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Polydnavirus-facilitated endoparasite protection against host immune defenses

(multi-gene families/insect immunity/intron conservation)

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ABSTRACT **The polydnavirus of *Campoletis sonorensis* has evolved with an unusual life cycle in which the virus exists as an obligate symbiont with the parasite insect and causes significant physiological and developmental alterations in the parasite's host. The segmented polydnavirus genome consists of double-stranded superhelical molecules; each segment is apparently integrated into the chromosomal DNA of each male and female wasp. The virus replicates in the nucleus of calyx cells and is secreted into the oviduct. When the virus is transferred to the host insect during oviposition, gene expression induces host immunosuppression and developmental arrest, which ensures successful development of the immature endoparasite. In the host, polydnavirus expression is detected by 2 hr and during endoparasite development. Most of the abundantly expressed viral genes expressed very early after parasitization belong to multigene families. Among these families, the "cysteine-rich" gene family is the most studied, and it may be important in inducing host manifestations resulting in parasite survival. This gene family is characterized by a similar gene structure with introns at comparable positions within the 5′ untranslated sequence and just 5′ to a specific cysteine codon (*C) within a cysteine motif, C-*C-CC-C-C. Another unusual feature is that the nucleotide sequences of introns 2 in the subfamily W*Hv*1.0/W*Hv*1.6 are more conserved than those of the flanking exons. The structures of these viral genes and possible functions for their encoded protein are considered within the context of the endoparasite and virus strategy for genetic adaptation and successful parasitization.**

General Overview of Parasite–Host Interactions and the Role of Polydnaviruses

Polydnaviruses not only have a unique taxonomic classification because of their segmented double-stranded DNA genomes but also exhibit an unusual relationship to two insects, an endoparasitic wasp and its host (1, 2). The virus is apparently symbiotically associated with the wasp where it is nonrandomly integrated into the chromosomal DNA of each male and female wasp in the wasp population. The virus replicates in specialized calyx cells of the female wasp at a specific time during late pupal development and in adults (3–7). After replication and assembly in the nucleus of the calyx cells, the polydnavirus is "secreted" into the wasp oviduct where it accumulates along with the wasp egg and a complex of oviduct secretions. Upon oviposition, the virus is transferred to a permissive host in which the endoparasite egg will develop. (Permissiveness in this context refers to a host that supports the development of the endoparasite.)

Significant developmental and physiological changes are induced in the parasitized host insect: phenomena that have been generally described as evidence of wasp-induced "host regulation" or "parasite-directed host manipulation" (8–10). The biological and biochemical manifestations of parasite-induced or -directed host manipulation are quite complex and vary significantly according to the specific parasitic wasp and host insect species; these changes are referred to as "immunosuppression" and "developmental arrest" (11, 12).

Immunosuppression and Developmental Arrest

The endoparasite's strategy for survival involves avoidance of host recognition as foreign or inhibition of the ensuing immune reaction (12–17). Since encapsulation is apparently the major host defense against parasite egg invasion, host immunosuppression is usually attributed to the wasp's ability to avoid or suppress encapsulation. For the endoparasite *Campoletis sonorensis* Edson *et al.* (18) showed that purified viable polydnavirus was responsible for suppressing the host insect's (*Heliothis virescens*) ability to encapsulate the wasp egg. Theilmann and Summers (19) also showed that both immunosuppression and developmental arrest are induced in *H. virescens* by purified polydnavirus. Inhibition of encapsulation also correlated with a decrease in circulating hemocytes and reduced plasmatocyte attachment and "spreading" (20).

Upon recognizing a foreign object, hemocytes form a cellular sheath (capsule) around that object and neutralize it (2). Although the identity of all hemocyte classes involved in the encapsulation process is not clear, most of the attention in investigating polydnavirus-induced immunosuppression has been given to the role of granulocytes and plasmatocytes (2). Granulocytes and plasmatocytes are the two principal hemocyte classes known to participate in forming the capsule (14). Granulocytes are thought to be the first hemocyte cell type to reach the foreign object (13, 14). Upon recognizing the invading object—for example, an endoparasite egg—granulocytes degranulate, releasing chemotactic factors that attract more granulocytes and plasmatocytes. Plasmatocytes attach and spread on the surface of the foreign body, for example an endoparasite egg. The role of the host prophenol oxidase cascade and/or other humoral factors in recognition of the endoparasite egg and initiation of encapsulation is not well defined (13).

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Abbreviation: hpp, hours postparasitization.

Diverse Strategies for Successful Parasitization

In the general spectrum of host–parasite relationships, there is considerable parasite-induced host variability resulting in perturbed host physiology, biochemistry, and developmental behavior (8, 21, 22). There is a wide range of speculation that the parasite has the capacity to modulate or regulate host systems (8, 10, 23). It is clear that the *C. sonorensis* polydnavirus induces developmental arrest in *H. virescens* larva (18, 24, 25), which is manifest as a significant reduction in weight gain and the delay of pupation to extend host larval life for a period of 9–12 days (19, 26). The mechanisms employed by polydnaviruses to alter host development have been comprehensively documented (8, 9) and must be studied pursuant to the identification of viral-induced or viral-expressed products that function in that role.

Parasitic wasps introduce several oviduct and venom gland secretions along with the wasp egg into the host. There are endoparasitic Hymenoptera that apparently alter the host's immune and developmental processes by parasite venom gland secretions (11, 27–30) or by both venom secretions and polydnaviruses (31–33). Edson *et al.* (18) showed that successful parasitization of *H. virescens* is dependent on *C. sonorensis* polydnavirus gene expression. Endoparasite eggs that are washed in physiological saline and injected into *H. virescens* alone or in combination with UV-inactivated *C. sonorensis* polydnavirus are encapsulated and killed. Preincubation with monoclonal antibodies specific for viral envelope proteins neutralized the effects of the virus on *H. virescens* (34). However, injecting *C. sonorensis* eggs with either the calyx fluid from the wasp oviduct or gradient-purified *C. sonorensis* polydnavirus results in the successful development of the endoparasite. So, *C. sonorensis* polydnavirus gene expression is both necessary and sufficient for inducing *H. virescens* immunosuppression and developmental arrest and, therefore, is essential for parasite survival.

Polydnaviruses: General Features

The major characteristic that distinguishes polydnaviruses from other animal DNA viruses is a segmented genome of multiple, covalently closed circular, double-stranded DNAs (6, 35, 36). Polydnaviruses have only been isolated from species of endoparasitic wasps belonging to the hymenopteran families of Braconidae and Ichneumonidae. The DNA genomes of viruses from different species of wasp endoparasites differ in the number of DNA segments and their molar abundance and sizes. A comparison of different polydnavirus species reveals from <10 to >25 segments in the genome (2, 6, 37). It is difficult to determine polydnavirus genetic complexity because not only do segments vary in number and size but also related sequences are found on the same segment and on different segments. The best estimates of the genome sizes are from 75 kbp to >250 kbp (6). The structure of the genome and nature of the virus life cycle clearly show that it is genetically very complex.

The nucleocapsid of the *C. sonorensis* polydnavirus is prolate ellipsoid in shape and has two envelopes (37): one envelope is obtained in the nucleus and the other as the virus buds through the calyx cell membrane into the lumen of the oviduct. The genome consists of ≈28 segments. Each DNA segment is given an alphabetical designation from A to W in order of increasing size. The segments are not present in equimolar ratios, but the agarose gel profiles of the banding patterns are qualitatively and quantitatively constant. Despite the genomic complexity, several rare and abundant genomic segments were cloned in their entirety, which allowed the mapping and sequencing of individual viral genes to be described in this paper (4, 25, 38). The functional significance of the different molar abundance of the viral segments is not clear at this time, but it may reflect the mode of replication and/or recombination among the multiple segments.

Viral DNA is integrated into the chromosomal DNA of male and female wasps. The virus also exists in an episomal form in female tissues and even in male wasps, though to a much reduced level (4). It is not known whether the linear or the episomal forms of the virus are the templates for viral replication.

Polydnavirus Expression in the Host

Although there is no detectable replication of the polydnavirus in *H. virescens*, viral DNA persists in infected tissues (19, 39), and there is abundant expression of several viral mRNAs. Fleming *et al.* (40) provided the first evidence that polydnavirus genes are transcribed in parasitized *H. virescens* larvae. At least 12 size classes of viral mRNAs are detected by Northern analysis in parasitized *H. virescens* during the course of endoparasite development (9–12 days), with some viral mRNAs detected as early as 2 hr postparasitization (hpp) (25, 38, 41–44). This led to the genomic mapping of the most abundant viral mRNAs to polydnavirus segment W (38). The mapping and sequencing of a number of viral genes and computer-assisted analysis of nucleotide and predicted amino acid sequences provided several additional insights regarding the function of the viral expression products. At this point, the most significant of these observations can be summarized as follows: (*i*) Transcription in the segmented genome is multipartite in that a specific cDNA probe will hybridize to different genomic segments. Comparisons of the DNA sequences of these cross-hybridizing genes revealed that there are related yet different genes on the same genomic segment and, very likely, on other segments. (*ii*) Different cDNA probes hybridized to different but sometimes partially overlapping sets of viral DNA segments. (*iii*) Almost all of the *C. sonorensis* polydnavirus genes mapped and studied to date belong to gene families. (*iv*) The expression of these genes apparently occurs in a host-specific manner, with some genes expressed only in the parasitized host and some only in the wasp, whereas others are expressed in both insects.

The purpose of our studies is to identify the viral expression products responsible for parasite survival of the host immune response. To do this, we have focused on identifying those viral genes that are abundantly expressed early (2 hpp) and continue during parasitization. We have been successful in mapping, cloning, and sequencing several of these genes and their corresponding cDNAs. We have cloned and expressed these cDNAs in the baculovirus expression system, and we have now formulated a working hypothesis to explain the role of a subset of these gene products.

Viral Multigene Families

So far, most of the *C. sonorensis* polydnavirus genes that are expressed in *H. virescens* are grouped into two gene families: "repeat" (25) and "cysteine-rich" (45) gene families. An additional "venom-related" gene family was identified based on immunological relatedness (34). The organization of several genes into families in *C. sonorensis* polydnavirus is yet to be described in other polydnaviruses. Although genes of gene families exist in other animal viral genomes (46–49), it is unusual to find different multigene families in the same virus genome.

Cysteine-Rich Gene Family

The cysteine-rich gene family is presently defined by the viral genes W*Hv*1.0, W*Hv*1.6, and V*Hv*1.1 [nomenclature: segment, W or V; host, *Hv* (*H. virescens*); RNA size in kb] (45). These genes share a common gene structure including introns at

comparable positions and encode secreted proteins that contain one or two cysteine motifs of the pattern C-C-CC-C-C (38, 45). W*Hv*1.0 and W*Hv*1.6 each contain a single cysteine motif, and V*Hv*1.1 contains two motifs. The sequence encoding the cysteine motif is interrupted by an intron immediately preceding the codon of the second cysteine of the motif. Also, introns interrupt the 5′ untranslated leader at comparable positions. The conservation of the intron positions clearly indicates that the three viral genes have a common ancestry. W*Hv*1.0 and W*Hv*1.6 are more related to each other than either is related to V*Hv*1.1; therefore, we grouped these genes into two subfamilies. A very unusual feature in the W*Hv*1.0/W*Hv*1.6 subfamily is that their intron 2 sequences are more conserved, over their entire length, than their flanking exons (45).

Gene Structure of W*Hv*1.0, W*Hv*1.6, and V*Hv1.1*

The cysteine-rich gene family was initially discovered as two separate gene families (41). Transcripts of 1.0 and 1.6 kb (encoded by W*Hv*1.0 and W*Hv*1.6, respectively) and 1.1 and 1.4 kb (encoded by V*Hv*1.1 and a gene yet to be identified, respectively) from parasitized *H. virescens* hybridized, under high-stringency conditions, to two different *C. sonorensis* polydnavirus genomic clones. Multiple viral genomic segments hybridized to these genomic clones. Those results suggested that two different and potentially large gene families are encoded by a discrete set of viral genomic segments. The reduced homology of W*Hv*1.0 and W*Hv*1.6 to V*Hv*1.1 sequences (45) explains the lack of cross-hybridization to the 1.1- and 1.4-kb transcripts under high-stringency conditions (38, 41). Additional members of this subfamily probably exist on other genomic segments as indicated by Southern analysis (38, 41).

The relatedness of W*Hv*1.0 and W*Hv*1.6 was confirmed when the cDNAs for these genes were cloned and sequenced (38). Five regions (A–E) were found to be 68–80% identical at the nucleotide and amino acid levels. These conserved regions were separated by regions of very low similarity. Region A encompasses the 5′ untranslated leader and the first 16 amino acids of the predicted open reading frame. Region B encompasses a stretch of 27 amino acids immediately preceding the cysteine motif and the primary sequence delineated by the first three cysteine residues of this motif. Region C encompasses the sequence delineated by the last two cysteine residues of this motif and the C terminus. Regions D and E are located in the 3′ untranslated sequence, separated by over 400 bp. After we cloned and sequenced V*Hv*1.1 and identified the conserved cysteine motif and locations of introns in all three genes, we reevaluated and extended the data for W*Hv*1.0/W*Hv*1.6 (45).

The promoter, 5′ and 3′ untranslated sequences, and introns 1 and 2 of W*Hv*1.0 and W*Hv*1.6 share 80% or greater similarity (identity) (38, 45). This may be explained by a relatively recent intrasegment gene duplication event. By contrast, the sequences encoding the mature N and C termini of these two proteins have significantly diverged (44% and 52% similarity, respectively) (38). The molecular mechanisms that maintain conserved noncoding sequences, including introns, but allow significant divergence of exon sequences are not clear at this time. The length difference between the 1.0- and 1.6-kb transcripts is due to 577 nt in the 3′ untranslated sequence, which may have resulted from two insertions/deletions (38).

A distinctive feature of this gene family is the high degree of similarity (92%) in intron 2 sequences of W*Hv*1.0 and W*Hv*1.6. This similarity is higher than that (76%) of the immediately flanking exon sequences, which encode the cysteine motif (45). The nucleotide similarity spans the whole intron sequence with two insertions/deletions toward the 5′ and 3′ ends of the introns, which are required to optimize the sequence alignment. A high degree of sequence similarity among introns of recently duplicated genes was reported, but in those cases the exon sequences were conserved to the same degree (50, 51).

It is very unusual for introns to be more conserved than their flanking exons. Except for the conserved splicing signals at the 5′ and 3′ ends, and the branch site of the intron, the primary intron sequence is not thought to play an important role in splicing (52). Also, unlike the cases of other nuclear pre-mRNA introns, computer-assisted analysis did not reveal the presence of known control elements (53–58) or the presence of functional transcripts or independent genes (59–61). The high degree of sequence conservation may simply reflect the short time since the divergence of the two genes. Alternatively, these introns may have a functional role yet to be identified.

The conservation of the intron position with respect to the cysteine motifs of V*Hv*1.1 strongly indicates an intragenic duplication event. Introns 2 and 3 of V*Hv*1.1 show similarity to intron 2 in W*Hv*1.0 and W*Hv*1.6, which is limited to the 5′ and 3′ ends beyond the conserved splicing signals: these introns also show the same limited similarity to each other (45). Assuming that introns in these genes are subject to similar evolutionary forces, the limited similarity between introns 2 and 3 of V*Hv*1.1 indicates that the presumed intragenic duplication preceded the duplication of W*Hv*1.0 and W*Hv*1.6.

Alternative splicing is another control process involved in gene expression that can produce different proteins from the same gene (62, 63). We isolated a V*Hv*1.1-like cDNA, pcVR900 (34), which utilizes an alternative 3′ acceptor site of intron 1 and an alternative polyadenylylation site. That cDNA, however, is incomplete; it lacks an initiator methionine at an analogous position to V*Hv*1.1, and the 5′ hexanucleotide consists of thymidine residues not coded by the V*Hv*1.1 gene (S.D.D.-H., B. Webb, and M.D.S., unpublished results). By using an internal AUG codon, that cDNA has a predicted open reading frame of only 60 amino acids that bear no similarity to the 217 amino acids of V*Hv*1.1. It is possible that this cDNA may have resulted from a cloning artifact, but it is also possible that the corresponding transcript may be encoded by another V*Hv*1.1-like gene or may be produced by trans-splicing of a leader sequence. Alternative and trans-splicing are two mechanisms to generate genetic diversity that may be utilized by this virus.

Open Reading Frames of the Cysteine-Rich Genes

The cysteine motifs in this polydnavirus gene family are structurally analogous to the cysteine motifs of the ω-conotoxins. The ω-conotoxins are high-affinity ligands with different receptor specificity for voltage-sensitive ion channels (64, 65). The mature ω-conotoxins have three disulfide bridges that function as a conserved, highly compact, structural scaffold with hypervariable intercysteine amino acid residues. The *C. sonorensis* polydnavirus cysteine-rich gene family encodes secreted proteins that are apparently produced throughout the parasitization period. The hypervariability in amino acids of the cysteine motif of these genes suggests that these proteins may bind to related, yet different, targets.

The sequences encoding the cysteine motifs in W*Hv*1.0 and W*Hv*1.6 are 76% identical, counting a 33-bp deletion as a single mutational event; however, the two motifs are 58% identical at the amino acid level (45). The relative high similarity at the nucleotide sequence level and the decreased similarity at the amino acid sequence level are inconsistent with predictions of the neutral theory of mutation (66). The majority of the nucleotide substitutions in this domain are in codon positions 1 and 2 and multicodon positions, which cause amino acid replacement. A similar pattern of nucleotide substitutions is found in the sequence encoding the antigen

recognition sites of the major histocompatibility complex I and II genes (67, 68). Codon substitutions in the antigen recognition site sequences result in hypervariability that is biologically significant and is explained by overdominant selection or positive Darwinian selective pressure.

Nei and coworkers (69) developed a mathematical model to describe overdominant selection. Briefly, they determined the fraction of silent substitutions (synonymous changes per synonymous site, d_S) and then determined the fraction of replacement substitutions (nonsynonymous changes per nonsynonymous site, d_N). In the case of the antigen recognition sites, d_S is 2- to 4-fold smaller than d_N; however, d_S is significantly greater than d_N (5- to 9-fold higher) in the constant domains (67, 68). When the sequence encoding the cysteine motifs of W*Hv*1.0 and W*Hv*1.6 are compared to each other, d_S is only about 2-fold greater than d_N. But, when the codons invariant in these two motifs are excluded from the analysis, d_S and d_N become comparable (45). This analysis included the codons of all the motif amino acids because those involved in binding to the putative target are yet to be identified. Using all of the codons in the analysis may have masked overdominant selection, since amino acids not involved in binding to the putative target would not be subject to overdominant selection. Alternatively, no negative selective pressure is exerted on this motif. It is conceivable that both of these mechanisms are at work on different codons of the cysteine motif.

Aside from the cysteine motif, conserved regions in the proteins encoded by W*Hv*1.0 and W*Hv*1.6 are likely to reflect functional roles. Based on the rules of Von Heijne (70), the N-terminal 16 amino acids may function as a signal peptide for secretion. Recombinant W*Hv*1.0 and W*Hv*1.6 proteins are secreted into the medium of infected insect cells (43) and into the hemolymph of parasitized *H. virescens* (S.D.D.-H., B. Graham, and M.D.S., unpublished results). By analogy to the propeptides of conotoxins, the highly conserved precysteine domain (26 out of 27 identical amino acids) may be important for folding the cysteine motif into a structure compatible with optimal biological function. Alternatively, this domain may serve another function yet to be determined.

The predicted mature N termini of W*Hv*1.0 and W*Hv*1.6 are only 16% identical, whereas the sequences C-terminal to the cysteine motif are about 36% identical (38). The untranslated sequences in these genes are more conserved than the sequences encoding these regions. It is possible that the specific role of the N and C termini is largely independent of their primary sequence. Alternatively, the divergence may reflect possible different functions. These conclusions will be further clarified as other members of this subfamily are cloned and sequenced or as functional assays for these proteins are developed. The fact that the N and C termini are flanked by highly conserved sequences may indicate that homologous recombination among members of this family results in the exchange of these domains to generate novel combinations.

In addition to the two cysteine motifs, the open reading frame of V*Hv*1.1 has three degenerate motifs, EPEADGKT, DEAN, and SAT (45) characteristic of the "DEAD" family of ATP-dependent RNA helicases (71, 72). The invariant amino acids of these motifs are underlined. The translation initiation factor eIF-4A is the prototype of this family (71); however, other family members are involved in splicing (73). The order but not the spacing of these motifs is conserved in the V*Hv*1.1 open reading frame. The first and second V*Hv*1.1 motifs are analogous to the DEAD family "A" and "B" motifs, which are essential for ATP binding and hydrolysis (74–76). The third motif, SAT, is implicated in RNA unwinding (75). Whether these motifs are functional in V*Hv*1.1 remains to be seen. The presence of these motifs, separated by the N-terminal cysteine motif, may better be explained by convergent evolution than the acquisition of a host DEAD domain by the virus.

Venom-Related Gene Family

Webb and Summers (34) report that polydnavirus envelope proteins share cross-reacting epitopes with proteins from wasp venom glands. The conservation of epitopes on the virus and venom gland proteins may suggest functional or evolutionary relationships. Three isolates of monoclonal antibodies raised against venom gland proteins of *C. sonorensis* were selected because each cross-reacted with *C. sonorensis* polydnavirus envelope proteins and soluble wasp oviduct and venom gland proteins. The viral envelope proteins had different molecular weights compared to wasp venom and soluble oviduct proteins. Preincubation of purified *C. sonorensis* polydnavirus with these monoclonal antibodies neutralizes the effects of the virus when injected into *H. virescens*. The immunological relatedness of different viral envelope proteins to each other and to wasp venom proteins defines the venom-related gene family.

The viral genes encoding the envelope proteins and the genes for the venom secretions are yet to be isolated and studied. The existence of the venom-related gene family was also indicated by the isolation of cross-hybridizing cDNAs from a wasp and parasitized *H. virescens* libraries (34). However, that result may have been fortuitous because the presence of the sequence encoding the DEAD motifs in pcVR900 cDNA (B. Webb and M.D.S., unpublished results) may explain the cross-hybridization to the wasp cDNA. Sequencing of the wasp cDNA revealed the presence of all conserved features of eIF-4A and a significant overall similarity to mouse and yeast cognates (B. Webb and M.D.S., unpublished results). The wasp cDNA is likely to correspond to the wasp eIF-4A gene.

Repeat Gene Family

Early reports indicated that the *C. sonorensis* polydnavirus genome was made up of mostly unique sequences (35). However, Blissard *et al.* (41) demonstrated that different genomic segments hybridized to distinct subsets of viral segments. Further analysis indicated that sequence homology exists among the majority of genomic segments (44). Viral segment O^1 hybridized to 11 other DNA segments. Highly repetitive sequences believed to be largely, although not entirely, responsible for the extensive homology among the segments were identified on segments B, H, and O^1. An optimal sequence alignment revealed an imperfectly repeated consensus sequence ≈540 bp in length, with an average similarity of 60–70%. Shorter regions of sequences approach 90% similarity, and the two repeated sequences on segment O^1 share long stretches of sequence similarity. The 540-bp repeat elements are present as a single repeat, as on segment B, or in direct tandem arrays of two or more, as on segments H and O^1. Southern blot analyses of the viral genome under conditions of low stringency indicate that sequences related to the 540-bp element are conserved on most of the DNA segments.

Northern blot analyses indicate that sequences of the four repeated elements are present on transcripts expressed in *H. virescens* by 2 hpp (25). Steady-state RNA levels peak at 2–6 hpp and then decline over the next 8 days. These transcripts are not as abundant as the cysteine-rich transcripts but are still major viral expression products.

Sequence analysis of cDNA clones shows that the 540-bp consensus repeats on DNA segment B, H, and O^1 are contained within an open reading frame. These predicted open reading frames have been confirmed by *in vitro* translation and expression of recombinant proteins in bacteria. Putative signal peptides at the N terminus of the open reading frames are not predicted for any of the 540-bp repeat gene protein sequences.

These repeated elements share no significant homology to the cysteine-rich gene family. This family of genes also differs

from the cysteine-rich gene family in that its members do not contain introns and three of the four transcripts studied so far are also expressed in oviducts of the female *C. sonorensis*. This, combined with the lack of significant similarity in the protein sequence, suggests that this family will likely play a role in the *C. sonorensis* polydnavirus life cycle distinct from that of the cysteine-rich gene family.

Parasite–Virus–Host

Many parasites or pathogens use insects as reservoirs, vectors, or hosts. Parasites also utilize diverse strategies to survive host defense mechanisms and must have the ability to rapidly evolve in response to them. A fundamental characteristic of strategies for parasite survival is the capability of rapid adaptation to their host immune defenses. The genetic basis for diversity within these strategies is the evolution of new genetic entities that involve gene amplification and regulation of gene expression at multiple levels, which may vary according to the specific parasite–host system (77–79). These include not only the expression of molecules designed for the passive protection of the parasite but also other effector molecules, which in turn can alter control and/or processing of gene expression products. Impose upon this the numerous parasite–host insect species and plethora of cells, tissues, and host developmental factors and processes that the parasite may target for its selective advantage, and one can appreciate the considerable potential for "diversity."

The parasite–host interaction continuously exerts selective pressure(s) on both insects to survive. The host insect presents a particularly challenging environment for endoparasites because of the rapid development and differentiation that characterize parasite and host life cycles (21, 22, 80, 81). Endoparasites may exploit a variety of agents to suppress or avoid host defenses and to modify the normal development of the host to match their need: polydnaviruses, venoms, oviduct secretions, and protective materials coating the parasite egg or produced by the endoparasite as it develops within its host.

In a permissive host, not all immature wasps are successful in surviving the host's defenses, indicating that selection pressures bearing on the survival strategies of both insects involve some balance that favors the parasite. To the extent that failed endoparasite development is not due to a defect in the wasp egg, it is likely that the surviving endoparasite represents a genetic lineage associated with a competent polydnavirus. For the polydnavirus of *C. sonorensis*, experimental data demonstrate that polydnavirus gene expression in the parasitized insect is sufficient for parasite survival. Assuming that the polydnavirus genetic system is in a dynamic state of evolution, one must be curious about the molecular mechanisms by which the surviving wasps are able to retain the essence of the most effective polydnavirus genetics to favor the wasp's survival strategy.

Segmented Virus Genomes: Evolution and Functional Significance

The endoparasite *C. sonorensis* has evolved with the ability to generate extrachromosomal genetic elements in the form of multiple double-stranded, superhelical DNA molecules. These DNA molecules are amplified in the calyx cell nucleus, packaged into viruses, and secreted in a complex process of viral maturation, which also provides a complex double viral envelope. One viral envelope is assembled in the cell nucleus, and the other is obtained during budding from the calyx cell surface into the oviduct lumen. Viral envelopes, which are derived from cellular membranes, may mediate species-specific virus host cell and tissue interactions. This could be one important aspect of the species-specific endoparasite–host relationship fundamental to parasite survival.

Perhaps polydnaviruses are another variation upon diverse eukaryotic mechanisms of gene amplification and transfer of genetic information. Numerous examples of mechanisms of extrachromosomal gene amplification and maintenance of superhelical genomic DNA (82–96) and viral acquisition and adaptation of host genes to benefit the parasite or the virus (97–99) are reported in the literature. Also, portions of eukaryote genomes are believed to consist of sequences that correspond to transposable and mobile elements. Some of these are known to be widespread among insect orders (100). It is reasonable to consider that the heterodisperse genetic elements of a polydnavirus may represent mobile elements that have evolved to take a viral form during horizontal movement to another eukaryotic organism. Theoretically, this segmented complex of extrachromosomal DNA superhelices, and the genes encoded by them, could undergo rapid evolution (77, 93, 94, 101, 102).

It is also possible that polydnaviruses were originally virulent in *C. sonorensis*, but the virus–wasp relationship has evolved toward mutualism (103). Obligate symbiosis enables the host to acquire functions that improve its chances for survival; the mutualist also benefits by securing its passage to the host progeny. Polydnaviruses may be optimal mutualists in that their genomes are integrated into the wasp chromosomal DNA and, thus, transmitted vertically to each male and female in the wasp population (2, 6).

Gene Families: Evolution and Significance

A significant part of the *C. sonorensis* polydnavirus segmented genome is apparently organized into several families of genes. It is not yet known if the occurrence of gene families is a general phenomenon for polydnaviruses, but *C. sonorensis* polydnavirus clearly possesses the genetic capability to express a large number of gene families, each with a significant amount of potential genetic variation. So, what is the significance of these viral gene families? It is recognized that gene families are a major source of genetic variability and that families evolve by gene duplication followed by sequence divergence (104). Gene duplication may occur during inter- and intramolecular recombination among the several viral genomic segments during viral DNA replication and/or by chromosomal crossing-over during wasp sexual reproduction. This viral genetic system presents significant potential for a large number of duplicated, yet diverging, genes encoding a variety of related, yet different, proteins.

Virus gene families have the potential to rapidly generate diversity to counter adaptive changes in host immune defenses to benefit the wasp's strategy for survival. We are limited in our ability to assess the significance of multigene families in the virus until the function(s) of the various proteins are identified. However, a classic example of the use of gene families is the surface antigen variability in trypanosomes (101, 102). In response to host selection pressure, trypanosomes express variable surface antigens by activating different members of a gene family.

Positive Darwinian selective pressure or overdominant selection is implicated in the evolution of the circumsporozoite protein genes of the malaria parasites, glutathione *S*-transferase of *Schistosoma* (105, 106) and the hemagglutinin genes of human influenza viruses (107). The vertebrate immune system has the potential to produce an impressive repertoire of molecules that recognize invading foreign antigens. The antigen-binding sites of antibodies and surface receptors encoded by the major histocompatibility complex I and II genes were also shown to be under positive Darwinian selective pressure (67, 68, 108). Nucleotide substitutions in the codons of the antigen-binding sites acquire more replacement than silent mutations. The ability of the immune system to generate this diversity places significant selective pressure on

the parasite: parasite proteins are subjected to two opposing evolutionary forces that control amino acid substitution (101). The first force is for neutral selection favoring silent mutations that retain the function of that protein, particularly if it is a nonstructural protein. The second force is the pressure toward diversity to avoid recognition by the immune system.

We have applied these models to assess if overdominant selection underlies the hypervariability of the intercysteine amino acid residues of the cysteine motif of the *C. sonorensis* polydnavirus cysteine-rich gene family (45). Statistical tests applied to the sequence encoding these motifs in W*Hv*1.0 and W*Hv*1.6 are consistent with this possibility but are inconclusive. Our analyses, however, are limited by the lack of knowledge of the specific amino acids that interact with the target molecules. Once identified, more rigorous analyses are possible. In further support of a possible functional significance of the intercysteine amino acid hypervariability, the replacements in the intercysteine residues also change the overall charge distribution in this domain. Analysis of the cysteine motifs of the V*Hv*1.1 gene awaits cloning of other members of this subfamily.

The repeat gene family tandem repeats may increase both intra- and intermolecular recombination events, which result in gene or epitope amplification that is usually followed by sequence divergence. This could be another strategy of adaptation by the virus to keep pace with its evolving host. Because of its potentially large size and sequence diversity, the 540-bp repeat gene family will presumably play an important role. Based upon predicted properties, the 540-bp repeat proteins are believed to function differently than those of the cysteine-rich family.

Summary

Several *C. sonorensis* polydnavirus genes are expressed by 2 hpp and during parasite development. The abundance and temporal pattern of expression strongly suggest that these viral-encoded proteins provide important functions for successful parasitization. For that reason, we have investigated the identity and the molecular basis of the structure and function of these genes and their products. We have identified several members of the cysteine-rich and repeat gene families (25, 38, 41, 45). So far, it has been difficult to propose a role for the expression products of the repeat gene family. However, there is preliminary evidence that conserved sequence elements in a member of the repeat gene family on segment B may be involved in the integration/excision events of that segment (4). The hypervariability of the intercysteine amino acids of the polydnavirus cysteine-rich proteins and the analogy to the ω-conotoxins suggest that members of this gene family bind to different molecular targets in *H. virescens* to affect blood cell functions, the encapsulation process, or other host functions.

It is reasonable to propose that the cysteine-rich proteins may play an important role in preventing the recognition of foreign objects and/or the normal response of components of the immune system. Poxviruses encode soluble lymphokine receptors that are secreted from infected cells to act as decoys and prevent identification and killing of virus-infected cells (109). The cysteine-rich family of proteins may bind to analogous signals in the insect hemolymph and prevent the activation of the immune response. Alternatively, these soluble proteins may bind receptors on hemocyte surfaces and prevent recognition of the endoparasite as foreign or inhibit the normal response of those cells. In studies of parasite-induced immunosuppression of insects, a soluble "transformation" factor(s) is proposed to explain the altered behavior of plasmatocytes (20), which correlates with suppression of encapsulation. Our model is consistent with the presence of such factor(s). At later times when the host immune system appears to be significantly compromised, cysteine-rich proteins, through their hypervariable cysteine motif, may bind to related yet distinct targets to affect multiple host systems.

This paper is an assessment of our current knowledge of the structure and function of gene families of polydnavirus of *C. sonorensis* and their expression relative to their role in endoparasite survival. It is not intended to provide a comprehensive review of endoparasite–host relationships or the several polydnaviruses associated with them: the literature citations in the first part of this paper are quite sufficient in that regard. Only a few other polydnavirus–endoparasite–host systems are under experimental scrutiny. Polydnavirus-induced host alterations are being studied in *Manduca sexta* (8), *Trichoplusia ni* (110), *Spodoptera frugiperda* (111), *H. virescens* (112), and *Pseudoplusia includens* (39). However, the structural and functional organization of the polydnavirus genome of *C. sonorensis* is the most comprehensively studied at the molecular level. From these studies, we have sufficient information on gene structure and expression of several families of gene products to begin a more direct inquiry into their function. The possible involvement of the cysteine-rich gene proteins in targeting parasitized host systems to facilitate parasite survival is currently a testable model for the role of polydnavirus proteins in inducing host immunosuppression and developmental arrest. If successful, our knowledge of these functions may provide additional insights for the biochemistry and cell biology of insect cellular immune processes.

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