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Venom and viral expression products of the endoparasitic wasp *Campoletis sonorensis* share epitopes and related sequences

(host avoidance/insect immunity/gene family/polydnavirus/developmental arrest)

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ABSTRACT **Endoparasitic wasps of lepidopteran insects must induce changes in host immunity and development to survive. Depending on the species, this may require wasp venom proteins and/or a polydnavirus. We describe an immunological and genetic relationship between the *Campoletis sonorensis* polydnavirus and the wasp's venom gland. Monoclonal antibodies raised against venom glands recognized epitopes conserved on several polydnavirus proteins and on multiple wasp oviduct and venom proteins. The viral envelope proteins had molecular masses of 16, 20, 45, and 50 kDa, while a complex of at least five immunoreactive venom-gland and soluble oviduct proteins ranged in size from 24 to 36 kDa. Since the conserved epitopes were present on the viral envelope, neutralization assays were performed. Monoclonal antibodies added to purified virus blocked the normal viral inhibition of host growth and development. To determine whether venom mRNA and viral genes were also related, venom-related cDNA clones were isolated from the wasp oviduct with a venom-gland cDNA probe. Venom-related viral clones were then identified and selected from a viral genomic library and from a parasitized *Heliothis virescens* cDNA library. Venom-related mRNAs were expressed in the venom gland, the oviduct, and the parasitized host. We propose that the immunological relationship between venom and viral proteins, and the hybridization of venom and viral genes, may reflect an evolutionary relationship in which venom gene homologs were incorporated into the viral genome, thereby allowing viral expression of venom-related genes and enhancing parasite survival.**

Parasites possess diverse mechanisms for avoiding host immune defenses. In insect endoparasite–host relationships the parasitic wasp alters the immune and developmental systems of its larval lepidopteran host to produce an environment that allows parasite survival and development. Because of diverse species-specific differences, the mechanisms through which endoparasites disrupt host physiological systems are largely unknown. Endoparasitic hymenoptera have been described in which host immune and developmental systems are altered by parasite venom secretions (1, 2), by both venom and a polydnavirus (3–5), or by the polydnavirus alone (6–9). Polydnaviruses have an apparently obligate symbiotic association with some endoparasitic hymenoptera (10, 11) and are characterized by segmented, double-stranded, superhelical DNA genomes that are heterogeneous in size and genetic composition. The life cycle of the *Campoletis sonorensis* polydnavirus (CsPDV) involves unusual host-specific regulation and expression of viral genes and gene families. Based on molecular and genetic analyses, some polydnaviruses are stably integrated into the chromosomes of every male and female wasp and are apparently maintained in wasp populations by vertical transmission (12, 13). The CsPDV replication is detected only in ovarian calyx cells (14, 15) and viral DNA replication is not detected in the lepidopteran host.

Because venom secretions from one parasite and polydnavirus expression from another may induce similar physiological symptoms in parasitized insects that benefit parasite survival, Tanaka (2) proposed that the role(s) of endoparasite venoms and polydnaviruses may share a more common evolutionary relationship than has been thought. However, no experimental evidence has indicated that such a relationship between venom and polydnavirus proteins or genes exists. The injection of purified CsPDV into lepidopteran host larvae induces immunological and developmental disruptions similar to natural parasitization (6–8, 16), and *C. sonorensis* venom gland secretions are not essential for endoparasite development (17).

We now report that monoclonal antibodies (mAbs) raised against wasp venom-gland proteins also recognize epitopes on viral envelope proteins and on wasp soluble oviduct proteins. The same mAbs blocked virus-induced growth inhibition and developmental arrest of the lepidopteran host. We also show cross-hybridization between venom-gland mRNA, viral genomic DNA, viral mRNAs expressed in parasitized insects, and parasite chromosomal DNA. The data indicate that venom-related (VR) genes are expressed both by the parasite venom gland and oviduct in *C. sonorensis* female adults and by the polydnavirus in parasitized lepidopteran larvae. The presence of conserved epitopes on venom and viral proteins, the expression of related DNA sequences between the parasite venom gland and CsPDV, and the neutralization of virus-induced physiological symptoms by anti-venom gland mAbs suggest a functional role for VR proteins during parasitization and may indicate an evolutionary relationship between polydnavirus and venom genes.

MATERIALS AND METHODS

C. sonorensis and *Heliothis virescens* rearing conditions and CsPDV purification and DNA isolation from female oviducts have been described (18, 19). Venom glands were dissected in saline (19) and stored at −20°C. Soluble oviduct proteins were collected from the top 0.5-ml fraction of a 25–50% sucrose gradient used for CsPDV purification (18). Viral envelopes were prepared by the method of Tweeten *et al.* (20).

Immunological Methods. Polyclonal anti-CsPDV antiserum was produced in rabbits by subcutaneous injection of 100 μg of CsPDV with Freund's complete adjuvant and three similar virus injections at 2-week intervals in incomplete adjuvant. Venom antiserum and mAbs were produced by injecting 300 venom glands into the peritoneum of a mouse three times at

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Abbreviations: CsPDV, *Campoletis sonorensis* polydnavirus; mAb, monoclonal antibody; VR, venom-related.

2-week intervals and then fusing the spleen cells to Sp2/0 cells (21). Hybridomas were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum, 4 mM glutamine (Sigma), and 50 μg of gentamicin per ml at 37°C in 6% CO_2. Hybridoma supernatants were screened in 96-well microtiter plates in an enzyme-linked immunosorbent assay (ELISA) by coating each well with 1 μg of solubilized CsPDV or 0.1 μg of solubilized venom gland in ELISA wash buffer (25 mM sodium phosphate, pH 7.2/50 mM sodium chloride/0.5% Tween 80) (22). Hybridomas that were positive in both screens were cloned by limiting dilution (23). Cloned supernatants were then assayed on immunoblots. The IgG fraction of selected mAbs was collected from cell supernatants by affinity adsorption to protein A-Sepharose CL-4B (Sigma). For immunoblots, proteins separated in polyacrylamide gels were transferred to nitrocellulose in an American Bionetics "polyblot" apparatus (24). Antigens were detected using alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG (Promega) (25). CsPDV neutralizing assays were performed by incubating CsPDV (1.25 μg/μl) with affinity-purified mAbs (0.5 μg/μl) or control rabbit IgG for 1 hr in phosphate-buffered saline. Two microliters of the virus/antibody mixture was then injected into New stage (26) fifth-instar larvae. Larval growth and developmental stages were monitored daily until metamorphosis or death.

DNA/RNA Methods. RNA extraction and oligo(dT)-cellulose chromatography were performed as described (27). Venom-gland cDNA was synthesized from 5 μg of total venom-gland RNA by using a commercially available kit (Bethesda Research Laboratories). DNA and RNA blotting, hybridizations, and probe synthesis were performed as described (27–29). Reduced- and high-stringency hybridizations and washes have been described (27, 30). For sequential hybridization of blots, probes were removed by two 30-min washes with 15 mM sodium chloride/1.5 mM sodium citrate, pH 7/1% SDS at 100°C.

cDNA clones were isolated from existing λgt10 *C. sonorensis* oviduct and parasitized *H. virescens* cDNA libraries (15) and subcloned into Bluescribe vectors (Stratagene). Viral genomic clones were isolated by hybridization to VR cDNAs from a viral genomic library constructed in λZAP (Stratagene). CsPDV DNA was digested with 0.5 units of *Hae* III, *Tha* I, and *Rsa* I per μg of DNA at 37°C for 5 min, *Eco*RI linkers (Promega) were added, and inserts were ligated into λZAP.

RESULTS

Viral, Wasp Venom, and Oviduct Epitopes Are Conserved. Polyclonal antisera to CsPDV and wasp venom were initially used to compare *C. sonorensis* venom and viral antigens. Viral antiserum reacted with venom proteins and venom antiserum bound to viral structural proteins (data not shown). Interestingly, soluble oviduct proteins also crossreacted with polyclonal venom and virus antisera. To enhance the specificity of the immune response, mAbs were produced using *C. sonorensis* venom glands as antigens. To select mAbs that recognized the conserved epitopes, hybridomas were screened in ELISAs against purified viral structural proteins, soluble venom proteins, and soluble oviduct proteins. Only those hybridomas which reacted with both CsPDV and the soluble venom proteins were cloned. Three clones that produced IgG antibodies were selected and designated 3B11, 2D11, and 3G3. In immunoblots of venom proteins, each of the mAbs reacted similarly with approximately five venom proteins with apparent molecular masses between 24 and 36 kDa (Fig. 1*A*). The immunoreactive soluble oviduct proteins were similar to the venom proteins (Fig. 1*B*). By contrast, the viral immunoreactive proteins had molecular masses of 16, 20, 45, and 50 kDa (Fig. 1*B*). The data show that viral proteins share related epitopes with *C. sonorensis* venom proteins but differ in number and size. The conservation of venom epitopes among the virus, the oviduct, and the venom proteins suggested the existence of a family of VR proteins.

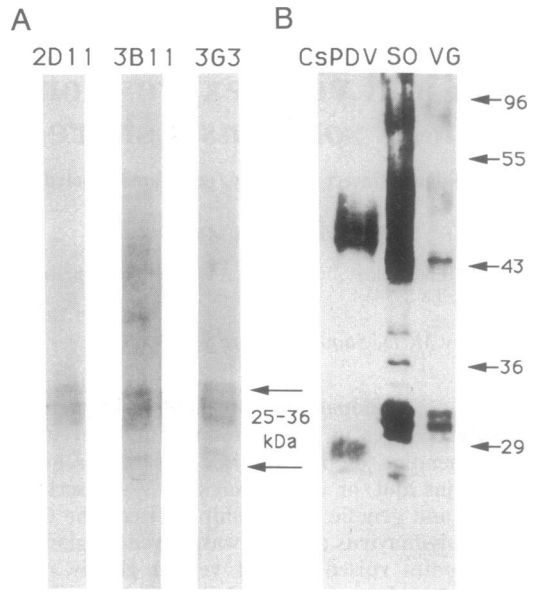

FIG. 1. Antigenic reactivity of mAbs selected for conserved viral and venom epitopes. (*A*) Individual mAbs 2D11, 3B11, and 3G3 detected epitopes from a complex of proteins in the venom gland with molecular sizes between 25 and 36 kDa. (*B*) The mAbs 2D11, 3B11, and 3G3 were pooled and used to probe an immunoblot of gradient-purified virus (CsPDV), soluble oviduct proteins (SO), and venom-gland proteins (VG). Ten micrograms of solubilized protein was loaded in each lane. Molecular size markers (kDa) are indicated at right.

Neutralization of CsPDV. Viral neutralization assays were performed by incubating 2 μg of CsPDV with 1 μg of the mAbs 2D11, 3B11, and 3G3 (Table 1). For control larvae, CsPDV was preincubated either without antibodies or with the IgG fraction from rabbit preimmune serum and was then injected into developmentally synchronized *H. virescens* larvae. In control larvae injected with purified CsPDV, weight gain was inhibited and development was arrested (Table 1). Treating CsPDV with mAbs that bound VR epitopes neutralized viral activity, since the treated virus did not inhibit larval growth or prevent metamorphosis (Table 1). No effects on larval growth or development occurred when only the mAbs were injected (data not shown). Since CsPDV injection normally causes a dramatic inhibition of larval growth and development (Table 1), the abrogation of this effect by prior incubation with any of the mAbs 2D11, 3B11, and 3G3 showed that each of these mAbs blocked the effects of the virus on the host insect.

Localization of VR Proteins on CsPDV. To study the VR viral proteins, the virus was fractionated into envelope and nucleocapsid. The envelope fraction was isolated with Nonidet P-40 and separated from the nucleocapsid by centrifugation in a glycerol gradient. The purity of the envelope and nucleocapsid preparations was visually verified by electron microscopy and by polyacrylamide gel electrophoresis of gradient fractions. After centrifugation of intact virus, viral proteins were detected in fractions 7–11 and in the gradient pellet, which was presumably due to virus aggregation. After Nonidet P-40 treatment, the envelope proteins remained at the top of the gradient while the nucleocapsid pelleted (data not shown). By protein immunoblot analysis, only those gradient fractions containing intact virus or the viral envelope reacted with anti-venom mAbs. No VR viral proteins were

Table 1. Neutralizing activity of VR mAbs

Day	Mock (n = 8)	CsPDV (n = 8)	R-IgG (n = 24)	VR mAbs (n = 30)
	Larval weight, mg			
0	112 ± 12	97 ± 11	110 ± 6	114 ± 14
1	129 ± 10	100 ± 14	114 ± 10	133 ± 5
2	184 ± 27	101 ± 18	127 ± 15	238 ± 8*
3	281 ± 24	122 ± 29	185 ± 16	301 ± 13*
4	344 ± 33	147 ± 35	221 ± 18	352 ± 10*
5	397 ± 44	170 ± 38	246 ± 16	328 ± 10*
6	†	186 ± 44	278 ± 15	338‡
	Percent wandering			
0	0	0	0	0
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	3.3
5	37	0	4.2	80
6	100	0	8.4	87†§

CsPDV injection suppresses host growth and inhibits development. Viral neutralization activity was indicated by normal larval growth and metamorphosis after injection of antibody-treated virus. Data from individual VR mAb neutralization experiments were similar and were pooled. Neutralization of viral effects on growth is indicated by normal weight gain after virus injection. Larval weight (mean ± SE) on days 0–6 following mock injection (mock), injection of 2 μg of CsPDV, injection of 2 μg of CsPDV preincubated with 1 μg of preimmune rabbit IgG (R-IgG), or injection of 2 μg of CsPDV preincubated with VR mAb 2D11, 3B11, or 3G3 is shown. n, Number of larvae in each treatment. Neutralization of developmental effects by VR mAbs is indicated by metamorphosis after virus injection. The term wandering is used to describe the initiation of metamorphosis and is indicated by morphological, physiological, and behavioral changes in the developing larvae.

*Significantly different from CsPDV injection ($P > 0.05$).

†All surviving larvae had initiated morphogenesis.

‡No SE since only two larvae had not wandered at this time.

§Four larvae died on days 5–6.

detected in the nucleocapsid pellet (Fig. 2). Thus, mAbs that blocked viral effects in the lepidopteran host recognized VR epitopes on the CsPDV envelope.

Cloned Viral DNA Sequences Hybridize to mRNA from Venom Gland and from Parasitized *H. virescens*. Since venom-gland tissue was difficult to isolate for standard cDNA cloning, VR cDNA clones were selected from an existing parasite oviduct cDNA library (15). To do this cDNA was synthesized from venom-gland mRNA and used as a hybridization probe to identify and select a crossreacting oviduct cDNA clone, pcCs1300. A viral genomic clone, pV1100, was then isolated that also hybridized to venom-gland mRNA. To determine whether these VR clones were related to any of the previously described viral genes, DNA dot blot hybridization analysis was performed with 34 cloned viral DNAs representing viral genes and DNA segments that are expressed in both the parasite and its host (15, 16, 31). Hybridization was observed to the viral clones partially encoding the abundant 1.1- and 1.4-kilobase (kb) viral mRNAs that are expressed in the parasitized host (data not shown and ref. 31). This indicated that VR CsPDV genes are also expressed after parasitization. To confirm this, a cDNA clone that hybridized to venom-gland mRNA was isolated from a 48-hr parasitized *H. virescens* cDNA library. This viral, VR cDNA clone, pcVHv900, also hybridized to the 1.1- and 1.4-kb mRNAs (data not shown).

Under reduced stringency, the oviduct cDNA and VR viral cDNA and genomic clones hybridized to venom-gland RNA, oviduct RNA, and viral DNA (Fig. 3*A*). Under stringent conditions the oviduct cDNA clone hybridized to parasite chromosomal DNA but did not hybridize to viral DNA (Fig. 3*B*). When the same blot was probed with the VR viral clones pV1100 and pcHvV900, they hybridized to both viral genomic and parasite chromosomal DNA (Fig. 3*B*). The VR viral clones hybridized strongly to a discrete set of CsPDV DNA segments. Hybridization to multiple but distinct sets of viral DNA segments has been observed in studies of CsPDV gene families (15, 31). Viral cDNA clones from superhelical segment W, representing mRNAs expressed in either the wasp or its host, also hybridize to more than one viral DNA segment but do so with genomic hybridization profiles unique for each cDNA clone (32). In hybridizations of VR viral clones to digested parasite chromosomal DNA, a unique set of off-sized bands was observed. The presence of off-sized viral DNA bands when compared with purified viral DNA is indicative of integrated viral forms in *C. sonorensis* chromosomal DNA (12). The VR viral clones also hybridized to the DNA bands detected in hybridizations to the VR oviduct clone (Fig. 3*B*, asterisk), demonstrating that some oviduct cDNA and viral sequences were related. These results indicate that the VR oviduct cDNA clone partially encodes a VR wasp gene that is expressed in the oviduct, and the viral genomic clones contain related sequences that hybridize to both the integrated chromosomal form of VR viral DNA and to the nonviral, wasp-encoded VR genes. When the VR viral genomic and oviduct cDNA clones were used as hybridization probes in an RNA blot of parasitized and unparasitized *H. virescens* larvae, the 1.1- and 1.4-kb mRNAs and an additional 2.7-kb mRNA were detected only in the parasitized larvae. This clearly shows that VR viral genes are expressed after parasitization (Fig. 3*C*). These results strongly indicate that a family of VR genes is expressed in the parasite venom gland and oviduct, and also from CsPDV in parasitized *H. virescens* larvae (Fig. 3 *A* and *C*).

DISCUSSION

We describe an antigenic relationship between the secreted wasp venom proteins and the polydnavirus structural proteins which are synthesized and assembled into virus parti-

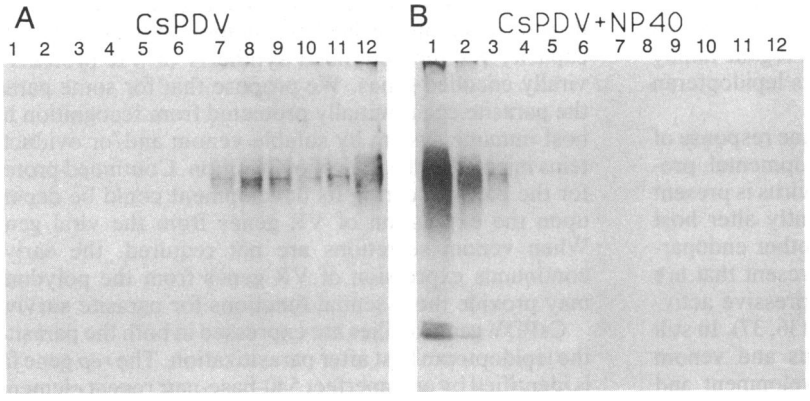

FIG. 2. Localization of VR viral proteins. Viral proteins were fractionated in a glycerol gradient by loading 200 μg of virus either before (*A*) or after (*B*) Nonidet P-40 treatment. Gradient fractions of 200 μl were removed and 20 μl of each fraction was electrophoresed in duplicate gels and then immunoblotted and probed with VR mAbs. Gradient fractions are indicated numerically from the top of the gradient. Gradient pellets were resuspended in 200 μl of phosphate-buffered saline and designated fraction 12.

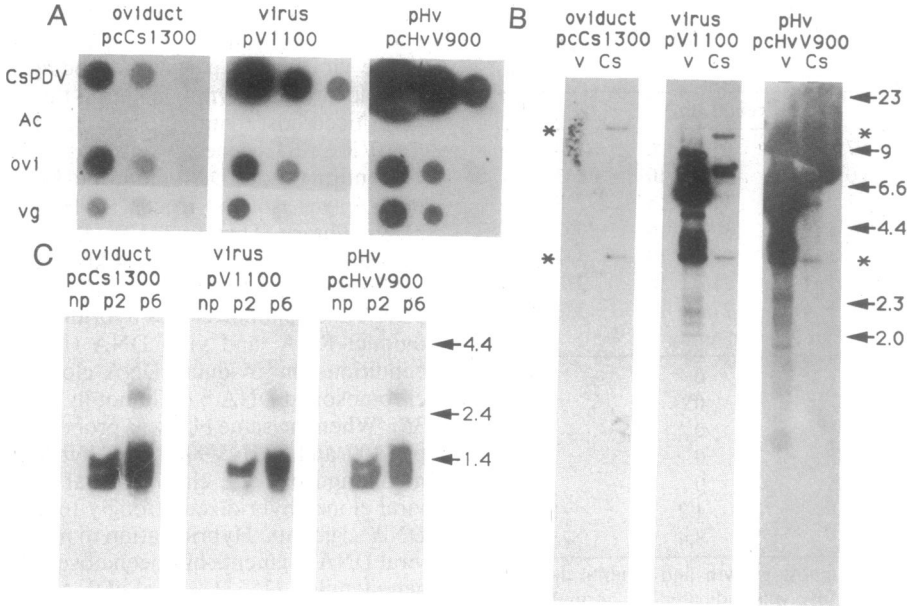

FIG. 3. RNA and genomic DNA blots probed with VR genes. (*A*) CsPDV and *Autographa californica* nuclear polyhedrosis virus (Ac) DNA (1 μg) and *C. sonorensis* RNA (1 μg) extracted from female oviducts (ovi) and venom glands (vg) were blotted to nitrocellulose and hybridized under conditions of reduced stringency to an oviduct VR cDNA (pcCs 1300), a viral genomic DNA clone (pV1100) and a viral, parasitized *H. virescens* cDNA clone (pcHvV900). Blots were washed and exposed for 48 hr (pcCs1300) or 16 hr (pV1100 and pcHvV900). (*B*) A genomic DNA blot of CsPDV (lane v) and *Hin*dIII-digested *C. sonorensis* DNA (lane Cs) was sequentially probed under stringent conditions with pcCs1300, pV1100, and pcHvV900. The blot was washed and exposed for 48 hr (oviduct), or 16 hr (virus, pHv). The wasp-specific VR DNA bands are indicated by an asterisk. *Hin*dIII-digested λ phage DNA size markers are shown in kilobase pairs. (*C*) Poly(A)$^+$ RNA was isolated from newly ecdysed fifth-instar unparasitized larvae (lane np) and from similarly staged parasitized larvae 2 hr (lane p2) and 6 hr (lane p6) after parasitization, electrophoresed, and transferred to nitrocellulose. The blot was then sequentially probed with pcCs1300, pV1100, and pcHvV900 at low stringency, washed, and exposed for 16 hr. RNA size markers are shown (Bethesda Research Laboratories) in kilobases.

cles from the specialized calyx cells of the wasp's oviduct. Secreted proteins from the venom gland and the oviduct share epitopes with viral envelope proteins that differ in number and apparent molecular weights. This suggests that viral proteins and venom proteins share some similar protein structure. Further, the mAbs that recognized the conserved venom, oviduct, and viral epitopes also blocked the virus-induced growth and developmental inhibition of *H. virescens* larvae that occurs during natural parasitization or after injection of purified virus.

Oviduct cDNA and VR viral cDNA and genomic clones were identified that, under reduced stringency, hybridized to both viral DNA and venom-gland RNA. Under high stringency, the oviduct cDNA clone hybridized only to *C. sonorensis* chromosomal DNA, while the VR viral clones hybridized to viral DNA and wasp chromosomal DNA. These viral clones hybridized both to mRNAs that are expressed in the venom gland and to the previously described 1.1- and 1.4-kb mRNAs that are abundantly expressed in the parasitized host (31). Although we have not yet established a direct relationship between the VR epitopes and VR DNA clones or mRNAs described in this study, the data show that the description of both the proteins and the DNA sequences as VR is valid and may indicate that at least one VR gene family is expressed in both the parasitic wasp and its lepidopteran host.

Endoparasitic hymenoptera evade the immune response of their hosts and frequently alter host developmental programs. In some species no detectable polydnavirus is present (33, 34) and venom secretions alone apparently alter host immunity and/or development (1, 2, 35). In other endoparasitic hymenoptera, virus-like particles are present that are associated with parasite-induced immunosuppressive activity but apparently lack detectable nucleic acid (36, 37). In still other parasitic species both the polydnavirus and venom secretions are required to suppress host development and immunity (2, 5). For example, Stoltz *et al.* (5) suggested that *Cotesia melanoscela* venom may facilitate viral expression by enhancing viral uncoating. Their data indicate that both secretions are important, if not essential, for successful parasite development. By comparison, venom-gland removal in *C. sonorensis* has no detectable effect on parasitization efficiency (17), and purified virus is capable of producing immunosuppressive and developmental effects (6, 7).

Our results establish that some venom and viral epitopes are conserved, and suggest that the expression products of parasite venom glands and polydnaviruses could be more closely related than previously indicated by the species diversity reported in the literature (2). That is, host venom-related genes may have become a part of the viral genome allowing both the supplementation and the eventual replacement of venom functions by virally encoded venom homologs, or conversely. The species-specific differences in which developmental arrest and/or immune suppression are caused by venom and/or polydnavirus expression may reflect different points in the evolutionary continuum of this relationship. Thus, during parasitization some parasites deliver not only venom and VR oviduct proteins, which may disrupt lepidopteran host physiology, but also the genetic capacity for the continued synthesis of VR proteins from virally encoded genes. We propose that for some parasites, the parasite egg is initially protected from recognition by the host immune system by soluble venom and/or oviduct proteins injected at the time of oviposition. Continued protection for the parasite during its development could be dependent upon the expression of VR genes from the viral genome. When venom secretions are not required, the early and continuous expression of VR genes from the polydnavirus may provide the essential functions for parasite survival.

CsPDV gene families are expressed in both the parasite and the lepidopteran host after parasitization. The *rep* gene family is identified by an imperfect 540-base-pair repeat element that

is present on the majority of the superhelical DNA segments as single or tandem repeats. Members of the *rep* gene family may be expressed in either the parasite or the lepidopteran host, or in both (15). Another gene family from CsPDV segment W encodes the related mRNAs of 1.0 and 1.6 kb that are expressed only in parasitized larvae (31). In our study, hybridization analysis showed that members of a putative VR gene family are present both in nonviral *C. sonorensis* chromosomal DNA and in viral genomic DNA. This indicates that VR gene expression occurs not only from the CsPDV but also from nonviral genes in the parasitic wasp. Our preliminary DNA sequence analysis of the VR oviduct and viral clones supports the dot blot and RNA gel blot analysis and shows that the hybridization reported herein is not due to highly repetitive sequences (unpublished data). Since the polydnavirus is integrated into wasp genomic DNA (12), the evolutionary potential for genetic transfer between the wasp and its virus clearly exists and may provide one explanation for the conserved antigenic relationships between venom and viral proteins.

Host-related viral proteins and functions have been described for many viruses (38–40). In vaccinia viruses, viral homologs of host genes regulating both cellular proliferation and the immune system have been identified. Viral expression of host-related genes may allow a virus to induce localized cellular proliferation (39), alter the immune response to the virus (40), or maintain the host in a developmental state that is advantageous for viral growth (41). In these examples viral expression of a host-related gene has a possible or apparent benefit only for the virus. However, CsPDV is unusual in that viral expression is essential for endoparasite development. This may represent a virus/host system in which a virally encoded homolog of a host gene is expressed to the apparent benefit of both the eukaryotic endoparasite host and the virus. The packaging of VR genes into a viral form may facilitate the mobility and targeting of these genetic units for expression in tissues that may be otherwise inaccessible to the parasite, thereby enhancing the regulation of host physiology and development.

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