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Virus with a Multipartite Superhelical DNA Genome from the Ichneumonid Parasitoid *Campoletis sonorensis*

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Virus was isolated from the lumen of the calyx region of ovaries in the parasitoid wasp *Campoletis sonorensis* (Hymenoptera: Ichneumonidae), and the nature of the viral DNA was analyzed. DNA purified from a homogeneous band of virus contained double-stranded superhelical molecules which were polydisperse in molecular weight. At least 25 different covalently closed circles were present, ranging in molecular weight from 4.0×10^6 to 13.6×10^6. The virus DNA was analyzed with restriction enzymes, and the nature of the genetic complexity was evaluated by Southern blot hybridization of native superhelical and relaxed circular virus DNA and of *Sal*I- and *Hin*dIII-digested DNA. The data suggest that most of the variously sized covalently closed DNAs were composed primarily of nonhomologous sequences. The different size classes of covalently closed viral DNAs did not appear to exist in equimolar concentrations. However, there was no evidence from observation of virus particles in the electron microscope or from virus fractionation experiments that a mixture of viruses was present in the calyx fluid. The results from this study suggest that the virus isolated from *C. sonorensis*, like those isolated from other endoparasitic hymenoptera, may belong to a new class of DNA viruses in which the genome is multipartite, with each DNA existing as a superhelical molecule.

The virus-like particles which occur in many species of endoparasitic hymenoptera belonging to the superfamily Ichneumonoidea (for a review, see reference 34) (12, 15, 16, 20, 21, 28, 29, 31, 42) are currently classified as subgroup C baculoviruses (18). Virus replication in the female parasitoid is intranuclear and restricted to the nuclei of the calyx (a specialized region of the oviduct) epithelial cells (20, 21, 29, 31, 34). Virus is secreted from the calyx cells and accumulates to a high concentration within the calyx lumen as part of a "calyx fluid." It is injected with the parasitoid egg into host caterpillars during oviposition. Certain experimental evidence indicates that a component of the calyx fluid protects the parasitoid egg from encapsulation within the hemocoel of its habitual host (19, 29–34, 40, 41). A component(s) of the calyx fluid may also be involved in mediating some of the physiological changes that occur in the host after parasitization and which are apparently of some ultimate benefit to the parasitoid (5, 6, 9–11, 14, 38, 39).

Vinson and co-workers initially demonstrated that the calyx fluid of *Campoletis sonorensis* prevents encapsulation of its eggs in its habitual host, *Heliothis virescens* (19), and may also mediate the "cross-protection" of eggs from an unrelated braconid parasitoid, *Cardiochiles nigriceps*, in the same host (41). More recently, Edson et al. (7) showed that purified virus from the calyx fluid of *C. sonorensis* (CsV) appears to be part of a symbiotic (mutualistic) relationship with the parasitoid, in that the virus may directly or indirectly protect the parasitoid egg and larva from encapsulation within the hemocoel of the host caterpillar *H. virescens*. In addition, the virus replicates within the parasitoid with no apparent pathological effect. In a separate study of a similar host-parasitoid relationship, purified calyx fluid particles from the parasitoid *Nemeritis canescens* were shown to prevent encapsulation of the eggs of this parasitoid in the hemocoel of its host, *Ephestia kuehniella* (3). The electron microscopic studies by Stoltz and co-workers showed that parasitoid viruses are transmitted to host caterpillar tissues (29, 31, 32). Although viral replication was not observed, virus enters the host cells and is uncoated within the nuclei of these cells (29, 31, 32). These data corroborate the experimental data from biological studies by showing that parasitoid viruses interact at a cellular level with host cells in some manner which may result in protection of the parasitoid eggs from encapsulation. It remains to be shown

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that purified parasitoid virus (and not some other component of the calyx fluid) is responsible for altering other aspects of host physiology which benefit the development of the parasitoid in the host after parasitization (5, 6, 9–11, 14, 38, 39, 41).

CsV is morphologically similar to viruses from the ichneumonids *Hyposoter exiguae* and *Hyposoter fugitivus fugitivus* (32) and replicates in the calyx cell nuclei of *C. sonorensis* (20). The virus is ovicylindrical and has a large internal electron-dense nucleocapsid measuring 330 by 85 nm. The nucleocapsid is surrounded by two concentric unit membranes, the inner derived de novo within the nucleus and the outer derived from the plasma membrane of the calyx epithelial cells.

Electron microscopy has been the major tool for studying parasitoid viruses in the parasitoid and in host caterpillars. The molecular biology of only two of these viruses has been studied in detail (15, 16), one from the braconid *Apanteles melanoscelus* and the other from the ichneumonid *Hyposoter exiguae* (HeV). A survey of the viruses from field-collected parasitoid species (30) shows that these viruses also appear to be unique in having a collection of superhelical (SH) polydisperse DNAs of various sizes as genomes.

To demonstrate that a multipartite DNA genome was not a characteristic unique to HeV, we needed to demonstrate that the DNA from at least one other virus had similar characteristics. In this paper we describe the isolation and biochemical characterization of virus from the calyx of the ichneumonid wasp *C. sonorensis* (CsV), with an emphasis on the organization of the genome and the nucleotide sequence homology among the different DNA molecules. Subclasses of viruses could not be detected by virus fractionation techniques. The virus DNA was shown to be polydisperse in size and consisted of predominantly nonhomologous sequences. This evidence supports our hypothesis that CsV and other viruses of endoparasitic hymenoptera could be considered candidates for a new family of viruses containing a multipartite DNA genome.

MATERIALS AND METHODS

Insects. *C. sonorensis* (Cameron) was reared from larvae of the host insect *H. virescens* (Fab). The host *H. virescens* was mass reared on an artificial diet as described by Vanderzant et al. (37). Fourth-instar larvae were parasitized by mated female parasitoids (7 days posteclosion, 5 days postmating). Parasitized larvae were reared individually. All insects were reared at 28°C in a light-to-dark photoperiod of 14:10 h. Adult wasps were fed a 33% honey-water solution.

Virus purification. Virus was purified from the calyxes of 2-week-old female wasps essentially as described by Krell and Stoltz (16). Ovaries from 50 to 100 female wasps were collected in 200 μl of cold phosphate-buffered saline (PBS) in a 1.5-ml Eppendorf microfuge tube. The ovaries were minced in the tube with sterile microscissors, and the cell debris was pelleted (15,600 × *g* for 5 s). The supernatant was removed and layered onto a 25 to 50% (wt/wt) sucrose gradient made up in PBS and centrifuged at 125,000 × *g* for 60 min at 5°C in a Beckman SW60 Ti rotor. The virus band was removed by fraction collection through the bottom of the tube, dialyzed against 0.1 × PBS for 24 h, and stored at −80°C.

Isolation of virus DNA. Purified virus from 100 female wasps was incubated for 15 min at 65°C and then for 30 min at 37°C in proteinase K (100 μg/ml), 5 mM EDTA, and 100 mM KCl. Sarkosyl was added to a final concentration of 4%, and the samples were incubated for 15 min at 65°C. The sample (500 μl) was mixed with CsCl to a final concentration of 1.55 g/ml, 100 μg of ethidium bromide (EtBr) per ml was added, and centrifugation was conducted for 60 h at 20°C and 130,000 × *g* in a Beckman SW60 Ti rotor. The resultant fluorescent bands were collected separately through the bottom of the tube. EtBr was extracted with isoamyl alcohol, and the sample was dialyzed extensively against 0.1 × SSC (1 × SSC = 0.15 M NaCl–0.015 M sodium citrate) and stored at −80°C.

AGE and purification of DNA from agarose gel bands. DNA was subjected to agarose gel electrophoresis (AGE) in 0.8% agarose gels as described by Smith and Summers (25). SH DNAs were used as molecular weight markers to calculate the molecular weights of the virus DNAs. These markers included the plasmid DNAs present in *Escherichia coli* V517 (17) and five well-characterized recombinant pBR322 plasmids in the molecular weight range of 3×10^6 to 10×10^6 (unpublished data; see legend to Fig. 3).

Different size classes of virus DNA were isolated from preparative agarose gels by the following procedure. SH DNA from purified CsV was fractionated by electrophoresis on a preparative agarose gel, and the major bands representing SH DNA were cut from the gel. The DNA was electroeluted from the gel slice (2, 26), extracted with phenol (saturated with 10 mM Tris–1 mM EDTA [pH 8.0]), and precipitated in alcohol. The precipitated DNA was suspended in 10 mM Tris–1 mM EDTA and electrophoresed in a second preparative gel. The slower migrating band representing the relaxed circular (RC) DNA of each preparation was excised, and the DNA was electroeluted and purified as described above. The RC DNAs were then labeled with [^{32}P]dATP.

For some experiments, DNA bands from a single track in an agarose gel were excised, and the DNA was recovered by the freeze-squeeze method (36). The DNA solution was then extracted with phenol and precipitated with ethanol before being labeled.

^{32}P DNA labeling and blot hybridization. DNA from purified virus and from preparative agarose gels was labeled to a high specific activity with [^{32}P]dATP (3,000 Ci/mmol; Amersham Corp.) and DNA polymerase I (New England Biolabs) by the nick repair method of Rigby et al. (24) as modified by Smith and Summers (27). SH and RC DNA from purified virus was fractionated in a preparative gel. The gel was prepared for transfer to duplicate nitrocellulose filters as described by Smith and Summers (27). After being blotted, the nitrocellulose filters were removed, washed in 1 M

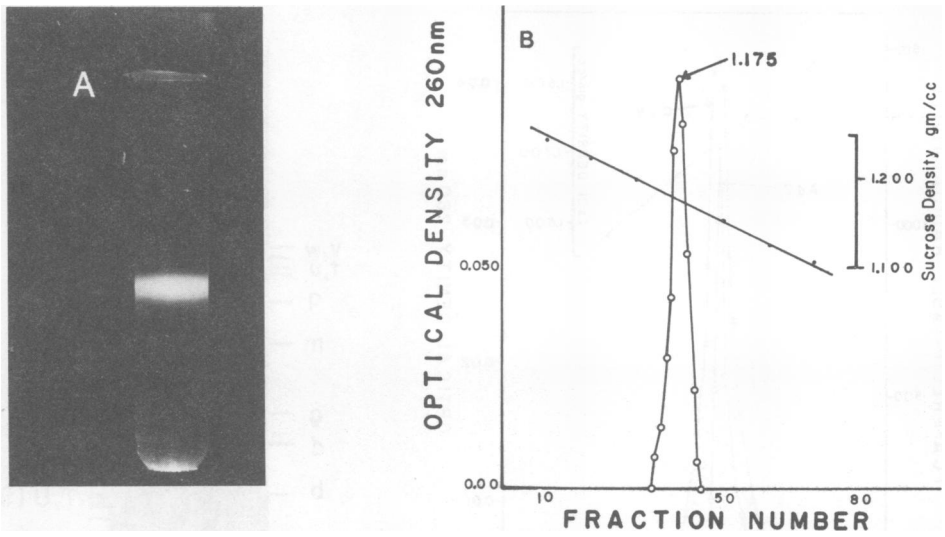

FIG. 1. Purification of CsV. Calyx fluid from 100 wasps was isolated as described in the text and layered on a 25 to 50% (wt/wt) sucrose gradient. Centrifugation was conducted at 35,000 rpm in a Beckman SW60 Ti rotor at 4°C for 1 h. (A) The virus band after centrifugation. (B) Densitometric profile of the gradient. Each 1-drop fraction collected from the bottom of the tube was mixed with 1 ml of distilled water, and the optical density at 260 nm was determined.

sodium acetate, air dried, and baked at 80°C for 2 h. Pretreatment and hybridization of the nitrocellulose filters was done as described by Wahl et al. (43). Hybridization with nick-repaired DNA was conducted as described by Smith and Summers (27). Autoradiography was carried out with Kodak XRP-1 film and a Dupont Cronex Lightning Plus intensifying screen at −80°C for 6 to 24 h.

Fractionation and analysis of virus band. CsV was centrifuged on a CsCl gradient (starting density, 1.214 g/ml in 1× PBS) for 60 h at 20°C and 130,000 × *g* and on a 25 to 50% (wt/wt) sucrose gradient as described above. Individual 1-drop fractions were collected from each gradient through the bottom of the tube. The CsCl and sucrose densities of the fractions were measured with an Abbe refractometer. Each drop (ca. 50 μl) was diluted with 1 ml of water, and the optical density was measured at 260 nm. In one experiment, CsV was fractionated on CsCl and sucrose gradients as described above. Each 1-drop fraction was diluted with 150 μl of 1× PBS, and the virus was pelleted by centrifugation for 15 min in an Eppendorf microfuge (15,600 × *g*). The virus pellet was suspended in 200 μl of PBS, digested for 1 h with proteinase K (100 μg/ml) at 37°C, and incubated at 60°C in 4% Sarkosyl for an additional 30 min. The DNA was phenol-extracted, lyophilized, and prepared for electrophoresis in agarose gels.

Polyacrylamide gel electrophoresis. Virus preparations and molecular weight markers (14,400 to 94,000; Pharmacia Fine Chemicals) were solubilized and prepared for electrophoresis in 10% Laemmli polyacrylamide gels as described by Summers and Smith (35). Some gels were stained by the periodic acid-Schiff reaction (8) to detect glycoproteins. The presence of lipoproteins was investigated by prestaining CsV with Sudan black B (23) and by staining gels with Oil red O (1).

Quantitation and buoyant density determination. CsV DNA was labeled in vitro with [^{32}P]dATP and centrifuged either separately or together with marker DNAs. Marker DNAs were adenovirus 2 DNA, *Micrococcus lysodeikticus* DNA, *Heliothis armigera* nuclear polyhedrosis virus DNA, phage PM2 DNA, and lambda DNA and were either unlabeled or labeled with [^{3}H]dATP or [^{32}P]dATP. DNA was subjected to isopycnic centrifugation in neutral CsCl in 10 mM Tris–1 mM EDTA at an initial concentration of 1.66 g/ml. Centrifugation was done at 130,000 × *g* for 48 h. Fractions were collected from the bottom of each tube and analyzed for trichloroacetic acid-precipitable radioactivity, optical density, and CsCl density. DNA concentrations were determined by the diphenylamine reaction (4).

RESULTS

Virus purification. In sucrose gradients, CsV formed a virus band at a density of 1.18 g/ml (Fig. 1). The virus was of homogeneous morphology and free of any particulate contamination when viewed by electron microscopy. Virus purified by isopycnic centrifugation in neutral CsCl formed a band at a density of 1.20 g/ml, with a shoulder below the main band. This shoulder appeared to consist of damaged particles and was not collected with the main band.

Characterization of DNA. The DNA of CsV banded at a density calculated at 1.71 g/ml in neutral CsCl (Fig. 2). This is equivalent to a guanine plus cytosine (G+C) content of 50%. It

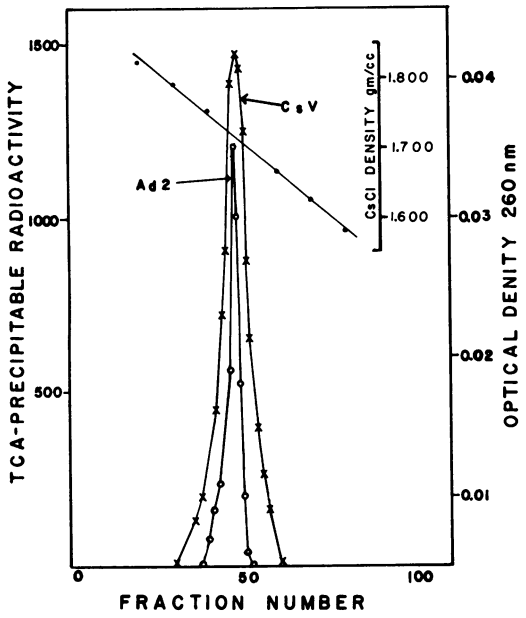

FIG. 2. Buoyant density of CsV DNA. Approximately 1.0 ng of ^{32}P-labeled DNA (10^8 cpm/μg) from purified CsV was mixed with 10 μg of adenovirus 2 DNA. The DNAs were centrifuged to equilibrium in neutral CsCl for 60 h at 40,000 rpm in a Beckman SW60 Ti rotor. Single-drop fractions were collected and analyzed for optical density at 260 nm (adenovirus 2) or trichloroacetic acid-precipitable radioactivity (CsV). Fractions were analyzed for CsCl density with a refractometer. The buoyant density of CsV DNA was calculated relative to the density of the adenovirus 2 DNA (1.716 g/cm^3).

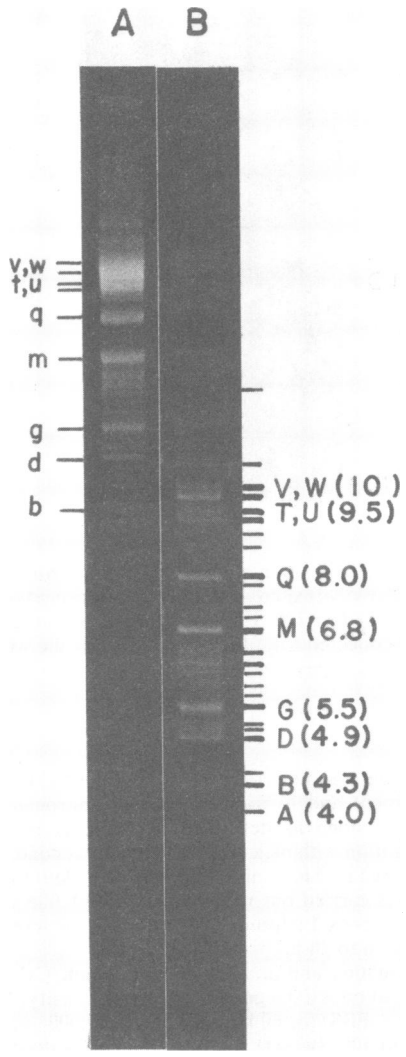

FIG. 3. AGE of SH and RC CsV DNA. SH and RC DNA from purified CsV was separated by isopycnic centrifugation on an EtBr-CsCl gradient as described in the text. The two DNA fractions were extensively dialyzed against 0.1 ×SSC–10 mM EDTA, and 1 μg of each was subjected to AGE in 0.8% agarose. Lane A, DNA from the upper RC band, and lane B, DNA from the lower SH band of the EtBr-CsCl gradient. The major SH DNA bands are labeled with uppercase letters to the right of lane B, and the major RC DNA bands are indicated to the left of lane A with the corresponding lowercase letter. The molecular weights (×10^{-6}) of the major SH DNAs are indicated in parentheses. The molecular weight of the SH DNAs was calculated by measuring DNA migration relative to known SH marker DNAs. The molecular weights of five recombinant pBR322 plasmids constructed in our laboratory were calculated to be (×10^6) 4.82, 5.17, 5.45, 8.4, and 9.6 by analysis of *Sal*I-digested SH plasmid DNA linear fragments (*Hin*dIII- and *Eco*RI-digested lambda DNAs were used as linear molecular weight markers). Plasmids from *E. coli* V517 (17), ranging from 1.36 × 10^6 to 4.82 × 10^6 molecular weight, were also used as markers.

was calculated by the diphenylamine reaction (4) that a maximum of 0.2 μg of virus DNA could be isolated per female wasp.

DNA from purified CsV was isolated in both SH and RC form on CsCl-EtBr gradients. Each of the RC and SH DNAs could be fractionated into at least 25 bands in agarose gels, with the SH DNAs migrating faster than the RC DNAs. The SH DNA preparations always contained some RC DNA, so that both SH DNAs and the corresponding RC DNAs were detected in a single agarose gel track (Fig. 3, lane B). The molecular weight of the DNAs ranged from 4.0 × 10^6 to 13.6 × 10^6, with an aggregate molecular weight of 170 × 10^6 (Table 1). The fluorescence of some of the bands was higher than that of other bands (Fig. 3). However, the fluorescence of the different DNA bands relative to each other remained constant over numerous electrophoretic fractionations.

Southern blot analysis of SH and RC CsV DNA. Southern blot hybridization experiments were initially performed to determine whether any

TABLE 1. Molecular weights of SH CsV DNAs calculated by mobility in agarose gels

SH band[a]	Mol wt ($\times 10^{-6}$)
A	4.0
B	4.3
C	4.5
D	4.9
E	5.1
F	5.2
G	5.5
H	5.6
I	5.8
J	6.0
K	6.2
L	6.4
M	6.8
N	7.1
O	7.3
P	7.8
Q	8.0
R	8.7
S	9.1
T	9.5
U	9.7
V	10.0
W	10.1
X	11.1
Y	13.6

[a] Letter designations as per Fig. 3, lane B.

sequence homology could be detected among the different-size DNAs. The purity of each DNA used as a probe was a major concern. Although SH DNA from a CsCl-EtBr gradient was fractionated in a preparative 0.8% agarose gel, the DNA bands in this gel could have been contaminated with comigrating nonrelated RC or linear DNA species. Therefore the major SH bands were excised from the gel, the DNA was eluted, and DNA from each band was run separately on a second preparative gel (Fig. 4). Less than 5% of the total DNA migrated more slowly to a position that corresponded to the RC form of the DNA (Fig. 4), and therefore the slower migrating band contained only DNA from the original SH band which was "nicked" during the isolation protocol to the RC form. If any RC or linear DNA was present in the SH band of the first gel, it should not have migrated to the same position as the slower-migrating RC DNA on the second gel. The DNA from each RC band of the second gel was then nick repaired and used as a probe in hybridization studies.

DNA was isolated from six different major bands as described above, and each probe was hybridized separately to six identical strips of nitrocellulose containing SH and RC viral DNA (Fig. 5). In all cases hybridization was observed with the homologous SH and RC DNA bands. Very minor hybridization was observed with two additional bands in all six strips (Fig. 5, arrows). The positions of these two bands corresponded to the positions expected for the SH and RC forms of a dimer of the homologous DNA. Moreover, the faster migrating of these two bands was not detected in the strip representing RC DNA and was therefore presumably SH DNA. Because of the difficulty of identifying a given autoradiogram band as SH or RC (both DNAs are present in any SH CsV preparation), labeled SH DNAs were hybridized with RC (Fig. 5, lane RC) and heat-denatured and renatured SH DNA on nitrocellulose Southern blots. Whereas SH DNA would quickly renature and

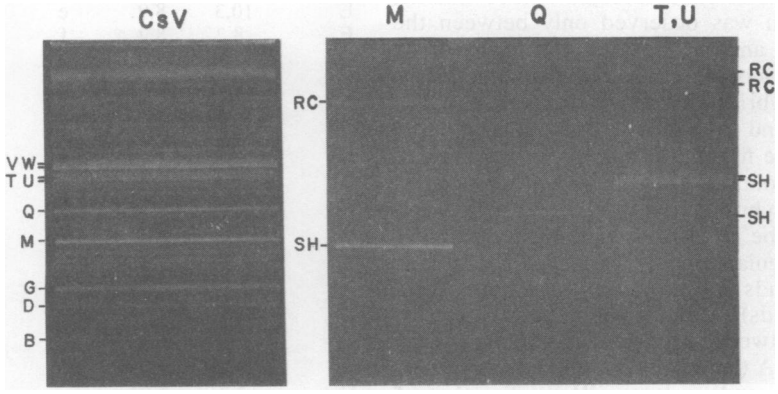

FIG. 4. Isolation and purification of SH and RC CsV DNA from agarose gels. The left panel (CsV) is a preparative gel containing mostly SH DNA from an EtBr-CsCl gradient of purified CsV DNA. The six major bands (B to T,U, indicated to the left) were excised from the gel, and the DNA was recovered by electroelution and then electrophoresed in a second preparative gel. The second electrophoresis of DNA from bands M, Q, and T,U are shown in the right panel (SH and RC bands of each DNA species are indicated). The bands representing the nicked form (RC) of SH DNA from each of the six major bands was then excised from the second gel, electroeluted, and labeled by nick repair for use as probes (see Fig. 5).

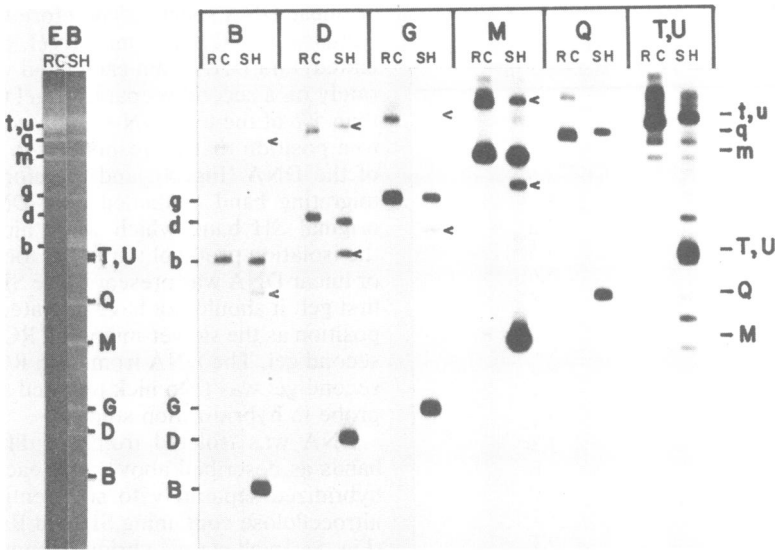

FIG. 5. Hybridization between purified ^{32}P-labeled RC DNAs and total CsV DNA. Identical nitrocellulose strips, each containing one lane of RC and one of SH DNA separated on a 0.8% agarose gel (EtBr-stained gel strip labeled EB) were prepared for hybridization as described in the text. The position of the bands representing SH (uppercase letters) and the corresponding RC (lowercase letters) DNAs used as probes are indicated. The nitrocellulose strips were labeled B to T,U to indicate which DNA was used as probe. The arrows indicate positions of minor hybridization.

migrate as before denaturation, any RC DNA present in the SH preparation would remain denatured and migrate as single-stranded DNA near the dye front. The autoradiographic data from this experiment corroborated the data shown in Fig. 5 in identification of the autoradiographic bands as SH or RC and the hypothesis that the minor hybridization was to bands representing SH and RC dimer DNA. With the exception of the DNA bands designated T,U and M, hybridization was observed only between the probe DNA and the SH and RC forms of its homologous monomer (and putative dimer). Little if any hybridization was observed between the probe and any other DNA bands on the nitrocellulose filters, thus showing that each of the DNAs contained largely unique sequences. Additional hybridization was observed between the T,U probe DNA and some higher mobility (lower-molecular-weight) bands (Fig. 5, lane T,U, SH bands K, O, P, and X and the equivalent RC bands). Minor hybridization was also observed between M probe DNA and a lower-mobility DNA (lane M) which may be a trimer.

Restriction endonuclease (REN) digestion of CsV DNA. Digestion of total CsV DNA with *Sal*I and *Hin*dIII followed by AGE resulted in 32 and 46 bands, respectively (Fig. 6). Some of these bands were in the same position as some bands representing the uncut RC DNAs and could have represented uncut circular DNAs (Fig. 6,

TABLE 2. Molecular weights of CsV restriction fragments

Digestion fragment[a]	Mol wt ($\times 10^{-6}$)		Digestion fragment[a]	Mol wt ($\times 10^{-6}$)	
	*Sal*I digestion	*Hin*dIII digestion		*Sal*I digestion	*Hin*dIII digestion
A			a	1.3	2.0
B			b	0.88	1.97
C			c	0.80	1.88
D			d	0.55	1.85
E	10.3	8.9	e	0.44	1.65
F	8.2	8.2	f	0.34	1.45
G	7.3	7.0	g		1.40
H	6.3	6.6	h		1.32
I	5.6	5.7	i		1.20
J	4.8	5.1	j		1.07
K	4.5	4.8	k		1.02
L	4.3	4.5	l		0.97
M	4.2	4.2	m		0.83
N	3.7	3.7	n		0.79
O	3.5	3.6	o		0.77
P	3.2	3.5	p		0.71
Q	3.0	3.4	q		0.56
R	2.8	3.3	r		0.51
S	2.7	3.0	s		0.48
T	2.4	2.9	t		0.42
U	2.3	2.8			
V	2.1	2.6			
W	2.0	2.5			
X	1.8	2.4			
Y	1.7	2.3			
Z	1.5	2.1			

[a] Letter designations as per Fig. 6, left panel.

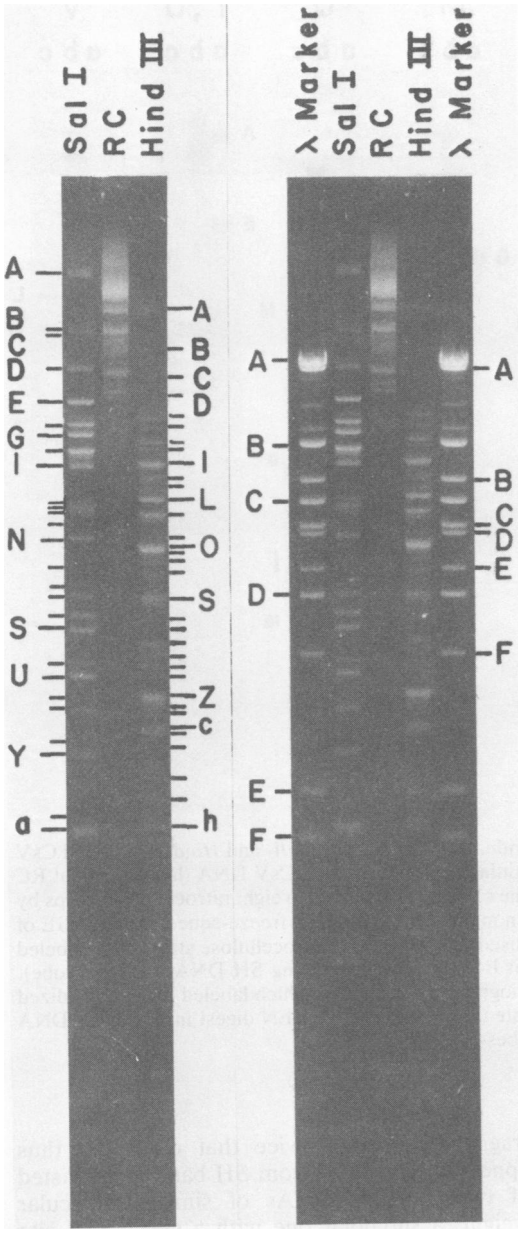

FIG. 6. AGE of REN digest of CsV DNA. RC DNA from purified CsV (lane RC) was digested with *Sal*I or *Hin*dIII. Each preparation (1 μg) was subjected to AGE in 1.0% agarose gels. A mixture of *Hin*dIII- and *Eco*RI-digested lambda DNA was used to give molecular weight markers (lane λ). The molecular weights for digested lambda DNA are as follows ($\times 10^{-6}$). For *Hin*dIII: band A, 15.4; B, 6.28; C, 4.35; D, 2.91; E, 1.50; and F, 1.27; for *Eco*RI: band A, 14.1; B, 5.02; C, 3.86; D, 3.64; E, 3.17; and F, 2.36. Some of the major bands in *Sal*I and *Hin*dIII digests of CsV DNA are indicated (upper- or lowercase letters; lowercase letters were used after band Z in both cases).

bands A, B, C, and D, lanes *Sal*I and *Hin*dIII). Assuming that bands A–D represent uncut RC DNA and that each of the other bands contains only unique linear DNA fragments, the aggregate molecular weight of the linear REN fragments and the DNAs in bands A–D of the REN digest gel is 135×10^6 for *Sal*I fragments and 140×10^6 for *Hin*dIII fragments (Table 2).

Since we could not correlate the *Sal*I and *Hin*dIII fragments with the SH DNA species, SH DNA was isolated from seven major bands and used as probes against undigested RC CsV DNA and *Sal*I- and *Hin*dIII-digested RC CsV DNA (Fig. 7). Table 3 is a summary of this hybridization data and correlates the major SH species to the *Sal*I and *Hin*dIII fragment bands. The DNAs from two major SH bands, B and G (we propose that there are two comigrating DNAs in band G; see below), were uncut by *Sal*I (Fig. 7, lanes B and G), whereas all seven appeared to be cut by *Hin*dIII. DNA from the

TABLE 3. Correlation of SH CsV DNA with *Sal*I and *Hin*dIII bands

SH CsV DNA[a]		*Sal*I[b]		*Hin*dIII[b]	
Band	Mol wt ($\times 10^{-6}$)	Band	Mol wt ($\times 10^{-6}$)	Band	Mol wt ($\times 10^{-6}$)
B	4.3			M	4.2
D	4.9	S	2.7	O	3.6
		U	2.3	o	0.77
				q	0.56
G	5.5	I	5.6	I	5.7
				O	3.6
				c	1.88
M	6.8	G	7.3	Z	2.1
				h	1.32
				k	1.02
				r	0.51
				s	0.48
				t	0.42
Q	8.0	H	6.3	M	4.2
		W	2.0	d	1.85
				g	1.40
				j	1.07
T,U	9.5	E	10.3	H	6.6
		H	6.3	J	5.1
		K	4.5	K	4.8
		N	3.7	O	3.6
		T	2.4	Q	3.4
				S	3.0
				V	2.6
				Z	2.1
V	10	S	2.7	L	4.5
		U	2.3	O	3.6
		Y	1.7	Z	2.1
		a	1.3		
		e	0.44		
		f	0.34		

[a] Data are shown in Fig. 3 and Table 1.
[b] Data are shown in Fig. 7 and Table 2.

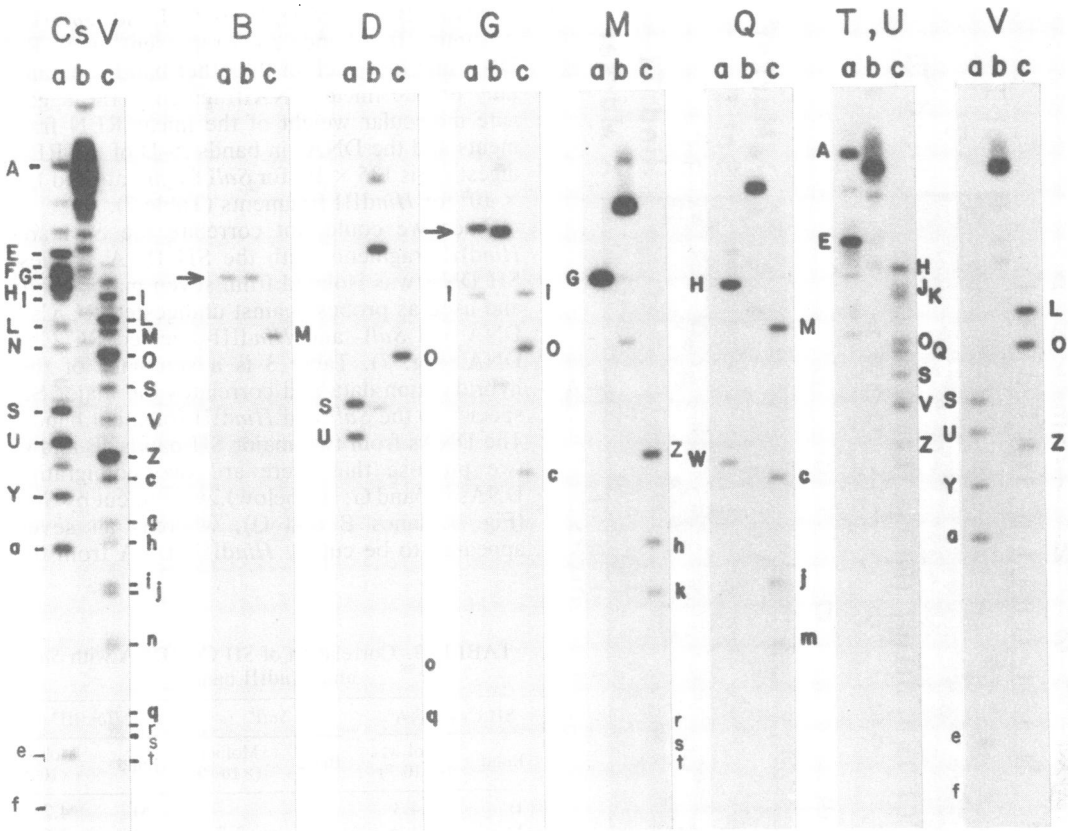

FIG. 7. Hybridization of CsV DNA (purified from individual SH bands) with *Sal*I- and *Hin*dIII-digested CsV DNA. Four replicates of the gel in Fig. 6, left panel, containing *Sal*I-digested RC CsV DNA (lane a), uncut RC CsV DNA (lane b), and *Hin*dIII-digested RC CsV DNA (lane c), were transferred to eight nitrocellulose strips by bidirectional transfer. SH DNAs were extracted from seven major bands (B–V) by freeze-squeeze after AGE of CsV SH DNA in a single lane, labeled by nick repair, and used as probes. The nitrocellulose strips were labeled according to the probe used (CsV, total CsV DNA probe; B–V, the corresponding SH DNA used as probe). Some of the major bands are indicated on the CsV autoradiogram. The bands to which labeled DNA hybridized are indicated beside the corresponding lane. Arrows indicate those bands of the REN digest in which the DNA hybridized to the equivalent RC DNA band in lane b (probes B and G).

major SH bands G and M had single *Sal*I sites, and DNA from SH bands G, M, and T,U had single *Hin*dIII sites. SH bands D and V had multiple *Sal*I sites, and SH bands D, M, Q, T,U, and V had multiple *Hin*dIII sites (Fig. 7).

When SH DNA from band G was used as a probe against *Sal*I-digested RC CsV DNA, there was hybridization with a band in a location equivalent to RC band G and with a (submolar) band whose location was that expected of a linear form of G DNA. When SH band G DNA was used as a probe against *Hin*dIII-digested CsV DNA, it hybridized with three DNA linear fragments: one with a molecular weight equivalent to G and two submolar DNAs with a composite molecular weight equal to that of G (the total aggregate molecular weight of all three fragments equaled twice that of G). It thus appeared that DNA from SH band G consisted of two different DNAs of similar molecular weight: a submolar one with a single *Sal*I site and one with none, and one DNA containing a single *Hin*dIII site and the submolar species with two *Hin*dIII sites (Fig. 7, lane G).

The REN digestion and hybridization data suggested that all of the major DNA bands except G contained a single species of DNA (the composite molecular weight of the fragments equaled that of the circular DNA). There was no evidence of minor hybridization to filter-bound, REN-digested DNA as there was to filter-bound SH and RC CsV DNA (Fig. 5).

Fractionation of virus band. CsV was centrifuged in a sucrose gradient and a CsCl gradient,

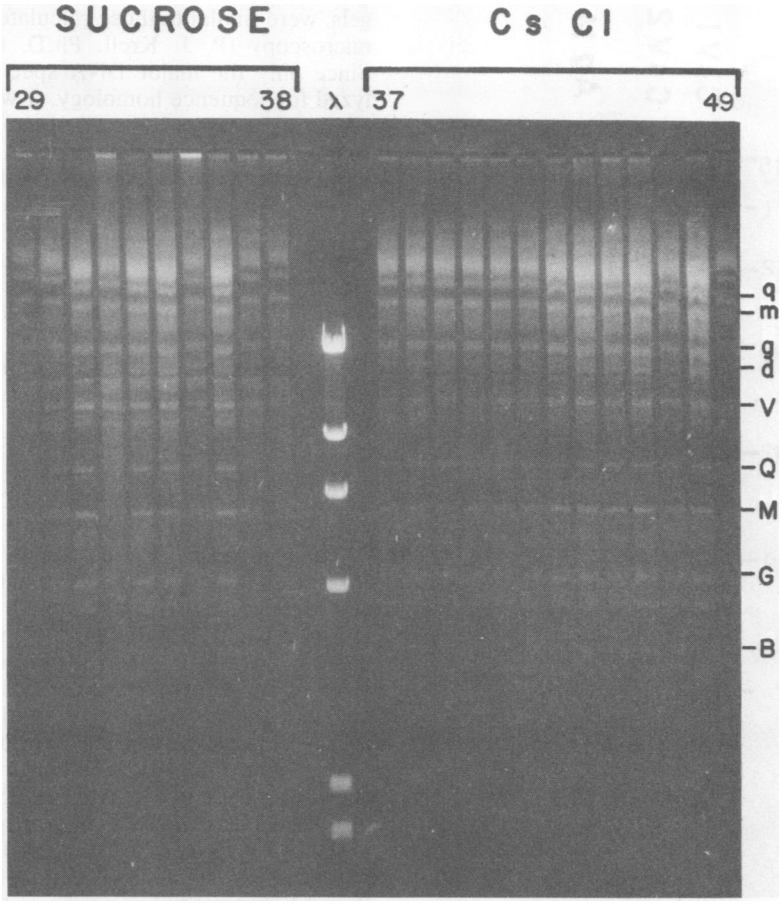

FIG. 8. Fractionation of CsV on sucrose and CsCl gradients. Calyx fluid was prepared for centrifugation on sucrose and CsCl gradients as described in the text. Single drops were collected from the bottom of the tubes after centrifugation, and virus DNA from each drop was prepared for AGE. The DNA banding patterns of fractions 29 to 38 (total, 68 fractions) from a sucrose gradient and of fractions 37 to 49 (total, 79 fractions) of a CsCl gradient are shown. Some of the major bands representing RC and SH DNA are indicated to the right. *Hin*dIII digestion fragments of lambda DNA were included as position markers in a lane near the center of the gel.

and fractions of the virus band were collected and analyzed to determine whether the virus preparation was homogeneous. The virus band was fractionated into 10 fractions in the sucrose gradient and 13 fractions in the CsCl gradient. The DNA gel pattern of each fraction was identical and resolved all of the SH and RC DNA bands observed for purified virus (Fig. 8).

Proteins. Polyacrylamide gel electrophoresis of purified CsV resolved approximately 25 polypeptides of which 6 to 7 were major polypeptides (Fig. 9). The molecular weight of the polypeptides ranged from 15,000 to 80,000. One polypeptide band (Fig. 9, GP) was stained by the periodic acid-Schiff reaction, whereas none of the bands was stained by either Sudan black B or Oil red O. The aggregate molecular weight was calculated to be 1.3×10^6, which, assuming that all proteins were of viral origin and that there were no overlapping coding regions, would require a minimum molecular weight of DNA of 26×10^6.

DISCUSSION

There is now sufficient data to show that the DNA genomes of the ichneumonid viruses, especially those of the subfamily Campopleginae, have some characteristics unique to DNA viruses. The most significant characteristic is the organization of the DNA as a complex of SH DNA circles. Viruses from several ichneumonid species share this feature (15, 16, 30; P. J. Krell, Ph. D. thesis, Dalhousie University, Halifax, Nova Scotia, Canada, 1980). Evidence from this study shows that each of the different SH DNAs of CsV is, for the most part, unique in nucleotide

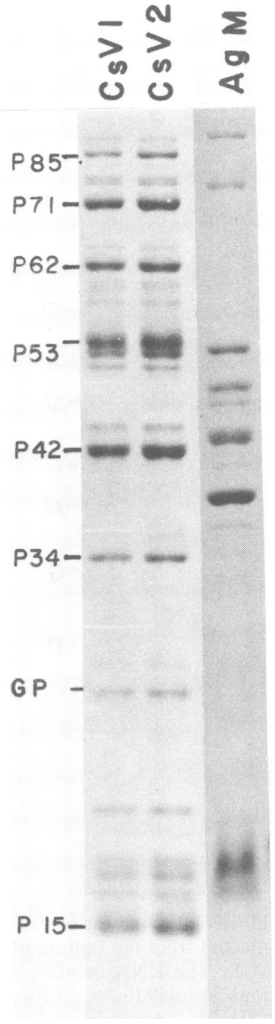

FIG. 9. Polyacrylamide gel electrophoresis of CsV polypeptides. CsV was purified from calyx fluid and subjected to polyacrylamide gel electrophoresis as described in the text. CsV1 and CsV2, Two preparations of CsV purified at different times. AgM, Polypeptides of the baculovirus *Anticarsia gemmatalis* nuclear polyhedrosis virus which were used as molecular weight markers (35). Some of the major polypeptides are indicated to the left with their molecular weights ($\times 10^{-3}$). GP, Position of a periodic acid-Schiff-staining band (25,000 molecular weight).

sequence, as was previously shown for the SH DNAs of HeV. One possible explanation for the presence of multiple DNA size classes would be that CsV was a mixture of defective particles containing deletions of the CsV genome. However, since the CsV SH DNA size classes are not homologous, we consider that CsV is not a mixture of defective virus particles.

The molecular weights of the CsV DNAs, calculated by mobility of the SH form in agarose gels, were similar to those calculated by electron microscopy (P. J. Krell, Ph.D. thesis, 1980). Since only the major DNA species were analyzed for sequence homology, it was difficult to obtain an accurate estimate of the total molecular weight of nonhomologous DNA. However, if we assume that each band of SH, RC, and *Sal*I- and *Hin*dIII-digested CsV DNA contains only one DNA species and that each DNA species is unique, then the aggregate molecular weight of the unique DNA of CsV is between 135×10^6 (from *Sal*I digest, Table 2) and 170×10^6 (from SH DNA, Table 1).

The Southern blot hybridization data showed that, with the exception of bands T,U and M DNA, the major SH DNAs shared little homology among themselves or with the minor DNA species. The hybridization of SH DNA with Southern blots of REN-digested DNA suggests that DNA from SH bands D and V may share a common sequence. Both D and V DNA probes hybridized to two identical *Sal*I bands (S and U) and one identical *Hin*dIII band (O) (Fig. 7, lanes Da, Dc, Va, and Vc). DNAs from the slower-migrating bands T,U hybridized with DNAs in some of the faster-migrating bands. The hybridization data from both circular and REN-digested DNAs suggest that some hybridization could have been artifactual, resulting from the presence in the gel of three forms (SH, RC, and linear) of at least 25 different-size species of CsV DNA and their putative dimers. However, the hybridization of T,U DNA with SH bands K, O, P, and X appears to be specific and suggests that either the DNAs in these minor bands are deletions of T,U DNA or they at least share some homology with T,U DNA. DNA from bands K, O, P, and X could therefore represent deletion or recombinant forms of the major (T,U) species.

In the experiments with REN-digested DNA, hybridization was observed only with linear fragments whose composite molecular weight was equivalent to that calculated for the corresponding SH molecules. This is consistent with the idea that the hybridization observed between the SH DNA probe and lower-mobility DNA (Fig. 5, arrows) was to dimers of homologous SH and RC DNA. Dimer DNAs are often generated in systems involving the replication of SH DNA, and in this case the dimer DNAs could easily be encapsidated. If the additional minor hybridization observed were between the SH DNA probes and a hypothetical higher-molecular-weight heterologous nondimer DNA, hybridization with REN fragments representing the higher-molecular-weight heterologous DNA in addition to the DNA fragments representing the homologous SH DNA might be expected, unless both the SH probe and the hypothetical heter-

ologous nondimer SH DNA had REN sites that resulted in digestion fragments of the same size. REN fragments of dimer molecules, on the other hand, would be the same size as the REN fragments of the circular monomer. In all cases, hybridization between SH DNA and REN fragments was with fragments whose molecular weight equaled that of a monomer (or half a dimer) of SH DNA. Hybridization studies with cloned REN fragments should yield more definitive answers.

The reason for the non-equimolar ratios of the different viral DNAs is presently unknown. Except for SH band G, each of the major SH bands analyzed appeared to contain only one DNA species. The presence of two or more virus subclasses in disproportionate amounts could account for the difference in the relative amounts of the different DNA species. There may have been, for example, a major subclass of CsV containing the major DNA circles and a minor subclass which contained the minor DNA circles. Although subclasses could not be detected by either CsCl or sucrose density gradient centrifugation, subclasses could still exist but may simply have had similar sedimentation characteristics.

From the observation that any one DNA circle has an insufficient coding capacity for all of the virus structural polypeptides, we suspect that several SH DNAs collectively code for these polypeptides. Moreover, it is probable that the virus genome codes for the additional nonstructural polypeptides required for virus replication and for polypeptides which could be involved in altering host physiology and affecting the encapsulation response of the host to the parasitoid. Several DNA circles therefore would have to be transcribed to account for all of these viral functions. It is tempting to speculate that the different DNA circles represent individual and independent transcriptional units. Such an organization of a DNA genome would indicate yet another and unique mechanism for virus gene regulation.

We do not yet know if the multipartite DNA genome of CsV or HeV is encapsidated in single virions or in a multicomponent virus. Braconid viruses such as *A. melanoscelus* virus (9) have a variety of nucleocapsid lengths and could easily be multicomponent. The smaller DNA circles could be encapsidated in the shorter nucleocapsids, and the larger ones could be encapsidated in correspondingly larger nucleocapsids. However, for the ichneumonid particles such as HeV and CsV, the nucleocapsids are of uniform size and large enough to encapsidate a full complement of circular DNAs. Two observations support this hypothesis. First, each nucleocapsid is large enough to accommodate a full complement of circular DNAs. The nucleocapsids of CsV are at least twice the volume of nucleocapsids of baculoviruses which encapsidate an amount of DNA equal to about 100×10^6 daltons, and each CsV virion could therefore encapsidate at least twice that amount of DNA. Secondly, the CsV could not be fractionated into subpopulations containing different DNA species by either sucrose or CsCl gradient centrifugation. Each fraction had virions which collectively have the full genome complement. We recognize that if there were in fact subpopulations, each containing different species of DNA circles, the methods we used may not have been adequate to resolve such populations. The only conclusion we can draw with any certainty is that if subpopulations of viruses are present in the CsV preparations, they must all have very similar sedimentation and density characteristics. In the absence of a suitable plaque assay procedure to separate single virions, the nature of the DNA encapsidation remains a moot question.

The present study and those on HeV (16) and other ichneumonid viruses (15, 30) show that the parasitoid viruses have a unique mode of genome organization in which the genome exists as a collection of SH DNA molecules of different lengths. They could therefore be classified as having a multipartite, double-stranded, SH DNA genome and represent a new family of DNA viruses. Each virus DNA could be encapsidated separately in individual nucleocapsids, as may be the case for the braconid viruses such as *A. melanoscelus* virus (15, 31). Such an organization for encapsidation has been shown for the RNA components of some RNA plant viruses and other small RNA viruses (13, 22). Alternatively, the full complement of virus DNAs could be encapsidated in each virion as are the double-stranded RNAs of reoviruses (44). Although the mode of encapsidation is unknown, it is clear that the parasitoid viruses have a multipartite DNA genome in which the major DNA components as studied herein share only minor sequence homology.

We are presently using cloned CsV DNAs to investigate the physical organization of the major DNAs and sequence heterogeneity among the CsV SH DNAs. With these studies we hope to overcome the inherent difficulties of working with a complex mixture of viral circular DNAs and be able to obtain a better understanding of the nature and complexity of the CsV genome.

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