boldface. In the two junction sequences, only short sequences of the viral LTR are shown.

gestion (Fig. 3A), two off-size, hybridizing fragments of equal intensity are observed. However, *Sac*I digestion produced two hybridizing fragments differing greatly in intensity, which results from the great majority of the segment (11.7 kb) being released as a single fragment. A smaller hybridizing region of W is produced from the other side of the *Sac*I site. The *Sma*I lies in the middle of segment W but produces a single high-molecular-weight band after digestion. Presumably, *Sma*I digestion produces two fragments of approximately equal size, but both of the flanking sites have not been mapped.

Enzymes that cut twice in extrachromosomal W (*Xho*I, *Sac*II, and *Kpn*I) would produce two off-size fragments and one cognate internal viral fragment that hybridized to labeled W (Fig. 4, lane 4 to 6). *Sac*II digestion generated two hybridizing bands. One represents a 7.6-kbp internal viral fragment, while the other fragment contains a small region upstream of the LTR shown in clone 115. The faint band at ~10 kbp, is probably due to hybridization to a 10.2-kbp *Sac*II fragment shown in clone 153 [Fig. 1A, Sc(3.5 to 13.7)]. A *Sac*II site lies just upstream of the LTR in clone 115 (Fig. 1), resulting in an almost perfect excision of segment W and the hybridization pattern of two rather than three bands.

*Kpn*I and *Xho*I digestion produced a hybridization pattern different from what was expected from the restriction map of the cloned segment W (Fig. 1A and 3A). This somewhat more complicated restriction pattern is due to segment polymorphism at *Kpn*I and *Xho*I sites [Fig. 3A, K(7.3) and Xh(2.6)]. The *Kpn* segment polymorphism is evident from comparison of the map of the segment W clones with the Southern blot of wasp genomic DNA (Fig. 1A, 3A, and 4). Digestion of segment W should produce a 6-kbp segment W internal fragment and two flanking fragments of 3.8 and at least 12.2 kbp. In the blot, we observe a faint 6-kb band, a faint hybridization to linearized extrachromosomal segment W (indicative of one *Kpn* site in segment W), a band that is over 16 kbp, and a 10-kbp band that is not predicted from the maps of the clones (Fig. 1A and 3A). This strongly hybridizing 10-kbp fragment would be produced by a restriction fragment polymorphism that eliminated the K(7.3) site (Fig. 3A) and accounts for this discrepancy in the blot. A segment W clone that contains a single *Kpn* site has been isolated but not extensively characterized (9a). Similar analyses of the *Xho*I sites indicates that the Xh(2.6) site is polymorphic, although the polymorphic segments appear to be present in approximately equal amounts in this case.

Detailed analyses of Southern blots and the mapped clones demonstrate that segment W is integrated at a single locus. In addition, patterns of the wasp genomic Southern hybridization support segment nesting. As previously observed, extrachromosomal W was consistently detected from the male wasp DNA preparations, suggesting that low levels of viral DNA replication occur in the wasp tissues other than ovaries (15). Enzymes that cut once in extrachromosomal W produced a single 15.8-kbp fragment (Fig. 4, lane 1 to 3). However, the 15.8-kbp fragment was still visible when the DNA was digested with *Xho*I and *Kpn*I, which cut twice in viral genome segment



B

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C     GTACTTGTTGACTTGTGCGTTATGAAGAATGGTTCACCCGGACCTGATTCTGCTGGATGAATCTA
A/D           A---A---------------A-----------------------------------
B     -G-----A-A--C-A--T--C--C------CA-------CA-AA----------TA----T-G--

C     AGGTCACCATCCGTTTAGGTTTGACACTGTGCTTGATATCCTGTGGTTACCCTGGCTCACAGTAA
A/D   -................--A----------------G--------------C----T---T-TC
B     -T--T-T---T-AA--G--------C-C---T-C----A---------CT--C-A--T-----C-

C     TAGTTACTAACTAATCGACAGATTGAAAAAGTTTTTCGATGTTCAAAGTTCTAGCATGAGGAACA
A/D   ----------G--C--A-----AA-----CC-A----A-----T------------C--A-----
B     -C-----A---C-C--A-TCA-A-A----CT-A------C---T---A--------CAT------

C     CAGCAGAAAGCGGGTAGGTGCTGTAGCCGTGTAATGAATGCCTGCTATGCATTCTGTGCTATAAT
A/D   ------G-C----------------A-G------C-----G--------------A-----C---
B     -----AG-C--.-----AC------A-G------C----------AT------.-A---C-----

C     CATCGCTTAGTCTGATGTATGCGGTGCTTAGTGACTTCACAGGCGTGGTACGGACGCAATGGCAA
A/D   ----------------------------------------C---------A--GC----------
B     ----------------T-G-------TGG-------CG---C---G-....A-TGA--G----

C     ATGTCTGTTAAAGTTGCTCTTG.CGTTGCGCTCGGGCTAGGAATTCCATTTGGGTTAGGGTTG
A/D   --------C-------------G--------G---------C-------------------A-
```

FIG. 3. Analyses of nucleotide sequence of CsPDV segment W. (A) Schematic representation of the repetitive sequences on integrated W. Six types of repetitive sequences are located on segment W, each labeled differently. Two types of repeats are located in LTRs. The four recombination repeats are named A, B, C, and D, A and D being identical. The involvement of the recombination repeats in generating R and M is illustrated. Once extrachromosomal W is generated by recombination within the LTRs, R and M could be further produced from W by recombination between recombination repeats. In addition, it is possible that M could be produced directly from the chromosomal template by recombination between repeats A and B. Only restriction sites used for Fig. 4 are shown, and abbreviations are as in Fig. 1A. Sp(0/15.8) is the arbitrary start site of the W sequence and the site at which segment W was originally cloned (3). Two polymorphic sites [K(7.3) and Xh(2.6)] are in boldface. (B) Comparison of the recombination repeats. Sequences are aligned by computer to indicate the significant homology between these repeats. The LTR repeat (A/D) and repeat B are compared to repeat C. Identical nucleotides are shown as dashes. Gaps (dots) are introduced to maximize the alignment. Repeats B, C, and A/D are located on viral segment W at positions 3583 to 3903, 6375 to 6766, and 9117 to 9483, respectively (the *Sph*I site is arbitrarily designated the start site).

W (Fig. 4, lane 4 and 6). This is an indication of segment polymorphism at Xh(2.6) and K(7.3) (Fig. 5A).

To conclusively demonstrate that viral segments W, R, M, and C^2 are nested, we cloned the band-isolated segments R and M and compared them to segment W. We have not attempted to clone C^2 because it is difficult to visualize in ethidium bromide-stained agarose gels. Hybridization results have shown that the WHv1.0 gene hybridizes only to segment W, indicating that the WHv1.0 gene is not present in R, M, and C^2. Our primary attempt to clone band-isolated R and M by *Pst*I and *Xba*I digestion produced subclones of R and M indicating deletions of WHv1.0 region from both segments (data not shown). Therefore, to isolate individual clones containing segments R and M in their entirety, viral DNA was digested with *Bam*HI, which cuts once in each of the WHv1.0 and -1.6 genes. Loss of the WHv1.0 gene in R and M would produce segments with a single *Bam*HI site. To eliminate clones that could result from DNA segments comigrating with R and M, *Bam*HI clones were screened with labeled W to identify two R and two M clones. The detailed restriction maps of the R and M clones matched extensively to that of segment W (Fig. 5A). Sequencing of a ~600-bp *Pst*I fragment (kbp 1.25 to 1.89 on W) on segments R and M showed that they are identical to the corresponding W sequence (data not shown). The portions of R and M where restriction maps did not correspond with W (~1.6 kbp on R and ~2.1 kbp on M) were subcloned for sequencing (Fig. 5A). The sequences were aligned with W and show deletions in R and M. Sequence comparisons showed that a 5,519-bp sequence between the recombination repeats B and A/D in segment W is absent in segment M, while the WHv1.0 region (2,803 bp) between the recombination repeats B and C in segment W is absent in R. To define the recombination junction in M and R, the retained copy of the recombination repeat was compared to the two recombination re-

TABLE 1. Direct imperfect repeats on viral segment W

Repeat type	Positions[a]		Length (bp)	Identity (%)
	Region 1	Region 2		
Recombination	3578–3886	6375–6685	311	75
	3587–3898	9117–9417	318	78
	6377–6766	9117–9483	383	90
Cys-gene	4155–5284	6672–7891	1,368	73
	4263–5912	11491–13204	1,891	81
	6775–7953	11598–12582	1,227	75
Cys-LTR	5474–5924	8311–8835	531	75
	5474–5924	12766–13204	526	77
	8311–8925	12765–13288	624	76
rep gene	2484–3073	9836–10430	598	66
	2183–3069	14277–15143	898	64
	9818–10502	14589–15269	693	64
Unique short	4155–4243	9388–9475	89	91
	8965–9115	13839–13985	151	69

[a] Relative to the arbitrary start site of segment W at *Sph*I.

peats in W that flank the deleted region (Fig. 5B). Interestingly, part of the M repeat is almost identical to the B repeat, while the remainder is almost identical to the LTR repeat (A/D). Similarly, part of the R repeat is identical to the B repeat, while the other part is identical to C repeat. These results suggest that homologous recombination occurs within the W recombination repeats to generate DNA segments R and M. Based on the sequence analysis of the recombination junctions, the molecular sizes of R and M were determined to be 13.01 and 10.29 kbp, respectively. The corresponding sequences on W, R, and M matched each other extensively, except for a few single nucleotide changes. Single nucleotide substitutions in the sequenced regions of R and M resulted in the loss of two restriction sites relative to the corresponding W map [Fig. 5A, K(7.3) and Xh(2.6)]. Restriction analysis of cloned viral genome segment W also revealed that some clones contain polymorphic restriction sites [e.g., K(7.3) and Xh(2.6)], which could result from polymorphic templates in the wasp genome (data not shown). Restriction fragment length polymorphisms have been detected in both bracovirus and ichnovirus genomes (40).

Theilmann and Summers cloned a CsPDV genome segment of 10.4 kbp that hybridized to band M (43, 44). However, this segment does not cross-hybridize to W even under lower stringency (30% formamide) (not shown). The physical map of the previously reported segment M is completely different from the M segment described in this report, indicating that they are comigrating segments. To avoid confusion, we named the segment M that was cloned earlier M^1 (43, 44).

DISCUSSION

The CsPDV genome contains many cross-hybridizing segments, some of which belong to distinct segment families (4, 5, 46). We have demonstrated that cross-hybridization between W, R, and M is the result of nesting of smaller segments (R and M) within a larger segment (W). A single locus in the wasp genome gives rise to all of these viral segments. The complex organization of segment W includes LTRs at the termini of the proviral segment and internal direct repeats of various lengths and homologies. These sequence repeats appear to have arisen through intramolecular duplications and subsequent divergence. Some repeats are associated with recombination events, some are associated with related gene families on the segment, and others are not associated with known activities but may be associated with duplication events. Based on segment W and its nested segments, we propose that CsPDV replication initiates from the integrated proviral segments by homologous recombination between the LTRs followed by intramolecular recombination at the internal repeats to generate the nested segments. Segment nesting and the existence of some segments in hypermolar ratios increase the copy numbers of some CsPDV genes which may be linked to functional requirements for high-level expression of some PDV genes (46).

Integrated PDV genome segments terminate in direct repeats. Both bracovirus and ichnovirus DNA segments are integrated in the genomes of their respective wasp hosts as proviruses. Bracovirus genome segment integration was suggested by genetic analyses (35, 38) and confirmed by molecular analysis of segment integration sites (18). Integration of ichnovirus genome segments has been shown for CsPDV and HfPDV. Southern blotting of digested wasp and viral genomic DNA identified off-size restriction fragments that were indicative of integration of DNA (14, 49). Each of the analyzed viral segments exists at a single chromosomal locus. In previous reports, segment nesting was shown by cloning and mapping segments that cross-hybridized (46, 50). Here we describe the first complete sequence of a PDV genome segment, analyze the proviral cognate to define a previously unsuspected LTR of over 1 kbp, and define the recombination loci of two additional segments produced by intramolecular recombination at internal repeat sequences in segment W. These studies clearly document that multiple segments are generated from a single PDV template segment.

The four integrated polydnavirus segments that have been analyzed terminate in direct sequence repeats in the wasp genome (16, 18, 50). In CsPDV, the integrated viral segments B and W both terminate in direct repeats, albeit the sizes and levels of homology between the repeats differ significantly. The LTRs of integrated segment W are relatively long (1,185 bp), and the sequences are 100% identical. In contrast, the terminal repeats of segment B are only 59 bp long and are imperfect (83% identity) (16). Similarly, the integrated bracovirus *Chelonus inanitus* PDV genome segment CiV12 terminates in two very short imperfect repeats of only 15 bp (18). The lengths and levels of homology of the LTRs could affect the efficiency

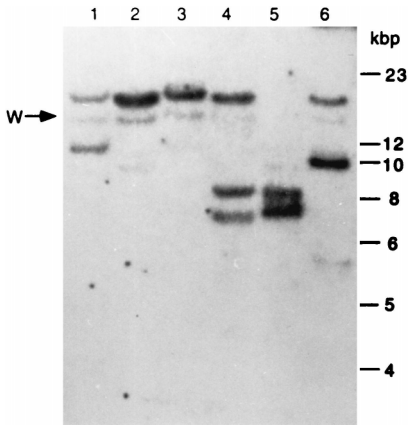
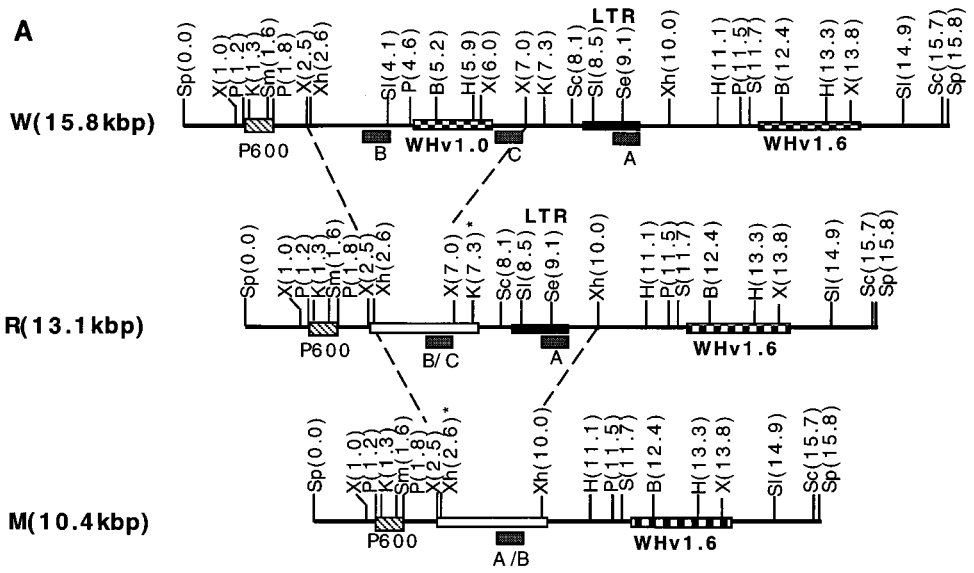


FIG. 4. Genomic Southern blots of male wasp DNA with labeled segment W. In each lane, 10 μg of DNA isolated from adult male wasps was digested and separated in a 0.8% agarose gel. Lanes 1 to 3 were digested with enzymes that cut only once in viral genome segment W: lane 1, *Sph*I; lane 2, *Sac*I; lane 3, *Sma*I. Lanes 4 to 6 were digested with enzymes that cut twice in W: lane 4, *Xho*I; lane 5, *Sac*II; lane 6, *Kpn*I. DNA from adult male wasps contain low amounts of extrachromosomal segment W, as indicated by the arrow.



B

Alignment of M sequence with the recombination repeats of W

```
                                                    -->repeat
B ----------------------------------------------------------------------------------------------------G
M TGTAATGAACCCAACCTCTACTTAATTAATTATGAATGGACTTGATAACCTATGTGTCATCAAGAATCATTCACCCCAAAATGATTCTGCTTAATGATTC
A -TCGT-CTTTATTGTAAAAC-CCCGA--CC-TG-C-CTC-ACATTC---TAG--C--T--G------GG-------GG-CC----------GG----A--

                                                                                                    *
B --------------------------------G-------------------------------------------------------------A-----T
M TACATGTTATCATTCAATTGGGTTTGACCCCATGTTCGATAACCTGTGGTTCTCCCGACTTACAGTCATTCGTTACAAACCACTCAATCAAATTAAAACC
A ----.................--A----A-TG--C-T---GT---------AC----G------T-TC--A-----T--GT------CAG--AG------

B ------G-C-------A---------ATG-----------A-----.-----AC-------------------------AT------.-----C-T---
M TATTTCAATGTTTAAAGTTCTAGCACGAAGAACAGCAGCAGGACGCGGGTAGGTGCTGTAACGGTGTAACGAATGCCTGCTATGCATTCTATGCTACAAT
A ------------------------------------------------------------------------G-------------------------

                                                      repeat<--
B -----------------T-G-------TGG-------CG-------G-....--T-A--G----TC-GC-GG-AG-GTGC-CAGCTCAC-ATGA-A-AAG
M CCATCGCTTAGTCTGATGTATGCGGTGCTTAGTGACTTCACACGCGTGGTACAGAGCCAATGGCAAAATGTCTGTCAAAGTTGCTCTTGGCGTTGCGCGC
A ----------------------------------------------------------------------------------------------------

B TCCGGGTT-GA-T-C-GCT-AC-CCAAAGATTTT-TTTA-G--TTTAATCTGCACTCCTGGT-GTTCGAA-C-CAC-TA-ATAAGT-GGTCC--TCTC-A
M GGGCTAGGCATTCCATTTGGGTAAGGGTAGGACATAAAGGATGACATCGAACGTTGTGCTCAGCGCTCTGATCGGTCACATGGTACAAACAACTATGGCG
A ----------------------------------------------------------------------------------------------------
```

Alignment of R sequence with the recombination repeats of W

```
                                          -->repeat
B ----------------------------------------------------------------------------------------------------
R TGTAATGAACCCAACCTCTACTTAATTAATTATGAATGGACTTGATAACCTATGTGTCATCAAGAATCATTCACCCCAAAATGATTCTGCTTAATGATTC
C -C-CC-TTGAA--TAACAATT-G-TAAG--CGGATGC-T-----T-G--T-G--C--T----------GG-------GG-CC----------GG----A--

B ----------------------------------------------------------------------------------------------------
R TACATGTTATCATTCAATTGGGTTTGACCCCGTGTTCGATAACCTGTGGTTCTCCCGACTTACAGTCATTCGTTACAAACCACTCAATCAAATAAAAACT
C ----G--C-C---C-GT--A---------A-T---C-T----T---------AC--T-G--C-----A---A-----T---T-A--G-CAG-T-G----AG
                                                                                    *
B ----------------------------------------------.----------------GG-G--AC--A-G-----AT-------.-A---C-----
R TATTTCGACGTTTAAAATTCTAGCACATGGAACAGCAGCAAGACGCGGGTAGACGCTGTAACCCTTTAGTGACTACCTGCCATGCATTCTGTGCTATAAT
C -T------T---C---G---------TGA------------GA-A---------GT------G--G-G--A---A-G------------------------

                                                      repeat<--
B -----------------T-G-------TGG-------CG---C---G-....A-TGA--G----TC-GC-GG-AGCGTGC-CAGCTCACGA-GAGAGAA-
R CCATCGCTTAGTCTGATGTATGCGGTGCTTAGTGACTTCACAGGCGTGGTACGGACGCAATGGCAAAATGTCTGTTAAAGTTGCTCTTGCGTTGCGCTCG
C ----------------------------------------------------------------------------------------------------
```

of the homologous recombination event, which may be correlated with the relative abundance of the CsPDV genome segments (21). Segment W is among the most abundant segments, whereas B is much less abundant.

Terminal repeats are a structural feature of many transposable elements. The W LTRs are relatively long and contain DNA sequences of great internal complexity, with many short direct repeats, inverted repeats, and palindromes. The W LTRs have some similarity with respect to length and internal complexity to the class II.2 type transposable elements (13), retroviruses and related class I retroposons (12). But unlike the class II.2 transposable elements, the PDV LTRs are in direct orientation. Integrated retrovirus and retroposons have LTRs in direct orientation but mobilize via an RNA intermediate (12, 19). We have been reluctant to use retroviruses as a model for PDV replication because of introns in PDV genomes. However, descriptions of the human endogenous retroviruses that express genes with introns (23) and have other similarities to PDVs are intriguing. Most prokaryotic and eukaryotic transposable elements excise from the host genome by way of homologous recombination between the two terminal direct or inverted repeats (12, 13, 20). In many cases, one copy of the terminal repeats is retained within the excised molecules. Homologous recombination between PDV LTRs could excise segments from the host chromosome. Some data that favor this hypothesis exist. Replication of the bracovirus *C. inanitus* PDV initiates with excision of the viral DNA in pupal and adult wasps (18). After excision of viral DNA, the wasp genomic DNA is rejoined with one copy of the imperfect terminal repeat left in the wasp genome. In all integrated viral segments analyzed to date, two terminal repeats exist in the integrated form, whereas only one is present in the encapsidated viral segment. Two clones of CsPDV DNA segment B carried different 59-bp terminal repeats, reflecting the alternative incorporation of the terminal repeats (16). Another feature of transposable element insertion in the host genome is the duplication of a short host sequence at the insertion site (12, 20). The LTRs of integrated W terminate in a GATC motif, which could represent a duplicated host sequence. However, such sequence is not evident at the segment B integration junctions. Analysis of additional segments is required to clarify some aspects of PDV replication and genomic organization. With only segment B and W integration sites sequenced, it is too early to critically compare integrated PDV sequences with other mobile DNA elements.

Generation of nested segments. Larger DNA segments may undergo intramolecular recombination between internal direct repeats to generate smaller DNA circles that will be duplications of portions of the larger segments. In CsPDV, homologous sequences such as the *rep* gene family have been found on the same as well as different viral genome segments (43). Moreover, the complete sequence of the 15,812-bp segment W has revealed six types of internal imperfect repeats (65 to 90% identity) of various lengths. Two of these repeat types are also found in the LTRs. Sequence analyses show that intramolecular recombination events that produce segments R and M occur within a repeat type that we have designated recombination repeat. Although C^2 has not been analyzed, a similar mechanism may be used to generate this segment. Similarly, the two HfPDV LTRs of segment U and an internal LTR are implicated in generation of the excision of segment U and production of the nested segment L by intramolecular recombination (50).

We propose a model for PDV DNA excision from the chromosome and for generation of nested segments via site-specific recombinations between LTRs and internal repeats (Fig. 6). In this model, integrated segment W with two LTRs and four recombination repeats (A to D) are illustrated. Segment W is excised from the wasp genome via recombination within the LTRs. The W template is amplified, possibly by a rolling circle type mechanism. Segments R and M are generated by recombinations between the B and C repeats and between the A/D and B repeats, respectively. Segment M could also be generated through an alternative pathway from R in which A/D and B/C repeats recombine. Segment C^2 may be produced from a recombination event involving another repeat type most probably between the repeats in the 3′ region of the WHv1.0 and WHv1.6 genes (Table 1). The failure of C^2 to hybridize to WHv1.6, WHv1.0, CsW1, or CsW2 probes (3, 4) suggests that the region between two cysteine-rich genes (with the *Sph*I site) is deleted during the recombination event that produces this segment. In all cases, homologous recombination between two repeats of a circular template generates two circular molecules with one repeat retained on each molecule. If homologous recombinations occur according to this model, many smaller segments will be produced. For example, smaller molecules of ~2.8 and 5.6 kbp would be produced from recombination events that produce R and M from the W template (Fig. 6). However, these smaller circles are not detected in viral genomic Southern blots (3, 4), possibly because these molecules lack currently undefined packaging signals for inclusion in the virion.

Site-specific homologous recombination events have been found in many systems (6). Generally, intramolecular recombination between directly repeated sites results in the excision, resolution, or deletion of the segments between the sites from the substrate molecule. Site-specific homologous recombination between inverted repeats usually leads to the inversion of the segment between the sites and may regulate gene expression (31, 45). The model that we propose resembles λ phage excision from the *Escherichia coli* chromosome (24) and transposition of Tn*3*-like transposons (17). Many higher plant mitochondrial genomes are also multipartite, consisting of DNA circles of variable sizes. In the tripartite *Brassica campestris*

FIG. 5. Comparison of nested viral segments W, R, and M. (A) Detailed physical maps of R and M showing extensive identity of regions to W, with abbreviations as in Fig. 1A. Dashed lines are used to align regions on segments R and M with segment W to indicate deletions in R and M. Recombination repeats A (same as D), B, and C are shown as hatched boxes. The recombination between B and C to produce segment R and recombination between A and B to produce segment M are further supported by sequence comparison in panel B. The open boxes on R and M are the regions that are different from segment W in physical maps and have been sequenced. In addition, the ~600-bp *Pst*I fragment on R and M (cross-hatched boxes; P600) were sequenced and are identical to the fragment on W. WHv1.0 and -1.6 genes are indicated as checkered boxes; LTRs are shown as solid boxes. The asterisks indicate single nucleotide substitutions that have abolished restriction sites K(7.3) and Xh(2.6) on R and M, respectively. (B) Sequence comparison between W, R, and M. Only alignments between regions around the recombination repeats are shown here. Recombination repeats B (3541 to 4035) and A (9071 to 9553) on W are used to align with recombined sequence on M, while recombination repeats B (3541 to 3934) and C (6337 to 6737) are used to align with the recombined sequence on R. A is the recombination repeat in the LTR which is identical to D. The recombination repeats on W and the recombined copy on M and R are marked by arrows. Dashes indicate identical nucleotides, and dots indicate gaps. Note the extensive homology of two parts of the M or R sequence, which are separated by an asterisk, to the corresponding recombination repeats. The recombined repeat is a hybrid of the two recombination repeats, not identical to either one. This suggests the homologous recombination between two recombination repeats on W generates R or M.

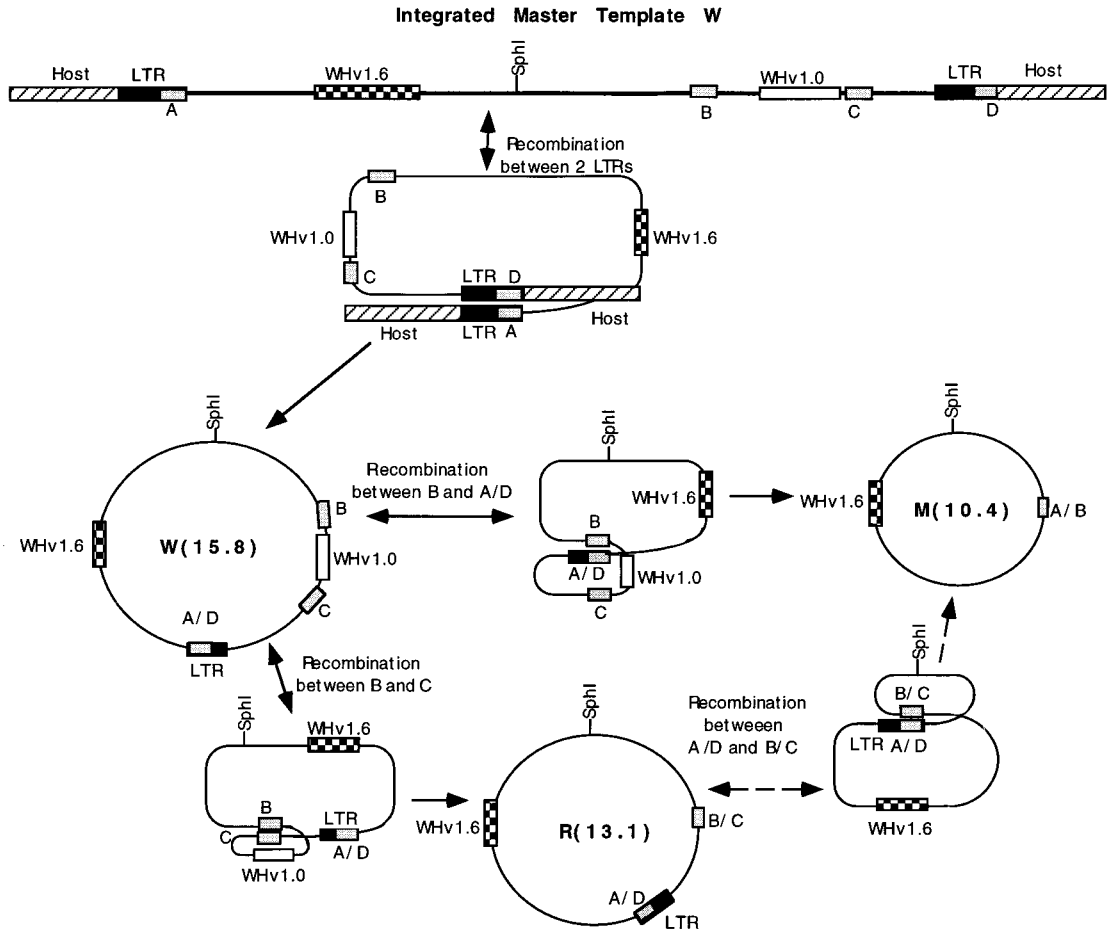


FIG. 6. A schematic diagram of CsPDV genome segment W integration and generation of the nested segments (not to scale). Wasp sequences are shown as hatched regions that flank the integrated W. Two LTRs and four recombination repeats (A, B, C, and D) are also shown. The WHv1.0 and -1.6 genes are shown as checkered boxes. Recombination between the two LTRs generates segment W. R is generated from circular W by recombination between repeats A/D and B. M can be produced from W and/or R by recombination between two repeats. During the recombination process, one copy of the recombining repeats is left in the circular DNA molecule.

mitochondrial genome, the 218-kbp DNA appears able to recombine intramolecularly between two direct repeats to produce smaller DNA circles that are partial duplications of the parent molecule (25). By analogy to these mechanistically related site-specific recombination systems, we speculate that a recombination system whose expression is developmentally regulated by ecdysone may exist within the wasp or CsPDV genome (47). Furthermore, this recombination system recognizes elements that lie within the LTRs and the internal repeats.

Segment nesting and PDV functions. PDV genomes contain nested segments belonging to distinct segment families. In CsPDV, two distinct segment families, W and V, are known, while in HfPDV, segments L and U are nested (50). Our preliminary Southern analyses indicate that several additional segment families exist in the CsPDV genome (unpublished data). Analysis of the bracovirus *Microplitis croceipes* PDV also indicates segment nesting (unpublished data). This is a particularly interesting result as these PDV genera are morphologically distinct and thought to be from evolutionarily independent lineages (39, 48). If PDVs are derived from morphologically similar free-living DNA viruses (e.g., bracoviruses from baculoviruses and ichnoviruses from ascoviruses), the evolutionary progenitor viruses would not have been segmented. Thus, segmentation would be a result of convergent evolution that may have resulted from the obligate association of the progenitor virus with parasitic wasps.

Segment nesting may be linked to PDV gene expression and function. In other biological systems, gene copy number is amplified as a means to increase gene expression (e.g., minisatellite chromosomes, polyploid and polytene cells, and gene amplification) (29, 33, 34). We suggest that a functional requirement for increasing the copy number of genes expressed in lepidopteran hosts has driven PDV genome segmentation and segment nesting. To date, the analyzed nested CsPDV genome segments (W and V) are present in hypermolar amounts and encode abundant secreted proteins (WHv1.0 and -1.6; VHv1.1 and -1.4) expressed only in parasitized insects (3, 4, 7, 8, 11, 32). Segment nesting differentially increases the copy number VHv1.4 and WHv1.6 genes (located on many nested segments) relative to their homologs found only on the parental segments (VHv1.1 and WHv1.0 on V and W, respectively). Since CsPDV does not appear to replicate in parasitized insects (42) and CsPDV promoters are of the early or constitutive type (9), the level of gene expression may depend largely on gene copy number. Therefore, segment nesting may be important for the generation of high copy number of functional genes in parasitized insects (14).

As a result of segment nesting, the sequence complexity of the CsPDV genome may be much less than estimated from

counting bands in agarose gels. The integrated segment W locus (~17 kbp including both LTRs) produces viral genome segments W, R, M, and C^2 (more than 47 kbp). The repetitive sequences on segment W suggest that this segment may have arisen through duplication and subsequent divergence possibly from sequences encoding a single ancestral cysteine-rich gene. These studies suggest that both real and apparent complexities exist in polydnavirus genomes and have significant implications for both functional and organizational studies of these fascinating viruses.

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