Campoletis sonorensis Endoparasitic Wasps Contain Forms of *C. sonorensis* Virus DNA Suggestive of Integrated and Extrachromosomal Polydnavirus DNAs

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Campoletis sonorensis virus (CsV) (Polydnaviridae) previously was detected only in the calyx epithelial cells and lumen of the oviducts from female *C. sonorensis* (Ichneumonidae) endoparasitic wasps (Norton et al., Cell Tissue Res. 162:195-208, 1975). Using dot-blot hybridizations, we detected low amounts of CsV DNA in male and female wasp head and thorax tissues and in male abdominal tissues. Low amounts of extrachromosomal viral DNA were detected in Southern blots of undigested male wasp DNA and in male DNA purified by isopycnic centrifugation. High-molecular-weight male wasp DNA digested with any of several restriction endonucleases and hybridized with cloned viral DNAs from CsV superhelices B and Q under stringent conditions contained CsV-specific DNA fragments that differed significantly in size and number from the hybridizing fragments detected in comparably digested viral DNA. Identical offsize restriction fragments were detected in digested female head and thorax DNA. These data suggest that at least CsV DNAs B and Q are integrated in *C. sonorensis* cellular DNA and that the virus may be transmitted through the germline.

Campoletis sonorensis virus (CsV) has only been observed to replicate in the calyx epithelial cells of the oviduct of female *C. sonorensis* (Hymenoptera, Ichneumonidae) wasps and accumulates in dense suspensions ("calyx fluid") in the oviduct lumen (9, 10, 13). Ovipositing female wasps inject the calyx fluid along with their eggs into *Heliothis virescens* (Lepidoptera, Noctuidae) host larvae, where CsV transcripts are detected within 2 h (5). Cross-protection studies and investigations of parasite survival in larvae artificially injected with purified virus instead of calyx fluid suggest that CsV compromises host cellular defense reactions such that the wasp egg is not encapsulated by host hemocytes (4, 13, 14). We hypothesize that CsV gene products may be involved in the abrogation of host cellular immune reactions and thereby protect the parasite egg or larva or both during endoparasitic development in the lepidopteran host.

CsV, the best-characterized virus in the family Polydnaviridae (Report of the International Committee on Taxonomy of Viruses, 5th International Congress of Virology, Sendai, Japan, 1 through 7 September 1984), has a complex genome composed of at least 28 superhelical DNA molecules which are present in nonequimolar ratios and range in size from 6 to 21 kilobase pairs (kbp) (8; G. W. Blissard et al., submitted for publication). In initial studies to characterize the viral genome, the six major DNAs which were used individually as probes did not cross-hybridize significantly with any of the other CsV DNAs, suggesting that the CsV genome is composed largely of nonhomologous sequences (8). These results and our studies of the expression of CsV in parasitized hosts suggests that the viral genome is multipartite (5, 8). Transcriptional mapping of some of the major CsV mRNAs found in parasitized host larvae further support the hypothesis that the CsV genome is multipartite (Blissard and Summers, submitted for publication).

Although it has been shown that CsV replicates in the

oviducts of female wasps and is expressed in parasitized hosts, the mechanism by which the virus is transmitted between wasp generations is not known. Previous electron microscopic studies suggested that CsV occurs only in *C. sonorensis* female wasps and that, similar to observations in other ichneumonid parasitic wasp-polydnavirus systems, all adult *C. sonorensis* females appear to contain CsV (13). These observations and the physical complexity of the CsV genome led us to speculate that the genetic relationship between the virus and parasite and the mechanism of CsV transmission might be more complex than one based on infection of developing parasite progeny with virus injected by the ovipositing female. Whether male wasps also contain the virus is unclear, since most of the published work has focused on virus morphology and replication in female wasps or the fate of the virus in parasitized hosts. We therefore examined *C. sonorensis* males for CsV-specific DNA to determine whether wasps of both sexes contain the virus. We also conducted Southern blot analyses to investigate the genetic relationship between the virus and the wasp and to understand the transmission of the virus better.

In this study we show that male *C. sonorensis* wasps contain CsV DNA. Virus-specific DNA also was detected in female wasp tissues which previously were thought not to contain virus. We present evidence suggesting that CsV DNA sequences are integrated in wasp cellular DNA.

MATERIALS AND METHODS

Insects. *C. sonorensis* (Cameron) and *H. virescens* (Fab.) were reared essentially as described previously (8). Individual parasitized host larvae were reared in separate containers; a single *C. sonorensis* wasp develops in each parasitized host. Adult female wasps are easily distinguished from adult males by the presence of a prominent ovipositor on the posterior abdomen. Newly eclosed adult wasps (≤ 24 h) were either extracted immediately or maintained in cages with other virgin wasps of the same sex.

Virus and viral DNA. CsV was isolated from oviducts

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excised from *C. sonorensis* females and purified on sucrose gradients as described previously (5, 8). Viral DNA was extracted from purified virus by proteinase K digestion and sodium lauryl sulfate disruption and purified on CsCl gradients (8). Only the superhelical DNA fraction was used unless otherwise stated.

Wasp cellular DNA. The sex of each individual *C. sonorensis* wasp was visually rechecked before extraction of DNA. DNA was extracted from pooled male wasps by one of three methods. Nucleic acids were extracted by brief homogenization in guanidine hydrochloride as described previously (method I) (5) or by proteinase K digestion of disrupted tissue by the method of Bradfield and Wyatt (method II) (1). A combination of methods I and II most consistently yielded high-molecular-weight DNA free of contaminating eye pigments (method III). Insects were frozen in liquid N₂ and ground in a mortar and pestle to a fine powder, which was suspended in 15 volumes of cold (−20°C) 6.6 M guanidine hydrochloride–25 mM EDTA–142 mM 2-mercaptoethanol (extraction buffer) per g of fresh weight. After the removal of cellular debris by low-speed centrifugation (1,700 × g, 3 min), total nucleic acids were ethanol precipitated in the presence of 250 mM sodium acetate. The pellet was digested with 200 µg of proteinase K per ml of 100 mM EDTA (pH 8.0)–0.5% sodium lauryl sarcosinate at 50°C for 3 h, phenol extracted as described previously (5), and ethanol precipitated. Total nucleic acids were treated with RNase A (10 µg per ml of 2 mM EDTA [pH 7.5]) at 37°C for 1 h, extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1), and ethanol precipitated. The resultant DNA was of high molecular weight and free of RNA as judged by electrophoresis in agarose gels.

Individual wasps or wasp body segments (head, thorax, abdomen) were extracted by crushing frozen (−80°C) tissues with a siliconized glass rod in 500 µl of cold (−20°C) extraction buffer containing 250 mM sodium acetate. The debris was removed by centrifugation at 15,600 × g for ≤30 s, yeast total RNA (Worthington Diagnostics, Freehold, N.J.) was added as a carrier, and the nucleic acids were ethanol precipitated. The pellet was digested with proteinase K, phenol extracted three times as described above, and ethanol precipitated. Restriction digests of these samples were treated with RNase A (10 µg/ml) at 37°C for 30 min before electrophoresis in agarose gels. Samples for dot blots were adjusted to 0.3 N NaOH and heated at 100°C for 3 min before application to nitrocellulose by the method of Kafatos et al. (7). Comparably treated controls containing only yeast total RNA were included in all dot blots of individual wasps or wasp body segments.

Viral DNA from male wasps. The amount of DNA homologous to CsV DNA present in individual wasps or body segments was estimated by hybridization of dot blots with ³²P-labeled CsV DNA and determination of the amount of bound viral DNA by scintillation counting of excised dots in the presence of a fluor (Econofluor; New England Nuclear Corp., Boston, Mass.). Values were corrected for background and compared with a standard curve derived from dot blots of CsV DNA (1 µg to 1 pg).

Viral DNA was isolated from male wasps by extracting total nucleic acids from ≥290 male wasps by extraction method III. After proteinase K digestion, half of the sample was centrifuged without further treatment, and 15 µg of CsV DNA was added to the remainder of the sample for a control. Test and control CsCl-ethidium bromide gradients were centrifuged on an SW60 Ti rotor at 142,000 × g for 96 h. Each gradient was fractionated from top to bottom in 200-µl

samples. After the refractive index of the fractions was determined, the samples were diluted and extracted with isoamyl alcohol, and the DNAs plus added carrier *Escherichia coli* tRNA were ethanol precipitated. Portions (40%) of each fraction were electrophoresed in 0.7% agarose gels. The nucleic acids were transferred to nitrocellulose filters and hybridized with ³²P-labeled CsV DNA. The experiment was repeated three times with total nucleic acids from two independently extracted samples of pooled male wasps.

Plasmid DNA. Viral DNAs for cloning were obtained by electrophoresing undigested CsV DNA in 0.7% agarose gels and electroeluting selected individual superhelical DNAs from excised gel slices. Purified superhelical DNAs were digested with restriction endonucleases and cloned in pUC8 vectors by standard methods. Clone pBE6600 was provided by D. Theilmann. Plasmid DNA was isolated by the method of Holmes and Quigley and purified on CsCl gradients (6). Because the complexity of the CsV genome makes it difficult to isolate individual superhelical molecules free of comigrating relaxed circular forms of other superhelices, radiolabeled cloned viral DNAs were hybridized to Southern blots of undigested CsV DNA to confirm the origin of each cloned DNA.

Restriction endonuclease digestion, agarose gel electrophoresis, and Southern blots. Restriction endonuclease digestions were done according to the specifications of the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.). The completeness of digestion of wasp cellular DNA samples was monitored by digesting duplicate wasp DNA samples containing added CsV DNA or vector-free cloned viral DNA. The control samples were electrophoresed in lanes adjacent to lanes with digested wasp cellular DNA (10 µg of DNA from pooled male wasps or DNA from a single male wasp in studies of individuals) or lanes with digested CsV DNA (250 ng and 1.5 µg). DNA samples were electrophoresed in 0.7 or 1.0% agarose gels and transferred unidirectionally to nitrocellulose by the method of Smith and Summers (12). Dot blots were prepared as described previously (7).

Radiolabeled DNA and hybridization. Vector-free cloned viral DNA used for probes was isolated from plasmids by restriction endonuclease digestion and electroelution from agarose gels. Superhelical CsV DNA or vector-free cloned viral DNA was labeled with [α -³²P]dATP to specific activities of 1 × 10⁸ to 5 × 10⁸ cpm/µg by the method of Rigby et al. (11). Radiolabeled DNA was diluted in hybridization buffer to 10 to 30 ng/ml. Hybridization buffer is composed of 50% formamide–5× Denhardt reagent–200 mM sodium phosphate buffer (pH 7.0)–5× SSC (1× SSC is 15 mM sodium citrate, 150 mM NaCl)–150 µg calf thymus DNA per ml. Blots of wasp cellular DNAs were hybridized with ≥200 ng of probe DNA. After hybridization at 43°C for 40 to 48 h, the blots were washed three times in 2× SSC–0.1% sodium lauryl sulfate at room temperature for 15 min and six times in 0.1× SSC–0.1% sodium lauryl sulfate at 55°C for 15 min. Autoradiography on XRP-1 or XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.) was done at −80°C. Short and long exposures of all autoradiograms of both male and viral DNAs were done to detect minor bands. However, representative data are shown in the figures, and exposure times for the pictured autoradiograms are given in the legends.

RESULTS

Presence of CsV-specific DNA in male and female wasps. To determine whether male wasps contain virus-specific DNA, ³²P-labeled CsV DNA was hybridized with dot blots of

FIG. 1. Wasp DNA homologous to CsV DNA. DNA was isolated from individual whole virgin male or female *C. sonorensis* adult wasps (24 h posteclosion) or from a single body segment (head, thorax, or abdomen) of an individual male or female and applied to nitrocellulose filters. Dot blots were hybridized with ^{32}P -labeled CsV DNA under stringent conditions.

cellular DNA isolated from individual male wasps. All samples of DNA separately extracted from individual male wasps (50 replicates) hybridized to readily detectable levels with CsV DNA probes (Fig. 1). This is probably not due to viral contamination of the insect's cuticle, since washing wasps in $0.1\times$ or $4\times$ SSC before DNA extraction did not decrease the amount of CsV-specific DNA detected in male wasps by autoradiography or scintillation counting of dot blots (data not shown). Each male *C. sonorensis* wasp contains approximately 10 ± 2 pg of DNA that is homologous to CsV DNA (mean of 25 replicates) (Table 1). When the head, thorax, and abdomen of an individual male wasp were extracted and blotted separately, the amount of CsV-specific DNA present in each male body segment correlated roughly with the average fresh weight of the body segment (Table 1, Fig. 1). Time course studies of individual male wasps extracted from approximately 12 h to 17 days posteclosion indicated that the amount of DNA homologous to CsV DNA per male wasp remains relatively constant throughout the wasp's adult life (three replicates per time point, two experiments) (Fig. 2). In contrast, female wasps (≥ 3 days posteclosion) contain approximately 140 ± 36 ng of DNA homologous to CsV DNA (mean of 21 replicates) (Table 1). The higher amount of ^{32}P -labeled CsV DNA bound by whole female DNA is likely due to virus in the calyx cells

TABLE 1. Fresh weight and DNA homologous to CsV DNA in male and female wasps

Body part	Male wasp		Female wasp	
	Fresh wt (mg) ^a	DNA homologous to CsV DNA (pg) ^b	Fresh wt (mg) ^a	DNA homologous to CsV DNA (pg) ^b
Whole body	3.8	10	4.7	1.4×10^5 ^c
Head	0.6	2	0.6	3
Thorax	1.9	4	2.0	16
Abdomen	1.3	4	2.1	1.4×10^5

^a Mean of ≥ 21 replicates per whole insect or individual body segment.

^b Values for whole bodies are means of 25 and 21 replicates for males and females, respectively. Values for individual body segments are means of four replicates per body segment per sex.

^c Adult females ≥ 3 days posteclosion (see the text).

FIG. 2. CsV-specific DNA in male and female adult wasps of different ages. DNA was isolated from individual virgin male or female adult wasps at the indicated times posteclosion and serially diluted 1:2 before application to nitrocellulose. Each row shows the serially diluted DNA from a single insect. Dot blots were hybridized with ^{32}P -labeled CsV DNA under stringent conditions.

and oviduct lumen, since it approximately equals the average amount of viral DNA (150 ng) per female which we routinely recover from purified virus from excised oviducts (unpublished results). Although abdomen DNA accounted for most of the female DNA that was homologous to CsV DNA (Table 1, Fig. 1), female head or thorax DNA also hybridized with viral DNA. The amount of CsV-specific DNA in female head or thorax tissues correlated with the fresh weight of the body segment and was very similar to the amount detected in male heads or thoraces (Table 1, Fig. 1). Time course studies of individually extracted whole females from ca. 12 h to 17 days posteclosion indicated that the amount of CsV-specific DNA (approximately 140 ng per female) remains relatively constant after 3 days posteclosion (Fig. 2). Adult female wasps ≤ 24 h posteclosion appear to contain approximately 87 ± 29 ng of CsV-specific DNA (mean of 10 replicates), suggesting that some virus replication occurs in the adult female during at least the first 3 days posteclosion (Fig. 2).

Physical structure of CsV DNA in male wasps. CsV DNA and two cloned CsV DNAs, pQB7200, a 7.2-kbp *Bam*HI fragment of CsV superhelix Q (12.2 kbp), and pBE6600, a 6.6-kbp *Eco*RI fragment of CsV superhelix B (6.6 kbp), were used as probes to examine the physical structure of the homologous DNA found in male wasps. The cloned DNAs were selected for the following reasons. (i) Each fragment was cloned from a superhelical DNA that is one of the most abundant DNAs in the viral genome. The two superhelices

FIG. 3. Extrachromosomal viral DNA in male wasps. Southern blots of undigested DNA isolated from male wasps were hybridized with ^{32}P -labeled CsV DNA, vector-free pQB7200, or pBE6600 DNA probes under stringent conditions. Lanes with male DNA contain DNA (10 μg) from 7.7 male wasps for CsV DNA and pBE6600 DNA (a) probes or DNA (20 μg) from 15.4 males for pQB7200 and pBE6600 (b) DNA probes. Control lanes contain similarly isolated DNA from one female wasp or 250 ng of undigested CsV DNA isolated from purified virus. Blots are from similar but different gels and cannot be compared directly. A second blot hybridized with pBE6600 DNA (b) but containing only a CsV DNA control is shown because of its superior resolution. Pictured autoradiograms of CsV DNA or female DNA controls were exposed for approximately 20 h without an intensifying screen, and lanes containing DNA from males were exposed for ≥ 7 days with an intensifying screen. Except for the relaxed circular and linear forms of viral superhelix B (B_{RC} and B_{LIN} , respectively), bands indicated by letters represent CsV superhelical DNAs designated according to the previously established nomenclature (8).

are well resolved in agarose gels. (ii) Superhelical DNAs B and Q did not cross-hybridize with one another or with other viral superhelical DNAs in previously reported studies (8). Our preliminary experiments showed that the two cloned viral DNAs do not cross-hybridize with one another (J. Fleming, unpublished results). (iii) pBE6600 contains the entire CsV superhelix B as determined by physical mapping of independently cloned *EcoRI* and *BamHI* DNA fragments of electroeluted superhelix B and blot hybridization (D. Theilmann and J. Fleming, unpublished results). (iv) pQB7200 and pBE6600 hybridize with cDNAs of mRNAs isolated from parasitized larvae, indicating that they contain sequences that are expressed in parasitized *H. virescens* hosts (J. Fleming, unpublished results).

Labeled CsV DNA hybridized to undigested high-molecular-weight cellular DNA isolated from individual male wasps or pooled male wasps (10 μg per lane, i.e., the DNA from approximately 7.7 male wasps per lane) in Southern blots, but CsV superhelical or relaxed circular DNAs were not detected even after prolonged exposure of autoradiograms (Fig. 3). CsV superhelical DNAs, however, were detected in controls of (whole) female wasp DNA (DNA from one female wasp per lane) isolated from pooled females by the same method (Fig. 3). *HindIII*-digested DNA from pooled male wasps or *HindIII*-, *Sall*-, or *BamHI*-digested DNA from individual males (≥ 3 replicates per enzyme) had restriction endonuclease fragment patterns similar to those of comparably digested CsV DNA when ^{32}P -labeled CsV DNA was used as the probe (data not shown).

^{32}P -labeled pQB7200 hybridized to undigested male wasp cellular DNA, but viral superhelical or relaxed circular DNAs were not detected in DNA isolated from pooled males

(20 μg or DNA from 15.4 male wasps per lane) even after prolonged exposure of autoradiograms (Fig. 3). pQB7200 hybridized strongly to CsV superhelix Q DNA (12.2 kbp) and weakly to CsV DNAs (in order of decreasing intensity) J (9.2 kbp), A_2 (6.3 kbp), and L_1 (9.8 kbp) in controls of undigested CsV superhelical DNA or total DNA isolated from female wasps (Fig. 3). The additional female DNAs which hybridized (Fig. 3) appear to be relaxed circular viral DNAs homologous to pQB7200 DNA, since comigrating bands were observed in other control lanes containing undigested relaxed circular CsV DNA from CsCl-ethidium bromide gradients.

Radiolabeled pBE6600 DNA hybridized with high-molecular-weight cellular DNA isolated from male wasps. In contrast to the results obtained with labeled CsV or pQB7200 DNAs, superhelical, relaxed circular, and linear (6.6-kbp) forms of CsV DNA B were detected in undigested male DNA (DNA from ≥ 7.7 males per lane) after prolonged exposure of autoradiograms (Fig. 3). In CsV superhelical DNA controls, vector-free pBE6600 DNA hybridized (in order of decreasing intensity) to CsV superhelices B (6.6 kbp), R (13.3 kbp), and A_3 (6.5 kbp). (A_3 appeared to migrate immediately below superhelix B and above A_2 ; although it is partially obscured by superhelix B in the figures, A_3 was consistently detected in shorter exposures of blots of CsV DNA hybridized with pBE6600. A_2 [6.3 kbp] is a CsV superhelical DNA that recently has been observed when higher amounts of DNA are electrophoresed than those used in a previous report [8] in which A_1 was identified [Blissard et al., submitted for publication].) The additional DNA bands homologous to pBE6600 from comparably extracted females comigrated with the relaxed circular forms of viral DNAs B, R, and A_3 .

To confirm that extrachromosomal viral DNAs are present in *C. sonorensis* males, total nucleic acids were isolated from pooled male wasps and centrifuged on CsCl-ethidium bromide gradients. CsV relaxed circular and superhelical DNAs as well as high-molecular-weight cellular DNA were detected in nucleic acids isolated from male wasps in all three experiments (Fig. 4). The DNA in fractions in which each of the three types of DNA, i.e., chromosomal, relaxed circular, or superhelical viral DNA, was most clearly resolved from the other types of DNA was digested with *Eco*RI, transferred to nitrocellulose, and hybridized with 32 P-labeled pQB7200 DNA to examine the physical structure of CsV-specific sequences in extrachromosomal and high-molecular-weight cellular DNAs present in male wasps. The probe hybridized with *Eco*RI fragments of 1.6 and 5.6 kbp in digested relaxed circular or superhelical DNAs or viral DNA controls. In contrast, 32 P-labeled pQB7200 hybridized with *Eco*RI fragments of 1.0, 1.6, and 2.5 kbp in digested high-molecular-weight cellular DNA (data not shown). As supported by additional blot hybridizations of digested high-molecular-weight cellular DNA (described below), these data indicate that male wasps contain both extrachromosomal (viral) DNAs and CsV sequences that are organized in a manner distinctly different from that in the intact virus.

The hybridization patterns of each cloned viral DNA with restriction endonuclease digested viral and wasp cellular

FIG. 4. CsCl gradients of male wasp DNA. DNA isolated from approximately 150 male wasps was centrifuged on a CsCl-ethidium bromide gradient which was fractionated (200 μ l/fraction) from top (fraction no. 1) to bottom (fraction no. 20, not shown). A portion of the nucleic acids precipitated from each fraction was electrophoresed in agarose gels and transferred to nitrocellulose filters, which were hybridized with 32 P-labeled CsV DNA under stringent conditions. Chromosomal (Ch), relaxed circular viral (RC), and superhelical viral (SH) DNAs in male wasp DNA are from an autoradiogram exposed for approximately 72 h with an intensifying screen. Autoradiograms of a CsV DNA control were exposed for approximately 8 h without an intensifying screen. The major superhelical DNAs in the purified CsV DNA control (125 ng) are designated by letter according to the previously described nomenclature (8). The regions of the gel which contain mostly relaxed circular or superhelical DNAs are indicated to the right of the control (CsV) lane.

FIG. 5. DNA homologous to CsV superhelix B in male wasps. Southern blots of *Eco*RI- or *Sal*I-digested male DNA (10 μ g per lane) or CsV DNA (V, 125 ng/lane) were hybridized with 32 P-labeled vector-free pBE6600 DNA (CsV superhelix B) under stringent conditions. Pictured autoradiograms of male DNA were exposed for 24 h with an intensifying screen (male or male_s). A 6.6-kbp fragment (the linear form of superhelix B) is detected in longer exposures (male_s, 96 h with an intensifying screen). Digested CsV DNA controls are from autoradiograms exposed for <12 h without a screen. The most intense bands in *Sal*I-digested CsV DNA represent relaxed circular and superhelical forms of CsV superhelix B (B_{RC} and B_{SH} , respectively), which lacks a *Sal*I site. *Hind*III-digested lambda DNA markers for the two gels (M_1 and M_2) are indicated.

DNAs were compared to determine whether CsV-specific DNAs are present in male wasps in both extrachromosomal and integrated forms. The size or number (or both) of DNA fragments in digested male wasp cellular DNAs which hybridized with either pQB7200 or pBE6600 differed significantly from those observed in digested CsV DNA (Fig. 5 through 7, Table 2). These differences were not likely to reflect partial digestion products, since all controls containing male DNA plus added viral or cloned viral DNA had patterns indicative of complete digestion.

Male wasp DNA digested with *Sal*I contained a single 18.3-kbp fragment which hybridized with labeled vector-free pBE6600 DNA, which lacks an *Sal*I site (Fig. 5, Table 2). A band comigrating with the superhelical form of CsV superhelix B (which lacks an *Sal*I site) also was detected in *Sal*I-digested male DNA after prolonged exposure of autoradiograms. The probe hybridized strongly with the relaxed

TABLE 2. Restriction fragments in CsV and *C. sonorensis* cellular DNAs homologous to cloned viral DNAs

Probe	Enzyme(s)	Sizes of homologous DNAs (kbp)	
		Male DNA	CsV DNA
pBE6600	<i>SalI</i>	18.3	B _{SH} , B _{RC} ^a
	<i>EcoRI</i>	4.2, 5.1	6.6
	<i>XhoI</i>	1.4, 5.6	1.2, 5.5
	<i>XbaI</i>	4.2, 4.8	6.6
	<i>EcoRI-XhoI</i>	1.4, 1.9, 2.4, 3.0	1.2, 2.4, 3.0
pQB7200	<i>PstI</i>	17.2	6.7, 8.5, 11.7, >23
	<i>BamHI</i>	10.2	4.8, 7.2, 8.9
	<i>SalI</i>	3.2, 6.5	3.2, 9.5
	<i>EcoRI</i>	1.0, ^b 1.6, ^c 2.5 ^c	1.0, ^b 1.6, ^c 5.6 ^{b,c}
	<i>XhoI</i>	1.3, 4.5	1.2, 4.5, 7.6
	<i>EcoRI-SalI</i>	1.0, ^b 1.6, ^c 2.3, ^b 2.5, ^c	1.0, ^b 1.6, ^c 2.3, ^b 3.2 ^c
	<i>XhoI-SalI</i>	1.3, 1.5, 3.0	1.3, 1.5, 3.0, 7.5
	<i>EcoRI-XhoI</i>	1.0, 1.3, 1.6, 2.5, 4.1	1.0, 1.3, 1.6, 1.7, 4.1

^a B_{SH}, Viral superhelix B; B_{RC} viral relaxed circular B.

^b Hybridized with pQBS2400.

^c Hybridized with pQBS4800.

circular and superhelical forms of CsV superhelix B and weakly with two fragments of approximately 6.1 and 7.9 kbp in *SalI*-digested viral DNA. Hybridization of ³²P-labeled pBE6600 with male wasp cellular DNA digested with restriction endonucleases (*EcoRI*, *XhoI*, *XbaI*, or combinations of these enzymes) for which one or more sites exist on cloned CsV superhelix B resulted in the detection of a number of homologous DNA fragments that differed significantly from those detected in comparably digested CsV DNA (Table 2). The results obtained with *EcoRI* illustrate these differences. *EcoRI*-digested male DNA contained two fragments of 4.2 and 5.1 kbp homologous to the pBE6600 probe. In contrast, *EcoRI*-digested CsV DNA contained a single 6.6-kbp homologous fragment (linear CsV B) (Fig. 5, Table 2). Long exposures of autoradiograms resulted in the detection of a faint 6.6-kbp fragment in *EcoRI*-digested male DNA (Fig. 5, lane male₁). However, fragments of 4.2 and 5.1 kbp were not detectable in *EcoRI*-digested CsV DNA even after prolonged exposure of autoradiograms. Identical results were obtained with male wasp DNA isolated by each of the three extraction methods employed, indicating that the observed differences are unlikely to result from changes in the DNA during isolation. *SalI*- or *XhoI*-digested DNA from individual male wasps contained the same restriction fragments homologous to pBE6600 as comparably digested DNA from pooled males (data not shown).

The differences between digested viral and male wasp DNAs that were detected with pQB7200 probes also were significant. The viral DNA in pQB7200 lacks a *PstI* site, but the ³²P-labeled pQB7200 probe hybridized strongly with four *PstI* fragments (>>23 kbp and 6.7, 8.5, and 11.7 kbp) and weakly with fragments of 2.0 and 3.1 kbp in *PstI*-digested CsV DNA (Fig. 6, Table 2). In contrast, *PstI*-digested male DNA contained a fragment which migrated only slightly faster than the 23.1-kbp lambda *HindIII* DNA fragment and had an approximate size of 17.2 kbp (Fig. 6, Table 2). Similarly, labeled pQB7200 hybridized strongly with a 7.2-kbp fragment in *BamHI*-digested CsV DNA versus a 10.2-kbp fragment in *BamHI*-digested male DNA (Fig. 6, Table 2). The probe also hybridized moderately with *BamHI* fragments of 4.8 and 8.9 kbp and weakly with a 6.2-kbp *BamHI* fragment in digested viral DNA. The 4.8-kbp fragment also was detected in digested male DNA after long exposures.

Male DNA digested with *SalI*, *EcoRI*, or *XhoI*, for which there are single sites on pQB7200, or with combinations of these enzymes also contained restriction fragments that differed significantly in size and number from those detected in digested viral DNA with pQB7200 probes (Table 2). The results with *EcoRI* and *EcoRI-SalI* illustrate some of these differences (Fig. 7). pQB7200 DNA hybridized with 1.6- and 5.6-kbp *EcoRI* fragments of viral DNA, indicating that *EcoRI* sites closely flank the *BamHI* sites on CsV superhelix Q. A 1.0-kbp *EcoRI* fragment was detected in viral DNA in longer exposures. The probe hybridized strongly with fragments of 1.6 and 2.5 kbp and weakly with a 1.0-kbp fragment in *EcoRI*-digested male DNA. A 5.6-kbp *EcoRI* fragment was detected in male DNA only in autoradiograms exposed for long periods. Double digests of CsV DNA with *EcoRI* and *SalI* resulted in the predicted 1.6-, 2.3-, and 3.2-kbp fragments homologous to pQB7200 DNA as well as a faint band of 1.0 kbp. Male wasp DNA digested with *EcoRI* and *SalI* contained readily detectable fragments of 1.6, 2.3, and 2.5 kbp which were homologous to pQB7200 (Fig. 7, Table 2). The 3.2-kbp fragment observed in viral DNA was not detected in male DNA even after prolonged exposure of autoradiograms. Similar results were obtained with *EcoRI*- and *EcoRI-SalI*-digested DNA isolated from individual males (data not shown).

The 2.35- and 4.8-kbp *BamHI-SalI* fragments of pQB7200 (Fig. 7) were subcloned (pQBS2400 and pQBS4800, respec-

FIG. 6. DNA homologous to CsV superhelix Q in male wasps. Southern blots of *BamHI*- or *PstI*-digested male DNA (10 µg) or CsV DNA (V, 125 ng) were hybridized with ³²P-labeled vector-free pQB7200 DNA (7.2-kbp *BamHI* fragment of CsV superhelix Q) under stringent conditions. Pictured autoradiograms of blots of male DNA were exposed for 24 h with an intensifying screen, and control viral DNA blots are from an autoradiogram exposed for 24 h without a screen. A physical map of pQB7200 is shown in Fig. 7 for comparison. Markers of lambda DNA digested with *HindIII* are indicated.

FIG. 7. DNA homologous to CsV superhelix Q in male wasps. Southern blots of *EcoRI*- or *EcoRI-SalI*-digested male DNA (10 μ g) or CsV DNA (V, 250 ng) were hybridized with 32 P-labeled vector-free pQB7200 DNA (7200) or labeled subcloned *BamHI-SalI* fragments of pQB7200 (pQBS2400 [2400] and pQBS4800 [4800]) under stringent conditions. Pictured autoradiograms of viral and male DNA were exposed for <6 and 24 h, respectively, without screens. The hatched area on the physical map indicates a 1,300-base-pair *BglII-SalI* fragment to which a 900-base viral mRNA detected in parasitized *H. virescens* host larvae has been mapped (J. Fleming, unpublished data; Blissard, et al., in press). Markers of lambda DNA digested with *HindIII* are indicated.

tively) and used as probes against viral and male DNA digested with *EcoRI* and *SalI* to determine which regions of the pQB7200 probe were homologous to the various hybridizing fragments in male DNA. Labeled pQBS2400 hybridized with fragments of 1.0 and 2.3 kbp in both viral and male DNA (Fig. 7, Table 2). 32 P-labeled pQBS4800 hybridized with a 1.6-kbp fragment that also was present in both male and viral DNA. The pQBS4800 probe hybridized with a 3.2-kbp fragment in viral DNA as predicted by the physical map of pQB7200 (Fig. 7), but this fragment was barely detectable in autoradiograms of digested male DNA exposed

for prolonged periods. In contrast, pQBS4800 hybridized strongly with a 2.5-kbp fragment in *EcoRI-SalI*-digested male DNA (Fig. 7, Table 2). Male DNA fragments of 3.7 and 5.0 kbp, which were detected with pQB7200 probes only after long exposures, hybridized to readily detectable levels with pQBS4800 probes. These data suggest that the central 3.2-kbp *EcoRI-SalI* fragment in the 7.2-kbp *BamHI* fragment of CsV Q (i.e., pQB7200) is altered in male wasp DNA, although other differences in the flanking regions may also exist. Additional digests suggest that the 1.7-kbp *EcoRI-XhoI* fragment homologous to pQB7200 is altered in males

FIG. 8. CsV-specific DNA in female wasp DNA. Southern blots of undigested CsV DNA (V, 125 ng) or DNA from heads and thoraces of female wasps (15 μ g) and blots of *Eco*RI-digested CsV, male (whole body), and female (head and thorax) DNAs (125 ng, 15 μ g, and 15 μ g, respectively) were hybridized with 32 P-labeled vector-free pBE6600 or pQB7200 DNA under stringent conditions. All pictured autoradiograms except the lane of undigested CsV DNA hybridized with pBE6600 were exposed for approximately 41 h without a screen. The lane showing undigested CsV DNA hybridized with pBE6600 DNA is from an autoradiogram exposed for 18 h without a screen. Letters next to lanes containing undigested DNAs designate viral superhelical DNAs. Offsize *Eco*RI DNA fragments in male and female DNA are marked by arrows. Fragments migrating close to the offsize restriction fragments but found in both female and viral DNA are marked with an asterisk. Undigested DNAs homologous to pQB7200 or pBE6600 and migrating below CsV superhelix A_1 are marked by dots. Markers of lambda DNA digested with *Hind*III are indicated.

(Table 2), but further mapping is required to locate the altered region on the 3.2-kbp *Eco*RI-*Sal*I fragment more accurately.

Physical structure of CsV DNA in female wasps. DNA was isolated from pooled female head and thorax tissues to compare the organization of CsV-specific DNAs in males and females. Female wasp head and thorax tissues contain low amounts of CsV-specific DNA as determined by dot-blot hybridization (Fig. 1, Table 1) and therefore should contain fairly low amounts of extrachromosomal viral DNA that might obscure offsize restriction fragments if they are present in females as well as males. When labeled pBE6600 and pQB7200 DNAs were hybridized with undigested female DNA, all of the superhelical and relaxed circular DNAs detected in control viral DNA also were detected in female head and thorax DNA (Fig. 8). Undigested female head and thorax DNA contained a few additional, hybridizing DNAs that migrated slowly in agarose gels and that were not detected in undigested CsV DNA isolated from purified virus. Each probe also hybridized with undigested viral and female DNAs which migrated below the smallest previously detected CsV superhelix (A_1) (8) and which were not visible

in ethidium bromide-stained gels (Fig. 8). (Comparable bands were observed in undigested female and viral DNAs in longer exposures of autoradiograms shown in Fig. 3, but were not observed in undigested male DNA even after prolonged exposure.)

*Eco*RI-digested female head and thorax DNA contained fragments of 4.2 and 5.1 kbp homologous to pBE6600 and fragments of 1.6 and 2.5 kbp homologous to pQB7200 (Fig. 8). Fragments of identical size were detected in control *Eco*RI digests of equal amounts (15 μ g) of male (whole insect) DNA. Except for the 1.6-kbp fragment, which is also found in digested viral DNA, the intensity of these offsize fragments in digested male and female DNA was approximately equal (Fig. 8). Digested female head and thorax DNA also contained *Eco*RI DNA fragments of the same size as those detected in digested CsV DNA (fragments of 2.4 and 6.6 kbp homologous to pBE6600 and fragments of 1.6, 5.6, and 8.2 kbp homologous to pQB7200). In contrast to results with an equivalent amount of (whole insect) male DNA (15 μ g), the fragments identical to those in viral DNA were more intense than the offsize restriction fragments in female head and thorax DNA and were readily detectable after short

exposures, confirming that female heads or thoraces contain more extrachromosomal viral DNA than comparable male tissues as shown above with undigested wasp DNAs (Fig. 8). The low-molecular-weight DNAs detected below A_1 in undigested viral and female DNA and homologous to pQB7200 were not observed in *EcoRI*-digested DNAs. However, the mobility of the low-molecular-weight DNA detected below A_1 with pBE6600 probes was not significantly affected by *EcoRI* digestion (Fig. 8).

DISCUSSION

C. sonorensis male wasps contain CsV-specific DNA. DNA from all individually extracted male wasps, which are easily distinguished from females, and DNA from heads or thoraces of either male or female wasps hybridized with CsV DNA under stringent conditions. Significantly, two forms of CsV-specific DNA appear to be present in male and female wasps: (i) a form which is identical to that isolated from purified virions, and (ii) a second type which our Southern blot analyses suggest may represent integrated viral sequences, intermediates of recombination between viral superhelices, or some other differently organized form of viral DNA.

The size or number (or both) of restriction endonuclease DNA fragments detected in digested male wasp cellular DNA with either of two cloned viral DNA probes differed significantly from those detected in similarly digested CsV DNA (Table 2). Significant differences were detected with enzymes for which there are no sites on the probe DNAs as well as with enzymes which cleave the cloned DNAs one or more times. In preliminary experiments, *EcoRI*-digested female head and thorax DNA also contained offsize restriction fragments identical to those detected in male DNA, and these offsize fragments were present in the DNAs of the two sexes in similar amounts. The consistent detection of altered restriction patterns relative to viral DNA strongly suggests that at least some CsV DNAs are integrated in wasp cellular DNA. Moreover, the viral DNAs appear to be integrated in wasp DNA in the same configuration in males and females. The occurrence of CsV-specific DNA in all body segments of male and female wasps, its presence in very low amounts that correlate roughly with the tissues' fresh weight, and its persistence throughout the adult life of male wasps also are consistent with an integrated form of CsV DNA. Because the genome size of *C. sonorensis* wasps is not known, we could not determine the number of copies of the putatively integrated viral sequences in male wasp DNA with certainty. However, our preliminary experiments using the genome size of another hymenopteran, *Apis mellifera*, as an estimate of genome size (approximately 3.5×10^5 kbp per haploid genome) suggest that integrated DNAs homologous to CsV superhelices B and Q are present in male and female wasp cellular DNA in low copy number (2). It will be of interest to determine whether other CsV DNA probes besides B and Q also detect offsize restriction fragments and hence probably are integrated, since the cloned B and Q DNAs contain sequences that are expressed in parasitized host larvae and therefore may not be representative of all CsV superhelical DNAs. However, to prove that any of the CsV DNAs are in fact integrated in chromosomal DNA, cloning and nucleotide sequencing of *C. sonorensis* DNAs will be necessary to demonstrate that cellular DNA sequences flank viral DNA sequences at putative junction regions (3).

In addition to the virus-specific DNA detected as offsize restriction fragments, male and female wasps also contain a

form of CsV DNA that appeared to be identical to the DNA isolated from purified virus. Whether this extrachromosomal viral DNA in male wasps is episomal or present in virions is unclear. Although immunoelectron microscopic studies and Western blot analyses will indicate whether male wasps contain CsV particles, physiological experiments will be necessary to distinguish between virus particles acquired *per os* during endoparasitic development and virus replicated *in situ*. Nevertheless, the presence of this extrachromosomal form of CsV DNA suggests that virus replication possibly may occur in both males and females, albeit at markedly different levels in the two sexes. If replication does occur, the offsize restriction fragments alternatively may reflect the existence of replicative or recombinational intermediate forms from which the various viral superhelices found in virions are derived. Since the structural relationship between the different CsV superhelical DNAs is not well understood and now appears to be more complex than the initial report (8) indicated, a more detailed examination of the structure of the viral genome and its replication strategy will be necessary to evaluate this alternative hypothesis for the offsize restriction fragments. However, it should be noted that based on the intensity of the virus-specific fragments, the form of virus-specific DNA yielding offsize restriction fragments is considerably more abundant in males than the extrachromosomal or virion-derived form (Fig. 5), whereas the converse is true of female head and thorax DNA (Fig. 8). In spite of these marked differences in the amount of the extrachromosomal form in the two sexes, the intensities of the offsize restriction fragments in male and female DNA were roughly similar (Fig. 8). If the offsize restriction fragments are derived from intermediates of viral DNA replication or recombination and if they are present in amounts that correlate directly with the level of replication or recombination, one might expect a greater disparity in the relative intensity of the offsize restriction fragments in male and female DNA than was observed. In contrast, one would expect roughly similar amounts of the offsize restriction fragments per microgram of male or female DNA if integrated viral DNAs were to give rise to the offsize restriction fragments.

Differences in restriction patterns of male and viral DNAs were detected only with the cloned viral DNA probes. The hybridization patterns of digested CsV DNA and digested DNA from individual or pooled male wasps were similar when ^{32}P -labeled total CsV DNA was used as the probe. All of the CsV superhelical and relaxed circular DNAs were detected in extrachromosomal viral DNA isolated from several hundred pooled males and purified CsCl-ethidium bromide gradients (Fig. 4). (We conjecture that only superhelix B was detected in our Southern blots of undigested male DNA [Fig. 3], because of the extremely low amount of extrachromosomal DNA per male, the much smaller amount of DNA that was electrophoresed, and the probably somewhat greater nicking of the larger superhelices during the more extensive purification of the DNA not centrifuged on CsCl gradients.) The large size and complexity of the CsV genome (8) result in complicated restriction fragment patterns. Regardless of whether the CsV-specific DNAs are present in males in extrachromosomal, integrated, or recombinational intermediate forms, complicated restriction patterns would be observed, if all of the viral DNAs were present. Where a complex pattern exists, subtle differences would be detectable only with specific probes. It should be considered that even where pBE6600 and pQB7200 probes were used, some of the restriction frag-

ments detected were similar to those in the viral controls (Table 2). Hence, the use of a complex probe such as total viral DNA may have obscured subtle differences such as offsize restriction fragments and may account for the similarity of restriction patterns when ^{32}P -labeled CsV DNA was used as the probe.

The hybridization of the two cloned viral DNAs with viral superhelical DNAs besides those from which they were cloned was unexpected, since Krell and his co-workers reported limited or no cross-hybridization of CsV superhelices B and Q with other viral DNAs (8). We recently have observed more extensive cross-hybridization of other cloned viral DNAs with CsV superhelical DNAs than the previous report would predict (Blissard and Summers, in press; and D. Theilmann and J. Fleming, unpublished results). Because hybridizations in the present study were done under stringent conditions, we currently are unable to account for this discrepancy with the previously published study (8). These data suggest, however, that additional work including physical mapping of the viral DNAs and C_0T analyses should be done to clarify the level of internal sequence homology within the CsV genome.

In addition to the unexpected cross-hybridization of the probes with the previously described superhelices, each cloned viral DNA probe hybridized with one or more diffuse DNA bands that migrated below superhelix A_1 in undigested female wasp or CsV DNA. Two observations suggest that these DNAs probably are not partially degraded forms of viral DNAs. First, the relative mobilities of the diffuse DNA bands homologous to each of the two probes differ (Fig. 8). In addition, the migration of these DNA bands relative to superhelix A_1 and to each other is consistent in different blots of several different CsV DNA samples (compare Fig. 3 and 8; data not shown). Second, *EcoRI* digestion did not alter the mobility of the diffuse DNA band homologous to pBE6600, but apparently did cleave the DNAs homologous to pQB7200. The physical structure of these DNAs is not known and is currently under investigation.

Our data indicate that the relationship between the virus and parasite is more complex than was thought formerly and that CsV may be vertically transmitted through the germline. It is noteworthy that the same offsize restriction fragments were detected in all samples of cellular DNA isolated from pooled male wasps over a period of 2 years. If the offsize restriction fragments originate from integrated DNAs, this suggests that sequences homologous to CsV DNAs B and Q are stably integrated at specific sites in chromosomal DNA. Although this may indicate site-specific integration or a high degree of inbreeding of our *C. sonorensis* colony, it alternatively may reflect an integration event that occurred much earlier in the coevolution of the endoparasite and its polydnavirus. Vertical transmission of CsV is an attractive hypothesis since integration of viral DNA in wasp DNA would be a highly efficient transmission mechanism and would effectively maintain the apparently species-specific association between a given polydnavirus and a particular endoparasite species. These considerations may be important since CsV and other polydnaviruses may play an essential role during wasp endoparasitic development and thus may ultimately determine the wasp's reproductive success. However, ovipositing female wasps inject host larvae with significant amounts of polydnaviruses (D. Theilmann and M. Summers, manuscript in preparation), and wasp progeny may acquire virus per os and become infected during endoparasitic development. Per os transmission would suggest that the offsize restriction fragments are

more likely to originate from replicative or recombinational forms but would not necessarily rule out repeated site-specific integration in this unusual biological system. Biochemical investigations with additional ichneumonid wasp polydnavirus systems therefore are necessary to evaluate vertical transmission through the germline and per os acquisition of the virus as alternative mechanisms of transmitting polydnaviruses to ichneumonid wasp progeny. Such studies should help to clarify whether integrated or extrachromosomal viral DNAs act as templates for CsV replication and possibly may identify novel replication mechanisms for this unusual virus, especially in view of the physical complexity of the CsV genome. Regardless of the virus transmission mechanism used, our data clearly show that both male and female wasps contain CsV DNA, yet a comparatively high level of virus replication occurs only in the calyx epithelial cells of pupating and adult females (9, 10). The basis for this pronounced tissue specificity of CsV replication is not known and warrants further investigation.

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