

## $\text{I}\kappa\beta$ -Related *vankyrin* Genes in the *Campoletis sonorensis* Ichnovirus: Temporal and Tissue-Specific Patterns of Expression in Parasitized *Heliothis virescens* Lepidopteran Hosts†

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**Polydnnaviruses (PDVs) are unusual insect viruses that occur in obligate symbiotic associations with parasitic ichneumonid (ichnoviruses, or IVs) and braconid (bracoviruses, or BVs) wasps. PDVs are injected with eggs, ovarian proteins, and venom during parasitization. Following infection of cells in host tissues, viral genes are expressed and their products function to alter lepidopteran host physiology, enabling endoparasitoid development. Here we describe the *Campoletis sonorensis* IV viral *ankyrin* (*vankyrin*) gene family and its transcription. The seven members of this gene family possess ankyrin repeat domains that resemble the inhibitory domains of the *Drosophila melanogaster* NF- $\kappa\beta$  transcription factor inhibitor ( $\text{I}\kappa\beta$ ) cactus. *vankyrin* gene expression is detected within 2 to 4 h postparasitization (p.p.) in *Heliothis virescens* hosts and reaches peak levels by 3 days p.p. Our data indicate that *vankyrin* genes from the *C. sonorensis* IV genome are differentially expressed in the tissues of parasitized hosts and can be divided into two subclasses: those that target the host fat body and those that target host hemocytes. Polyclonal antibodies raised against a fat-body targeting *vankyrin* detected a 19-kDa protein in crude extracts prepared from the 3 days p.p. fat body. *Vankyrin*-specific Abs localized to 3-day p.p. fat-body and hemocyte nuclei, suggesting a role for *vankyrin* proteins in the nuclei of *C. sonorensis* IV-infected cells. These data are evidence for divergent tissue specificities and targeting of multigene families in IVs. We hypothesize that PDV *vankyrin* genes may suppress NF- $\kappa\beta$  activity during immune responses and developmental cascades in parasitized lepidopteran hosts of *C. sonorensis*.**

Polydnnaviruses (PDVs) share obligate symbiotic associations with parasitoid wasps in the families *Braconidae* (bracoviruses, or BVs) and *Ichneumonidae* (ichnoviruses, or IVs) (38, 70, 81, 84). The BV and IV genera are evolutionarily distinct (87, 88), yet similarities in DNA composition, structure, and function suggest that the two PDV genera may be convergently evolving. PDV genomes are the only known viral genomes comprised of multisegmented double-stranded DNA (81, 84, 86). PDVs are vertically transmitted as proviruses within the genomic DNA of their corresponding wasp hosts (6, 15, 22, 26, 55, 58, 72). Excision and replication of proviral segments occurs exclusively in virogenic stroma within the calyx cell nuclei of female wasps (52, 71, 90, 89, 83). Virus replication is initiated during late pupal stages following ecdysteroid-induced differentiation of the female ovaries (26, 46, 52, 70, 89). Viral DNA segments are packaged into double-unit membranes (IVs) or single-unit membranes (BVs) and released by budding (IVs) or lysis (BVs) from calyx cells into the extracellular calyx fluid (71, 73, 83, 89). Virions are then transferred to the parasitoid's lepidopteran hosts with eggs, ovarian proteins, and venom during parasitization, where they infect local tissues, express encoded genes, and induce pathologies essential to the survival of the developing endoparasitoid (38, 64, 70, 75, 81, 84). These pathological alterations include disruption of host development, immuno-

suppression, and mobilization of host protein stores for parasitoid utilization (2, 3, 13, 17, 30, 41, 50, 62, 64, 66, 74, 85).

PDV genomes are unique among extant viral groups in that they harbor large multigene families involved with immunosuppression and immunoevasion in parasitized hosts but lack genes involved in virus replication within the encapsidated genome (38, 81). This unusual feature of PDV genomes likely results from the symbiotic associations these viruses have with the parasitic hymenoptera. PDV multigene families are the subject of speculation in terms of their evolutionary origins and importance for parasitoid-host relationships. It is suspected that possession of gene family variants may be an attribute essential for lepidopteran host adaptation, host range expansion, and amplification of gene copy numbers in parasitized larvae (12, 38, 75).

The *Campoletis sonorensis* ichnovirus genome contains 24 unique segments encoding 248 kb of nonredundant DNA (unpublished data). Earlier publications have described the existence of three gene families within this genome: the *cys-motif*, *rep*, and *vinnexin* gene families. These multigene families have been characterized based on similarities in sequence, protein structure, expression patterns, and function (9, 10, 13, 14, 16, 19, 29, 38, 41, 68, 78, 79, 81). Here we describe the existence of a fourth gene family, the *viral ankyrin* or *vankyrin* gene family, in *C. sonorensis* IV. The *vankyrin* gene family includes seven genes encoded on two *C. sonorensis* IV genome segments, P (GenBank AY029396) and I<sup>2</sup> (GenBank AF362517). Members of this gene family encode open reading frames (ORFs) similar to the ankyrin repeat domains of the *Drosophila melanogaster* dorsal/NF- $\kappa\beta$  transcription factor inhibitor cactus, a member of the  $\text{I}\kappa\beta$  gene family (7, 25).

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*C. sonorensis* IV infection of *Heliothis virescens* larvae causes severe physiological changes in the host, including developmental arrest, a suppression of cellular and humoral immunity, and a decrease in host growth-associated hemolymph proteins such as arylphorin (13, 41, 62, 63, 64, 66, 85). Increased susceptibility to pathogenic attack has been partly attributed to decreased induction of cecropin and lysozyme activities in hosts of *C. sonorensis* (65). It is well documented that the promoters for many insect antimicrobial and antifungal peptide genes (*lectin*, *dipterocin*, *cecropins*, *drosocin*, *drosomycin*, *defensin*, *attacin*, and *lysozyme*) contain variations of the insect NF- $\kappa$ B transcription factor consensus DNA binding sequence and that the Toll and immune deficiency signaling pathways are essential to the induction of these genes during insect humoral immune responses (18, 20, 33, 35, 36, 45, 47, 49, 76). Several genes involved in cellular immunity and developmental cascades (e.g., *zernkult*, *twist*, *snail*, and *rhomboid* from *Drosophila*; interleukin-1 [IL-1], IL-6, tumor necrosis factor alpha [TNF- $\alpha$ ] in mammals) are also under NF- $\kappa$ B-mediated transcriptional control (7, 24, 25, 80). The presence of I $\kappa$ B-related genes in PDVs is intriguing, as it indicates the possibility that these NF- $\kappa$ B-mediated activities may be targeted for disruption by PDV infections. Such activities could benefit parasitization by preventing lectin-mediated recognition and hemocytic destruction of the endoparasitic egg and larvae as well as altering host development to support endoparasite survival and development.

In this paper, we describe the expression patterns for all members of the *C. sonorensis* IV *vankyrin* gene family and the location of vankyrin proteins in *H. virescens* tissues and cells. Our data present strong evidence for differential expression and tissue-specific targeting of proteins encoded by this multigene family.

#### MATERIALS AND METHODS

**Insects and parasitizations.** *C. sonorensis* parasitoids and their lepidopteran host, *H. virescens*, were reared at 27° with a 16-h light:8-h dark photoperiod as described previously (37). For parasitization, equal numbers of wasps and third-instar *H. virescens* hosts were placed in a petri dish for 4 h at room temperature (RT). For temporal transcriptional analyses, wasps were allowed to sting third-instar *H. virescens* hosts twice to ensure parasitization (i.e., immediately after the second sting = time zero h postparasitization [p.p.]).

**PCR.** Oligonucleotide primer pairs were designed for specific amplification of portions of the *H. virescens actin* gene from cDNA and each *vankyrin* gene from a genomic DNA library created from digestion and ligation of *C. sonorensis* IV viral DNA into a compatibly digested pZERO-1 vector (Invitrogen, Carlsbad, CA) (15, 29). The following specific primer pairs were designed for all *vankyrin* genes on *C. sonorensis* IV segments P and I<sup>2</sup> and for *H. virescens actin*, respectively: *P-vank-1* (forward [FW], 5' GAG GAT CAT ACA GGT GCT ACT AGA G 3'; reverse [RV] 5' TCT TTG TTG CAC GTT ATC AAC GAC G 3'); *P-vank-2* (FW, 5' TTC AAA AAC TCT TCA CCC GAA AGC C 3'; RV, 5' CAG GTG AGT TTA ATA CGG TTC AGT A 3'); *P-vank-3* (FW, ATC ACT CAC GGT GCT GTA CCG 3'; RV, CGC AAA ATT CTC ATC TCT TCA 3'); *P-vank-4* (FW, 5' CGA GAT TGC CCG AAC TGG ATT C 3'; RV, 5' GTC GCA AAA TTC TCA TCA TCT CTT G 3'); *I<sup>2</sup>-vank-1* (FW, 5' ATG GTA AAA ACT GCA TCC ACC TGG C 3'; RV, 5' CAC ACC GTA AGT GGA ACA GTT CG 3'); *I<sup>2</sup>-vank-2* (FW, 5' CTC GTT GGT AGA AAA CAG ATT ACT G 3'; RV, 5' CAT TCT CTA TAA TCG CCA TTT GAC 3'); *I<sup>2</sup>-vank-3* (FW, 5' GCA AAG CTG TTC GGT ACA AAC TGG 3'; RV, 5' TTT GAT GTG CCG TAA GTC CAG CGA 3'); *H. virescens actin* (FW, 5' GAT GTG GCG GCG TTG GTG GTC 3'; RV, 5' GTT GGA AGG TGG AGA GGG AAG C 3'). Standard 45-round PCR amplification reactions (2 min of 95°C hot-start denaturation, 30 s of 95°C denaturation, 45 s of 55°C annealing, 2 min of 72°C elongation, 45 cycles, 10-min 72°C final extension, 4°C hold) were performed according to Hilgarth and Webb (29) with recombinant *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA).

Resultant PCR products were used for Northern blotting, reverse transcription-PCR, and relative quantitative real-time PCRs as described below.

**Northern blots.** Total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions from male and female *C. sonorensis* adults (10 adults/isolation), nonparasitized *H. virescens* larvae, and parasitized *H. virescens* larvae (three third-instar larvae/time point) at different time points (2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 1 day, 2 days, 3 days, 4 days, 5 days) following parasitization. Parasitized larvae remained in the third instar throughout the time course as *H. virescens* host development arrests within 24 h of *C. sonorensis* oviposition. Purified total RNA (30  $\mu$ g/lane) was electrophoresed on 1% Tris-acetate-EDTA-agarose gels and transferred to nylon filters (Immobilon N+; Millipore, Bedford, MA) as described previously (14). <sup>32</sup>P end-labeled probes corresponding to *H. virescens actin* and specific members of the *C. sonorensis* IV *vankyrin* gene family were synthesized from PCR-generated DNA products using the Klenow fragment of DNA polymerase I. RNA filters were blocked in prehybridization buffer (6 $\times$  SSC [1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5 $\times$  Denhardt's reagent, 0.5% sodium dodecyl sulfate [SDS], 50% formamide) for 4 h at 42°C and subjected to hybridization with the radiolabeled probes (24 to 30 h). The filters were washed twice in 2 $\times$  SSC–0.1% SDS, 42°C, for 15 min and twice in 0.2 $\times$  SSC–0.1% SDS, 65°C, for 15 min. Following washes, filters were exposed to X-ray film for 3 to 7 days at –80°C. Northern blots were performed in duplicate on two different sets of RNA samples to verify signals for each gene variant.

**3' Rapid amplification of cDNA ends (RACE) and relative quantitative real-time PCR (rqRT-PCR).** Oligo(dT)-primed cDNA synthesis reactions were performed according to Hilgarth and Webb (29) using Superscript H Reverse Transcriptase (Invitrogen, Carlsbad, CA) on selected total RNA pools from above in addition to total RNA purified from the pooled individual tissues of 20 3-day parasitized and nonparasitized *H. virescens* larvae. Reactions were performed according to the manufacturer's protocol on 2  $\mu$ g of total RNA from selected pools. RNA pools were treated with DNase I for 15 min prior to cDNA synthesis to remove contaminating viral DNA and verified for absence of contaminating DNA by PCR. Oligonucleotide primers specific to all viral genes were designed, and standard 40-cycle PCRs (54°C annealing temperature) were performed in triplicate on cDNA pools to detect the expression of viral genes. Products were separated on 1% agarose gels and visualized by ethidium bromide staining. rqRT-PCRs on *vankyrin* genes were performed according to Stagliano et al. (69). cDNA pools created for 3'RACE reactions (1  $\mu$ l cDNA synthesized from 2  $\mu$ g total RNA/reaction) were subjected to 45 rounds of PCR in the presence of SYBR green dye (Bio-Rad, Hercules, CA) according to manufacturer's instructions. The fluorescence intensity was monitored after each PCR cycle with mean threshold cycles determined for all unknowns and a serially diluted cDNA standard testing positive for amplification of each gene. Reactions were performed in triplicate for standards and cDNA from each unknown tissue and time point postparasitization. Starting quantities for all unknown cDNA samples were calculated on the basis of the linear standard equation formulated from starting quantities and mean log threshold fluorescence values obtained from standards. Mean starting quantities of unknowns were calculated from the three independent runs, normalized to *actin*, and plotted for each cDNA sample to determine the mean relative starting quantities present in each unknown. Reactions were performed on a Bio-Rad MyiQ Single Color Real-time PCR Thermalcycler.

**Bacterial expression and antibody production.** PCR products specific to the entire ORFs of the *P-vank-1* and *P-vank-4* genes were amplified from cDNA of *H. virescens* larvae 3 days after parasitization with the following restriction enzyme tagged oligonucleotide primers: *P-vank-1* (FW, KpnI 5' CCA GGT ACC GAC AAT GGA GAT TTC TCA 3'); *P-vank-4* (FW, KpnI 5' CCA GGT ACC GGG AAC AAT GAA TAT TGC 3'); *Qt-OligoT Reverse* (5' CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT [16] 3') (23). Products were ligated into the pGEM T-easy vector (Promega, Madison, WI), and corresponding KpnI- and PstI-generated restriction fragments were subcloned into the pMal-c2e bacterial expression vector (New England Biolabs [NEB], Beverly, MA), generating maltose binding protein (MBP)-*P-vank-1* and MBP-*P-vank-4* N-terminal fusions. ORFs were verified for the absence of errors by sequencing on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Proteins were expressed in *Escherichia coli* and purified by affinity chromatography to MBP using amylose resin according to the NEB Protein Fusion & Purification System manual. Purified fusion proteins were used to generate *P-vank-1* and *P-vank-4* specific polyclonal antibodies in rabbits.

***H. virescens* tissue dissections.** Nonparasitized control and 3 days p.p. *H. virescens* larvae were anesthetized by immersion in ice-cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>  $\times$  7H<sub>2</sub>O, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) for 20 min prior to dissection. To collect hemocytes, 40

parasitized and control insects were punctured with pulled glass capillaries and bled into 200  $\mu$ l of ice-cold PBS. Hemocytes were collected by centrifugation at 800  $\times$  g and washed three times in PBS. Fat body, nerve cord, epidermal layer (including muscle, trachea, and epidermis), and digestive tract (including gut and malpighian tubules) tissues were dissected under a stereo light microscope and placed in ice-cold PBS. Purified tissues were centrifuged at 800  $\times$  g for 5 min and washed three times with PBS. Prepared tissues were subjected to total RNA extraction, crude protein preparations, and immunofluorescence analyses as described below.

**Western blots.** Crude protein extracts were prepared from tissues of 40 3-day *C. sonorensis*-parasitized and 40 nonparasitized *H. virescens* larvae. Tissues were dissected into 1 ml of PBS, centrifuged at 800  $\times$  g for 5 min, and washed three times with PBS prior to protein extraction. Washed tissues were resuspended in 200  $\mu$ l of lysis buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 30 mM NaCl, 0.25% Tween 20, 10 mM EDTA, 10 mM EGTA, pH 7.0) and sonicated (3 times, 15-s intervals) to release proteins. Samples were centrifuged at 16,000  $\times$  g for 10 min, and supernatants were analyzed for protein content by Bradford assay. Proteins (5 to 20  $\mu$ g) were loaded and separated on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to Immobilon-P (Millipore) membranes using transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, 20% methanol, pH 8.3) and a Bio-Rad Semi-dry transfer cell. Membranes were blocked in 5% BLOTTO (BLOT-Quickblocker; GENO-TECH, Inc.)-TTBS (100 mM Tris-HCl, 0.9% NaCl, 0.1% Tween 20, pH 7.5) for 1 h and then incubated overnight at 4°C in a 1:5,000 dilution of P-vank-1 polyclonal antibody (Ab) or a 1:1,000 dilution of P-vank-4 polyclonal Ab. Membranes were washed (three times for 10 min in TTBS) and incubated for 45 min in a 1:5,000 dilution (in BLOTTO) of alkaline phosphatase-conjugated secondary antibody (goat-anti-rabbit immunoglobulin G [IgG]; Sigma, St. Louis, MO). Subsequently, blots were washed two times in TTBS for 10 min and twice in alkaline phosphatase substrate buffer (0.1 M NaCl, 5 mM MgCl<sub>2</sub>, 100 mM Tris-HCl, pH 9.5) for 15 min prior to colorimetric assay. Antibody-protein complexes were then visualized by exposure to a chromogenic substrate of alkaline phosphatase (nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate; Sigma, St. Louis, MO).

**Immunofluorescence assays.** Hemocytes and fat body tissue from 3-day parasitized and nonparasitized *H. virescens* larvae were isolated (via bleeding and dissection, respectively) and washed three times for 10 min in PBS. Hemocytes were allowed to spread on microscope coverslips for 1 h in PBS. Cells were fixed in 3.7% formaldehyde-PBS for 20 min and permeabilized with PT (0.2% Triton X-100 plus PBS) for 30 min at room temperature. The hemocytes and fat body tissue were blocked for 1 h with PTB (PT plus 0.5% bovine serum albumin [BSA]) and incubated in a 1:100 dilution of primary Ab (specific to P-vank-4 or P-vank-1) for 45 min (hemocytes) at 37°C or overnight at 4°C (fat body). Following primary Ab incubations, cells were washed with PT and incubated with a 1:500 dilution of anti-rabbit IgG-Texas Red-conjugated secondary antibody (Molecular Probes, Eugene, OR) for 45 min at 37°C and counterstained for F-actin with a 1:30 dilution of fluorescein isothiocyanate (FITC)-phalloidin conjugate (Molecular Probes, Eugene, OR) in PTB for 30 min. For nuclear staining, cells were incubated with propidium iodide (1:3,000 dilution of a 1-mg/ml stock) for 20 min instead of primary Ab. Stained cells were centrifuged (fat body), washed 3 $\times$  in PT to remove excess dye, fixed to microscope slides, and analyzed for fluorescence signals using a Leica TCS-NT laser scanning confocal microscope.

**Nucleotide sequence accession numbers.** The following sequences were submitted to NCBI (accession numbers are in parentheses): *C. sonorensis* IV P vankyrin 1 (AY953130), *C. sonorensis* IV P vankyrin 2 (AY953131), *C. sonorensis* IV P vankyrin 3 (AY953132), *C. sonorensis* IV P vankyrin 4 (AY953133), *C. sonorensis* IV I<sup>2</sup> vankyrin 1 (AY953134), *C. sonorensis* IV I<sup>2</sup> vankyrin 2 (AY953135), and *C. sonorensis* IV I<sup>2</sup> vankyrin 3 (AY953136).

## RESULTS

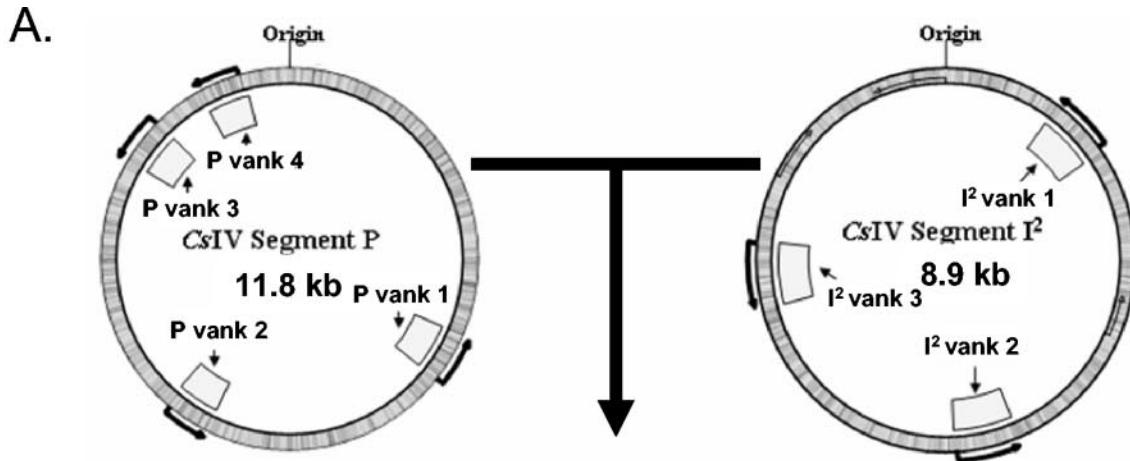
**Vankyrin sequence analyses.** National Center for Biotechnology Information (NCBI) database searches of the completed genome of *C. sonorensis* IV identified seven members of the vankyrin gene family on unique *C. sonorensis* IV segments P (GenBank AY029396) and I<sup>2</sup> (GenBank AF362517) (Fig. 1). There are four vankyrin genes on segment P (*P-vank-1*, -2, -3, -4) and an additional three encoded on segment I<sup>2</sup> (*I<sup>2</sup>-vank-1*, -2, -3) (Fig. 1A). The seven vankyrin genes carry consensus 5'-upstream TATA boxes (ranging from 37 to 168 bp upstream of the initiation methionine codons) and 3'-downstream poly-

adenylation signal sequences (ranging from 63 to 190 bp 3' downstream of stop codons). The seven ORFs (ranging from 480 to 510 bp) encode predicted proteins from 17.53 to 19.36 kDa in size (Table 1).

Alignments between *C. sonorensis* IV vankyrin members from both segments indicate that gene family variants in *C. sonorensis* IV possess similar DNA (Fig. 1A) and primary protein (Fig. 1B) structures. The majority of vankyrin genes exhibit higher identities between genome segments than within genome segments, suggesting that these two *C. sonorensis* IV genome segments may have arisen from a segment duplication event. The *P-vank-1* and *I<sup>2</sup>-vank-3* ORFs share the highest sequence identity (~90% at the nucleotide level), while *P-vank-4* and *I<sup>2</sup>-vank-3* exhibit the lowest sequence identity (~60% at the nucleotide level).

The vankyrin protein sequences lack secretory signal peptides (PSORT analyses) and exhibit variable degrees of similarity to the ankyrin repeat domains of *Drosophila* cactus and mammalian I $\kappa$ Bs (Blastx 10<sup>-5</sup> to 10<sup>-8</sup>). Most significant alignment occurs over ankyrin repeats 4 and 5 of *Drosophila* cactus, with lesser overlap exhibited over repeats 3 and 6 (Fig. 1B). Predicted vankyrin genes from the *Hyposoter fugitivus* IV, *Tranosema rostrale* IV, *Microplitis demolitor* BV, and *Cotesia congregata* BV genomes are similar to *C. sonorensis* IV and align in the same regions. Vankyrin proteins of *C. sonorensis* IV and other PDV genomes lack two N-terminal ankyrin repeats and N- and C-terminal protease sensitive domains involved with regulation of typical I $\kappa$ B protein activities (Fig. 1B). The absence of conserved protease sensitive regions suggests that these proteins could act as stable suppressors of NF- $\kappa$ B activities in parasitized lepidopteran hosts of *C. sonorensis*. Interestingly, this type of activity has been associated with an African Swine Fever virus (ASFV) I $\kappa$ B-like protein (A238L) that shares a similar structure (Fig. 1B) to *C. sonorensis* IV vankyrins (54, 56). Although they lack conserved protease sensitive domains, some *C. sonorensis* IV vankyrins do retain a small number of putative casein kinase II and/or protein kinase C phosphorylation sites (Table 1), indicating that different members of this gene family could show variation in their susceptibilities to phosphorylation-mediated regulation.

PROSITE analyses identified additional protein motifs of low and high probabilities of occurrence within the vankyrin sequences (Table 1). All proteins contain one potential central N-glycosylation site and at least one potential N-myristoylation site, although the molecular weight of the P-vank-1 protein on Western blots (see Fig. 6A to C) is not indicative of posttranslational modification. Of particular interest are metalloproteinase zinc-binding motifs (core sequence: N-HELAH-C) identified in the N-terminal regions (Fig. 1B, -ank repeat 3) of the P-vank-1 and I<sup>2</sup>-vank-3 genes preferentially expressed in the parasitized fat body. Similar structural motifs also exist in the N-terminal domains of the remaining five *C. sonorensis* IV vankyrin proteins and predicted vankyrin proteins from additional IV genomes (Fig. 1B, -ank repeat 3), but these family members lack the conserved second histidine residue in the motif typically necessary for binding Zn<sup>2+</sup>. Such domains suggest that a metal-dependent proteolytic activity could be associated with vankyrin proteins from IV genomes. Interestingly, vankyrin sequences from BV genomes lack conservation of the



ATGGATATTTCTGGAATTGAAAACCTCTTCGGTGCAGAACCGGCATCACGGGAAATAACATATTTCCAGGAGCTTGCCACGGCTGGATCCCTGACGCTGCTTTATCGGATTCGAGATA Majority  
 10 20 30 40 50 60 70 80 90 100 110

1 ATGGAGATTTCTCAAAATTCGAAAGCTTTTCGGTAAAACCCGGGTCACGAAAATAACATATTTCCAGGAGCTTGCCACGGCTGGATCCCTGACGCTGCTTTATCGGATTCGAGATA P vank 1  
 1 ATGGATATTTCTGGAATTGAAAACCTCTTCACCGGAAACCCAAATCGTGGGAGGAAACATATTTCCAGGAGCTTGCCACGCTGGATCCCTGACGCTGCTTTATCGGATTCGAGATA P vank 2  
 1 ATGGAGATTTCTGGAATTGAAAACCTTTTCGGTGCAGAACCGGCATCACGGGAAATAACATATTTCCAGGAGCTTGCCACGCTGGATCCCTGACGCTGCTTTATCGGATTCGAGATA P vank 3  
 1 ATGGATATTTCTGGAATTGAAAACCTCTTCGGTGCAGAACCCAAATCGTGGGAGGCTAAATATTTCCAGGAGCTTGCCACGGCTGGATCCCTGACGCTGCTTTATCGGATTCGAGATA P vank 4  
 1 ATGGATATTTCTGGAATTGAAAACCTTTTCGGTGCAGAACCCAAATCGTGGGAGGCTAAATATTTCCAGGAGCTTGCCACGGCTGGATCCCTGACGCTGCTTTATCGGATTCGAGATA I2 vank 1  
 1 ATGGAGATTTCTGAGATAAAGAAAACCTCTTCGGTGCAGAACCCAAATCGTGGGAGGCTAAATATTTCCAGGAGCTTGCCACGGCTGGATCCCTGACGCTGCTTTATCGGATTCGAGATA I2 vank 2  
 1 ATGGAAAATTTCTCAAAATTCGAAAGCTTTTCGGTGCAGAACCGGCATCACGGGAAATAACATATTTCCAGGAGCTTGCCACGGCTGGATCCCTGACGCTGCTTTATCGGATTCGAGATA I2 vank 3

ACTTCGACGAGCCATTCCATTCTCTCCGCAAGAGTTTAAACAATGATGGTGAAGATAAGTATCCACGTGGCAGCGAATACGCCACCGAGGGCGTCATGCAATAAAGGATCATCCAGGT Majority  
 120 130 140 150 160 170 180 190 200 210 220 230

116 ACATGACGAGCCATGAGCTCTTCCCTGCAAGAGTTAATGCTGATGGAGCTATAGTATCCATGTGGGGGCAAAACCGCACCGGAGGACGCTTGCCAGTGAAGATCATCCAGGT P vank 1  
 116 ACTTCGACGAGCCATTAGATTCCTACTGCAAGAGTTTCGACAACCATGGTGAAGACTGGATCCACGTCAGTGGCAGCGAATACGCCACCGAGGGCGTCATGCAATAAAGGATCATCCAGGT P vank 2  
 116 ACTTCGACGAGCCATTCACTCCGCTGATTTCTGCAAGAGACCGAACAAATGATGGTGAAGATAAGTATCCACGTGGCAGCGAATACGCCACCGAGGGCGTCATGCAATAAAGGATCATCCAGGT P vank 3  
 116 ACTTCGACGAGCCATTGGAATTCCTACTGCAAGAGTTTCGACAACCATGGTGAAGACTGGATCCACGTCAGTGGCAGCGAATACGCCACCGAGGGCGTCATGCAATAAAGGATCATCCAGGT P vank 4  
 116 ACTTCGACGAGCCATTGCATTTCTACTGCAAGAGTTTCGACAACCATGGTGAAGACTGGATCCACGTCAGTGGCAGCGAATACGCCACCGAGGGCGTCATGCAATAAAGGATCATCCAGGT I2 vank 1  
 116 ACATGCAATGCAATCATTCCATTCTTCCCTGCAAGAGACCGAACAAATGATGGTGAAGATAAGTATCCACGTGGCAGCGAATACGCCACCGAGGGCGTCATGCAATAAAGGATCATCCAGGT I2 vank 2  
 116 ACATTCGACGAGCCATGAGCTCTTCCCTGCAAGAGTTAATGCAATGCGAGACTATAGTATCCATGTGGGGGCAAAACCGCACCGGAGGACGCTGCAGTGAAGGATCATCCAGGT I2 vank 3

ACTAATGAACTGGGGGCAGATCTGAAATGCAAGAGACCGTTTGTGAACATTACTGTCTCCACGTCGCGGTTTACCACAAGGATTACGCCCTCGCAAAGTGGCTGTGCCAGCAG Majority  
 240 250 260 270 280 290 300 310 320 330 340

231 GCTACTGAGTTTGGGGCAAACTGAAATGCAAGAGATTCGCTCTCTGGAACCTTACTGTACTGCAATGTCGCGGTTTACGCGAGACGATTACTTCCCGCAAAGTGGCTGTGCCATCC P vank 1  
 231 ATGATGAACTGGGGGCAGATTTAAATGCAAGAGACCGTTTATTGAACATTACTGTCTCTCCACGTCGCGGTTTACCACAAGGATTACGCCCTCGCAAAGTGGCTGTGCCAGCAG P vank 2  
 231 ACTGGCTGACCTGGGGTGAATGCAAGAGACCGTTTCACTGAAACATGACTGTCTCTCCACGTCGCGGTTTACCACAAGGATTACGCCCTCGCAAAGTGGCTGTGCCAGCAG P vank 3  
 231 ATGATGAACTGGGGGCAGATTTAAATGCAAGAGACCGTTTGTGAACATTACTGTCTCTCCACGTCGCGGTTTACCACAAGGATTACGCCCTCGCAAAGTGGCTGTGCCAGCAG P vank 4  
 231 ATTAATGAACTGGGGGCAGATTTAAATGCAAGAGACTTTGTTGAATATGACTGTCTCTCCACGTTTCCGGTGCACCAATAAGGATTACGCCCTCGCAAAGTGGCTGTGCCAGCAG I2 vank 1  
 231 TCTTGTGGCAATGGGGTGAATGCAAGAGACTTTGTTGAATATGACTGTCTCTCCACGTTTACGTTTGGGCAAAAGATTACGCCCTCGCAAAGTGGCTGTGCCAGCAG I2 vank 2  
 231 ACTACTGGAATGGGGGCAATCTGAAATGCAAGAGATTCGCTCTCTGGAACCTTACTGTACTGCAATGTCGCGGTTTACGCGGAGGATTACTTCCACAATGTTGGCTGTGCCATCC P vank 3

CCAAATCTTGATATCAATGCAAGAGCTTCGATGGACTGACTGCATATGGAATGGCAATTCATAGAGGGGCACAAAAGAGATGATGAGAATTTTGGCAGCCGAAACGGTGTAAACTCA Majority  
 350 360 370 380 390 400 410 420 430 440 450 460

346 CCACAAATGATTTGGATGCAAGAGCTTGGGATGGACTTACGGCACATGAAAGCTGTTGATAACCTGCACAAAAGAGATGATGATATTTTCCGAAACCGAGCGTGTAAACAGAG P vank 1  
 346 CCAAATCTTGATATCAATGCAAGAGCTTTGATGGCTGACTGCATATGGACTTGGCCACCATAGAGGGGCGAAGAGATGATGAGAATTTTGGCTGCTGAACTGTTTAAACTCA P vank 2  
 346 CCAAATCTTGATATCAATGCAAGAGCTTCGATGGCTGACTGCATATGGACTTGGCCACCATAGAGGGGCGAAGAGATGATGAGAATTTTGGCAGTGAACCTTTGAAACTCA P vank 3  
 346 CCAAATCTTGATATCAATGCAAGAGCTTCGATGGCTGACTGCATATGGACTTGGCCACCATAGAGGGGCGAAGAGATGATGAGAATTTTGGCAGTGAACCTTTGAAACTCA P vank 4  
 346 CCAAATCTTGATATCAATGCAAGAGCTTTGATGGATGACTGCATATGGAAATGCAATCTCAGAGGGGCGAATCCAGATGATGATGATGATTTTGGCAGCCGAAAC---TGTTCCACTTA I2 vank 1  
 346 TCACGAAATCAATTTAGATGCAAAAGCTTGGGATGGACTTACGGCAATTCAAATGGGGATTTATAGAGAATGACAAAAGAGATGATGAGATTTTTCAAAGCCCTTGGTG----- I2 vank 2  
 346 CCACAAATGATTTGAAATGCAAGAGCTTTCGGTGGACTTACGGCACATCAAAATGGGGTTGATGTCTTGGCACAAAGATGATGATGATATTTTCCGAAACCGAGCGTGTATTCGGAG I2 vank 3

CCG-T-GTACA-AGCTGAAAAGTGCAXXXXXXXXXXXXXXXXXXXXXXXXXX Majority  
 470 480 490 500 510

461 CCGTGGTACAGAGCGGAAAAGTGAATGAAAAGTACATCGAATGACAATCAGCAT P vank 1  
 461 CGT---GTACA---GCTGAAAAGTGCA P vank 2  
 461 CCGTGGTACAGAGCTGAAAAGTGC P vank 3  
 461 CGT---GTACA---GCTGAAAAGTGCA P vank 4  
 459 CG---GTGTG I2 vank 1  
 452 CTCCACGTGCACAAACCGAAAAGTGAATGAAAAGTAAATCGAGTAATGAGAAAACAT I2 vank 2  
 461 CCGTGGTTCAGAGCGAAAAGTGAATGAAAAGTACATCGAATGACAATCAGCAT I2 vank 3

**B.**

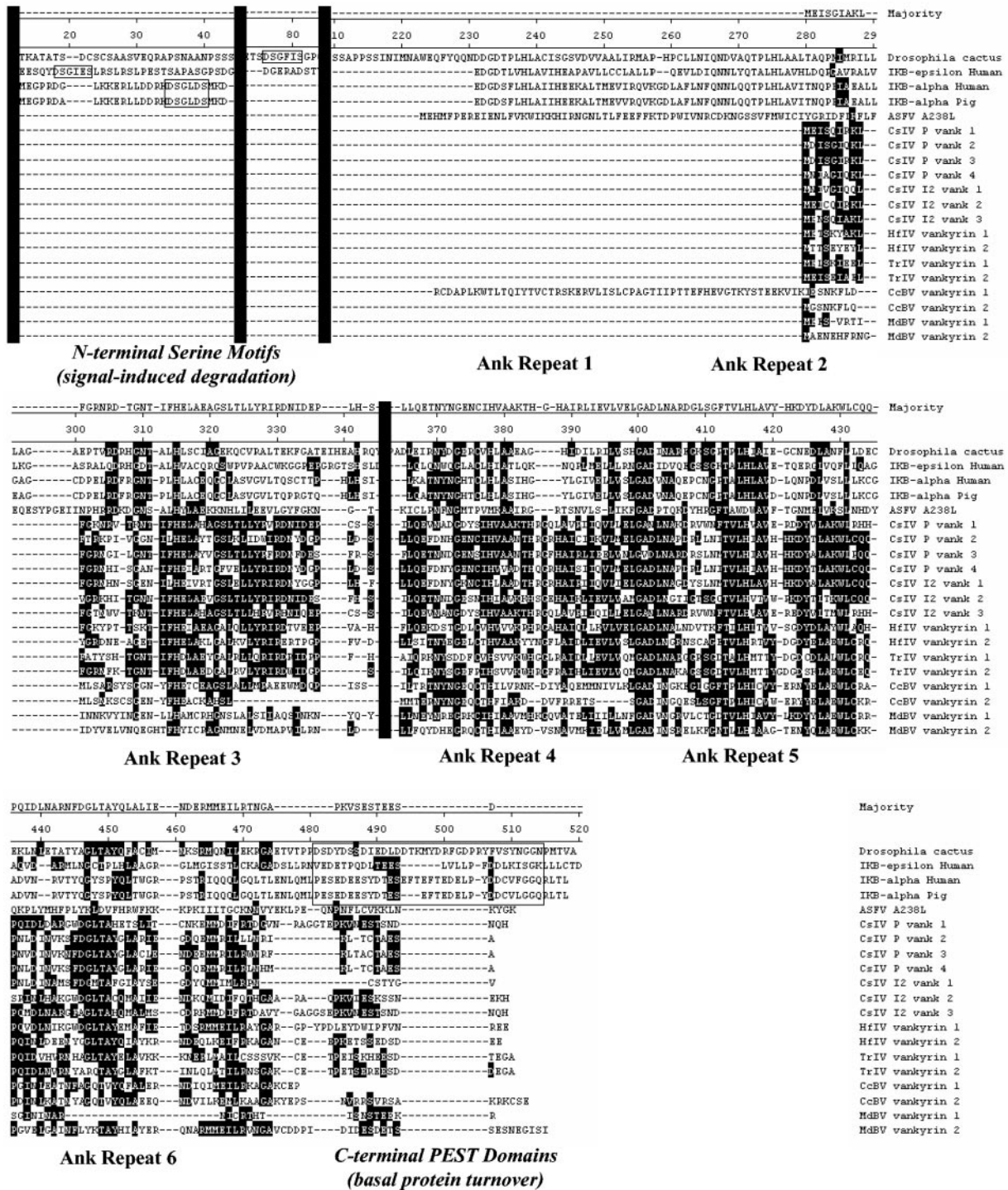


FIG. 1. A. Position of *vankyrin* (*vank*) genes on segments P and I<sup>2</sup> of the *C. sonorensis* IV (CsIV) genome, and DNA sequence alignment of the seven gene family variants. Black residues indicate matches to the *vankyrin* consensus sequence. B. Protein sequence alignment of the *C. sonorensis* IV *vankyrin* gene family with members of the Iκβ gene family and representative *vankyrin* homologs from the HfIV, TrIV, MdBV, and CcBV PDV genomes. *C. sonorensis* IV *vankyrin* proteins align with ankyrin repeat domains other PDV vankyrins and with those of typical Iκβs. Black residues indicate matches to the consensus sequence. Locations of *Drosophila cactus* ankyrin repeats 1 to 6 have been denoted according to Dushay et al. (18). PDV vankyrin proteins lack N- and C-terminal destruction domains (black boxes) that are sensitive to protease activity and are involved in the regulation of cellular activity. Sequences removed to optimize the alignment are indicated by vertical black lines. Alignments were created with MEGALIGN software (DNASTAR, Madison, WI). NCBI accession numbers: *Drosophila cactus* (A44269), Iκβε human (O00221), Iκβα human (A39935), Iκβα pig (CAA84619), ASFV A238L (NP\_042733), HfIV vankyrin 1 (AAS90270), HfIV vankyrin 2 (AAX24120), TrIV vankyrin 1 and TrIV vankyrin 2 (AY940454), CcBV vankyrin 1 (CAG17493), CcBV vankyrin 2 (CAG17492), MdBV vankyrin 1 (AAW51804), MdBV vankyrin 2 (AAW51781).

TABLE 1. Predicted properties of the *C. sonorensis* IV vankyrin proteins (PROSITE)

Protein	<i>Drosophila cactus</i>	P-vank-1	P-vank-2	P-vank-3	P-vank-4	I2-vank-1	I2-vank-2	I2-vank-3
Length (amino acids)	500	171	160	161	160	155	168	171
Predicted size (kilodaltons)	53.81	19.36	17.98	18.47	18.09	17.53	18.85	19.31
Ankyrin repeats	6	4	4	4	4	4	4	4
Motifs with low probability of occurrence								
Zinc-binding motifs	No	1	?	?	?	?	?	1
Motifs with high probability of occurrence								
Leucine Zipper motifs	No	No	No	No	No	1	No	No
N-myristoylation sites	6	2	1	2	1	2	5	2
N-glycosylation sites	2	2	1	1	1	2	1	2
Casein kinase II phosphorylation sites	5	2	No	1	No	1	2	1
Protein kinase C phosphorylation sites	6	1	3	2	No	1	No	1
Tyrosine kinase phosphorylation sites	2	No	No	No	No	No	No	No
Tyrosine sulfation sites	1	No	1	No	1	No	No	No
Amidation sites	No	No	No	No	No	No	1	No
Cyclic Amp-dependent phosphorylation sites	No	No	No	No	No	No	1	No

structural motif (Fig. 1B), indicating the potential for divergence in protein activities between the IV and BV genera.

**Temporal transcription of vankyrin genes.** Northern blots of total RNA isolated from *C. sonorensis* wasps and time-course parasitized *H. virescens* third-instar larvae detected six of the seven vankyrin genes in parasitized *H. virescens* hosts, but none in the wasp hosts (Fig. 2). Duplicate blots were loaded with equivalent amounts of RNA, treated under identical conditions, and probed with <sup>32</sup>P-labeled oligonucleotides of similar intensity and specific activity for each viral gene. Comparisons between signal intensities given off by each probe allow assessment of relative mRNA expression levels among the different gene family variants. Northern blots showed a similar temporal transcription pattern for the six detectable viral genes. Transcription of viral genes is restricted to the parasitized *H. virescens* host with ~800- to 830-base-pair viral mRNA transcripts first detected from 4 to 8 h p.p., reaching highest apparent levels by 24 h p.p., and persisting in parasitized hosts for up to 5 days p.p. Apparent mRNA expression levels for the different vankyrin genes varied over the time course. The *P-vank-1* and *I<sup>2</sup>-vank-3* variants exhibited the most intense signals on Northern blots. The *I<sup>2</sup>-vank-2*, *P-vank-2*, and *P-vank-3* signals were intermediate in relative expression levels, and the *P-vank-4* signal could only be detected following 5d of exposure (Fig. 2). The *I<sup>2</sup>-vank-1* mRNA could not be detected on Northern blots suggesting a significantly lower level of transcription for this gene in parasitized *H. virescens* hosts. However, expression of *I<sup>2</sup>-vank-1* was detected by 3'RACE and verified by rqRT-PCR. rqRT-PCR performed against the seven vankyrin genes confirmed the temporal transcriptional profiles occurring over the time course of parasitization and detected peak mRNA transcript levels for the seven vankyrin genes at 2 to 3 days p.p. in host larvae (Fig. 3). Using this method, transcripts all vankyrin genes could also be detected at low levels in female wasps.

**Tissue specificity of vankyrin transcription.** To delineate the tissue specificity of vankyrin gene transcription in parasitized hosts, total RNA was isolated from various tissues of 3-day-p.p. *H. virescens* third-instar larvae (corresponding to peak transcript levels identified for all genes) and subjected to rqRT-PCR analyses as performed for temporal profiling of gene transcription in parasitized larvae. The data show that some

vankyrin genes are preferentially expressed in the fat body, nerve cord, and epidermis of parasitized larvae, and others exhibit preferential expression in hemocytes from parasitized hosts (Fig. 4). The fat body and epidermal tissues in insects are tightly associated and difficult to separate in dissection, thus, signals apparent in the epidermal tissues may be a consequence of residual fat body retained on these tissues. The *P-vank-1*, *I<sup>2</sup>-vank-2*, and *I<sup>2</sup>-vank-3* genes exhibit the highest levels of transcription in the parasitized fat body relative to other tissues, while transcription of the remaining vankyrin family members (*P-vank-2*, *P-vank-3*, *P-vank-4*, and *I<sup>2</sup>-vank-1*) is restricted almost exclusively to parasitized, *C. sonorensis* IV-infected hemocytes (Fig. 4).

The tissue-specific transcription pattern among vankyrin genes is correlated with their apparent evolutionary relationships within the gene family. A phylogenetic tree constructed from the vankyrin consensus sequence indicates that the three vankyrin genes predominantly expressed in the parasitized fat body and nervous tissue (*P-vank-1*, *I<sup>2</sup>-vank-2*, and *I<sup>2</sup>-vank-3*) cluster and are ancestral to the other four genes predominantly expressed in hemocytes (*P-vank-2*, *P-vank-3*, *P-vank-4*, and *I<sup>2</sup>-*

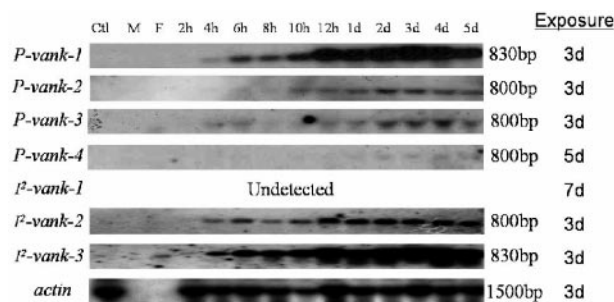


FIG. 2. Northern blots for *H. virescens actin* and six of the seven *C. sonorensis* IV vankyrin genes over the time course (2 h to 5 days) of *C. sonorensis* parasitization. Signals for individual vankyrin variants were detected after 3 to 5 days of exposure to X-ray film (right panel). An *H. virescens actin* probe verified that approximately equivalent amounts of total RNA were loaded in each well (~30 µg/lane). Ctl, nonparasitized control *H. virescens*; M, male *C. sonorensis*; F, female *C. sonorensis*; h, hours postparasitization; d, days postparasitization.

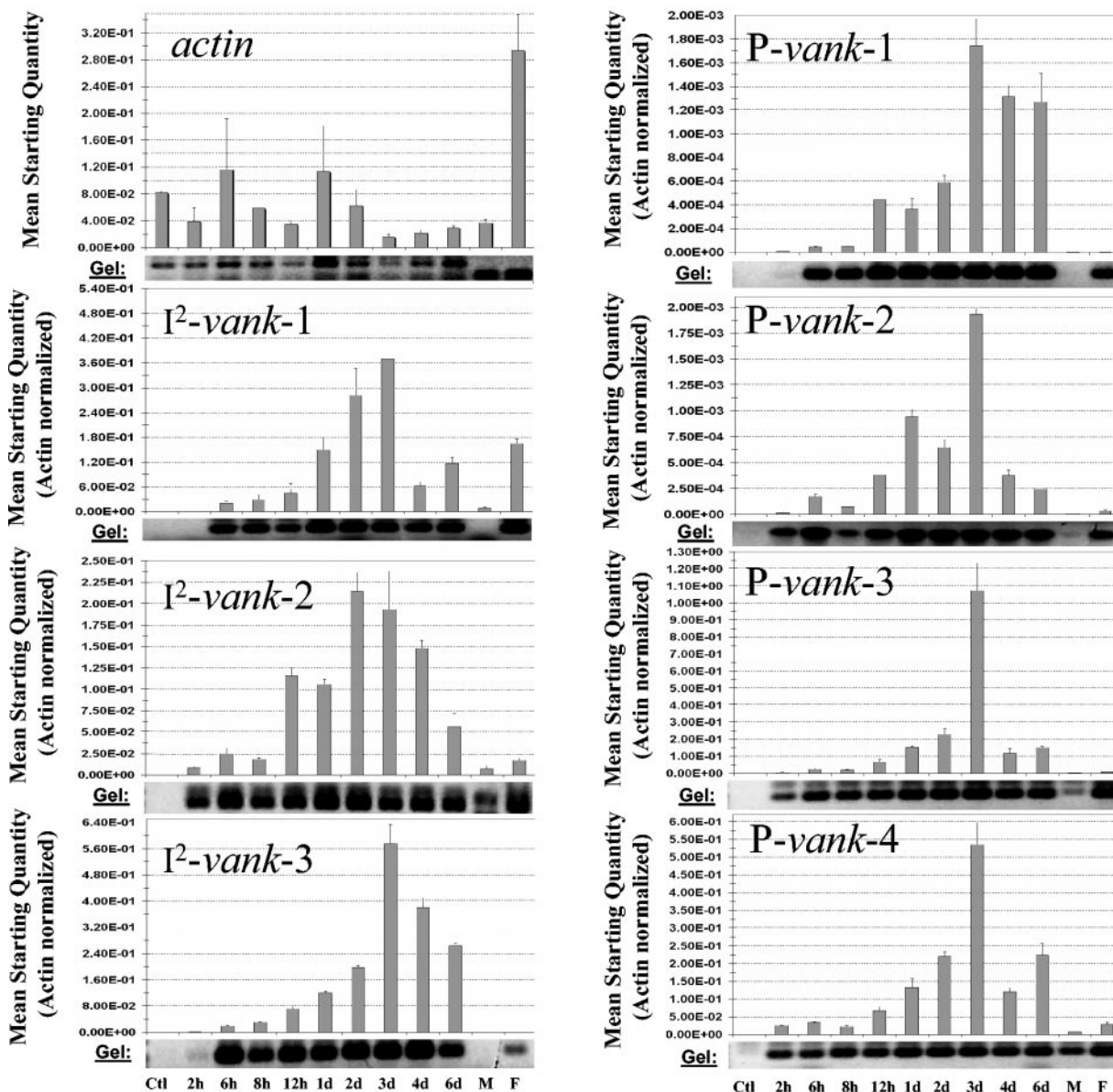


FIG. 3. Relative quantitative real-time PCR for *H. virescens actin* and the seven *C. sonorensis* IV *vankyrin* genes over a 2-h to 6-day time course of parasitization. All values for viral genes were normalized to *actin* controls to account for variations in cDNA pools. All viral genes were expressed by 2 h p.p. and showed peak transcription by 3 days p.p. in *H. virescens* larvae. Error bars represent +1 standard deviations from the mean starting quantity calculated for each time point. Ctl, control *H. virescens*; h, hours p.p.; d, days p.p.; M, male *C. sonorensis*; F, female *C. sonorensis*. PCR amplimers were run on 1% agarose gels (photos) to verify the presence of DNA fragments of the correct size. No viral-specific amplimers were detected for cDNA pools of nonparasitized *H. virescens* control larvae.

*vank-1*) that cluster in a separate group (Fig. 5). Interestingly, genes preferentially expressed in the fat body also appear to be expressed at considerably higher levels (Fig. 2) than their hemocyte-specific counterparts.

**Detection of the P-vank-1 protein.** To characterize vankyrin protein expression in parasitized insects, polyclonal antibodies were raised against the fat body/nerve cord-targeting P-vank-1 and hemocyte-targeting P-vank-4 proteins. The two polyclonal Abs reacted specifically and with similar intensities to the recombinant vankyrins against which they were raised (not shown). Results from Western blots were consistent with tran-

scription data in that they verified the existence of tissue-specific protein expression patterns of the P-vank-1 variant (Fig. 6). The P-vank-1 Ab reacted specifically with a 19-kDa protein of the appropriate size in 3-day-p.p. crude protein extracts from *H. virescens* fat body, epidermis, and whole body tissues (Fig. 6A). However, the P-vank-1 protein was not detected in the parasitized nerve cord, although rqRT-PCR detected transcripts for this gene in nervous tissue at approximately half the level to that identified in the parasitized fat body. The weaker signal apparent in the epidermis extract may result from residual fat body retained on epidermal tissues, as

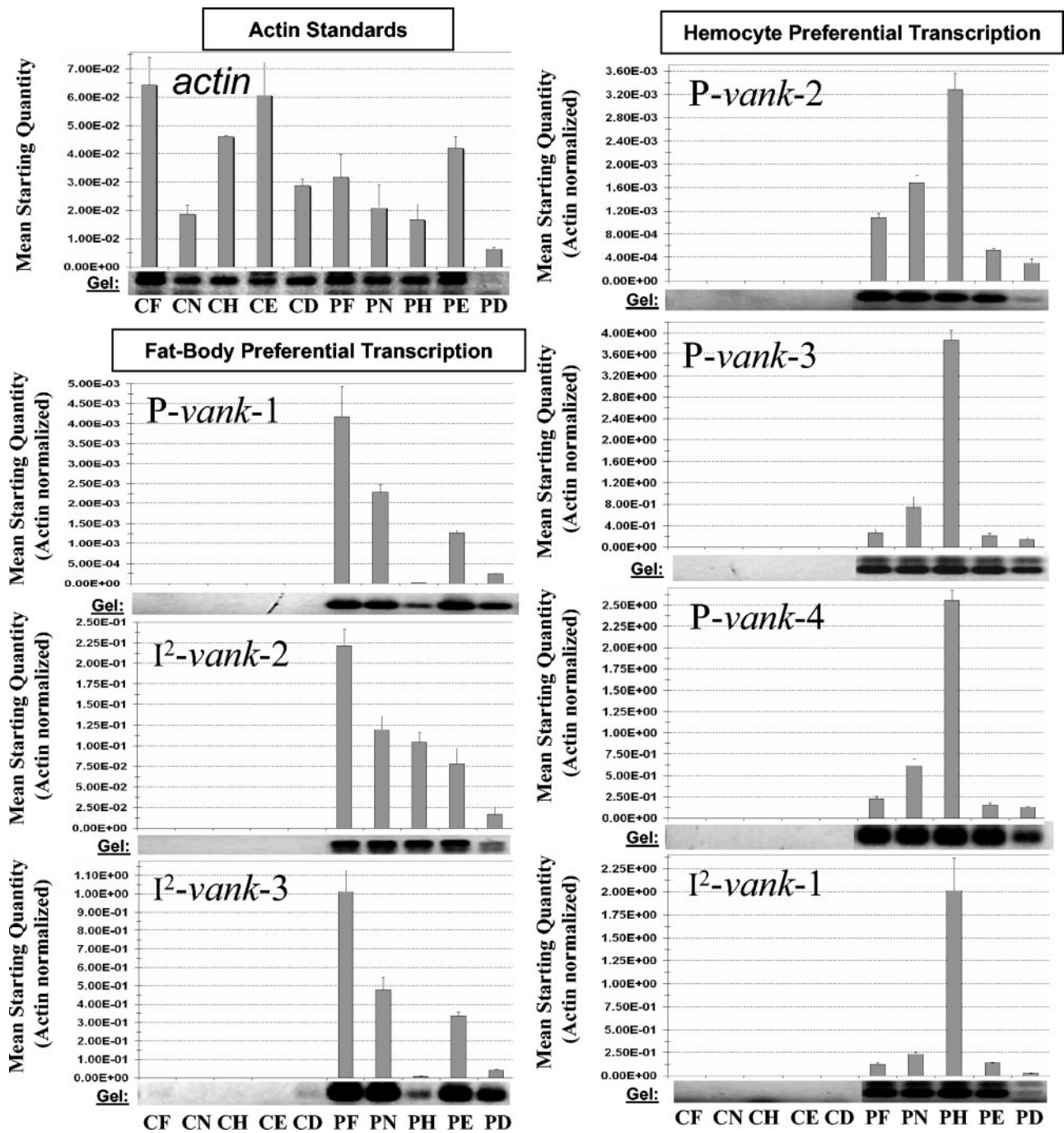


FIG. 4. Relative quantitative real-time PCR for *H. virescens actin* and the seven *C. sonorensis* IV *vankyrin* genes in selected tissues of control and 3-day-p.p. *H. virescens* larvae. All values for viral genes were normalized to *actin* controls to correct for variations between cDNA pools. Viral genes showed preferential targeting for transcription in different tissues of the parasitized insect. Expression of the *P-vank-1*, *I<sup>2</sup>-vank-2*, and *I<sup>2</sup>-vank-3* viral genes was higher in the parasitized fat body relative to other infected tissues. The *P-vank-2*, *P-vank-3*, *P-vank-4*, and *I<sup>2</sup>-vank-1* genes show highest levels of expression in the parasitized hemocytes. Error bars represent +1 standard deviation from the mean. CF, nonparasitized (np) fat body; CN, np nerve cord; CH, np hemocytes; CE, np epidermal layer (including trachea, muscles, and epidermis); CD, nonparasitized digestive tract (including gut and malpighian tubules); PF, 3-day-p.p. fat body; PN, 3-day-p.p. nerve cord; PH, 3-day-p.p. hemocytes; PE, 3-day-p.p. epidermal layer; PD, 3-day-p.p. digestive tract. PCR products for the viral genes were detected in all parasitized tissues examined (gels). No viral-specific amplicons were detected for cDNA pools of nonparasitized tissues.

these tissues are tightly associated and difficult to separate during dissection. P-vank-1 proteins can be detected within 1 day p.p. and through at least 4 days p.p. in crude extracts from p.p. *H. virescens* fat body tissue (Fig. 6B). The anti-P-vank-1 Ab

was highly sensitive, detecting protein in as little as 5  $\mu$ g of crude protein extract from the 3-day-p.p. fat body (Fig. 6C). Similar analyses with an antibody against the hemocyte-specific P-vank-4 protein did not yield conclusive results in Western



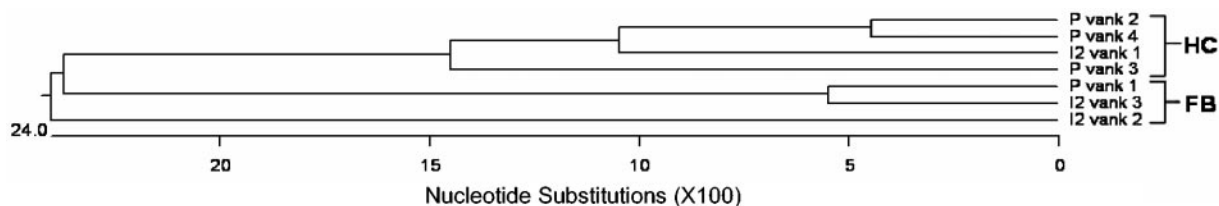


FIG. 5. Phylogenetic tree constructed via the Clustal W method of DNA alignment. The tree is rooted based upon assumption of the biological clock. HC, hemocyte-specific transcription; FB, fat body-specific transcription. Alignment parameters: gap penalty, 15; gap length penalty, 6.66; DNA weight matrix, International Union of Biochemistry. Tree was assembled with MEGALIGN software (DNASTAR, Madison, WI).

blots (data not shown), potentially due to much lower expression levels of this vankyrin variant in the parasitized insect, as evidenced in Northern blots (Fig. 2).

**Nuclear localization of vankyrins.** Immunofluorescence analyses using P-vank-1- and P-vank-4-specific Abs showed a nuclear localization pattern in 3-day-parasitized fat body tissue and hemocytes, respectively (Fig. 7). Fat body and hemocytes were stained with propidium iodide to verify identification of cell nuclei (Fig. 7). These data provide evidence for a possible functional role of vankyrin proteins in the nuclei of infected lepidopteran cells. Similar nuclear localization patterns have been found for a human I $\kappa$ B-like (I $\kappa$ B-L) protein (60). *C. sonorensis* IV infection is known to disrupt the actin cytoskeleton in hemocytes of parasitized insects (82), a phenomenon that is also apparent in the 3-day-p.p. fat body, as evidenced by fluorescein isothiocyanate (FITC)-phalloidin staining of F-actin (Fig. 7).

**DISCUSSION**

The data described in this paper characterize seven unique members of the *vankyrin* gene family in *C. sonorensis* IV. Expression of all genes was detected by 2 to 6 h p.p. in *H. virescens* hosts, with peak expression occurring by 2 to 3 days p.p. (Fig. 2 and 3). rqRT-PCR analyses also identified low levels of expression for the seven gene variants in female wasps. *C. sonorensis* IV DNA (and therefore gene copy number) is amplified severalfold in the ovaries of females prior to packaging and assembly of virions. Detectable levels of expression in female wasps may be a consequence of amplified gene copy numbers and amplified basal levels of transcription occurring in female parasitoids. The *C. sonorensis* IV *vankyrin* genes exhibit differential tissue-specific transcription patterns and can be divided into two classes: those that are expressed preferentially in fat body and nervous tissue (*P-vank-1*, *I<sup>2</sup>-vank-2*, *I<sup>2</sup>-vank-3*) and those that are predominantly expressed in infected hemocytes

(*P-vank-2*, *P-vank-3*, *P-vank-4*, *I<sup>2</sup>-vank-1*) (Fig. 4). Northern blots indicated that highest levels of RNA expression occur for the genes within the first subclass, with much lower levels of expression detected for members within the hemocyte-specific subclass (Fig. 2). P-vank-1 proteins were detected by 1 day p.p. in fat body protein extracts and in crude protein extracts from 3-day-p.p. *H. virescens* whole-body and epidermal tissues but were not detected in protein extracts from the parasitized nerve cord (Fig. 6A to C). The fat body and nerve cord are highly specialized tissues in the insect, and these data may indicate that although actively transcribed in the nerve cord, these genes may not be translated or retain detectable levels of protein in this tissue. Antibodies raised against the P-vank-1 and P-vank-4 proteins localized to the nuclei of 3-day-p.p. *H. virescens* fat body tissue and hemocytes, respectively, indicating a potential physiological role for vankyrin proteins in the nuclei of *C. sonorensis* IV-infected cells (Fig. 7).

Differential tissue-specific expression of the *C. sonorensis* IV *vankyrin* family suggests that variants within this gene family target different tissues and potentially provide specialized functions within parasitized insects. Interestingly, the P-vank-1 and I<sup>2</sup>-vank-3 proteins are also predicted to encode a consensus metalloproteinase zinc-binding motif (Table 1). The remaining five vankyrin variants also encode the core zinc-binding domain yet lack the second conserved histidine residue typically associated with the motif (Fig. 1B). Similar structures are also apparent in predicted *vankyrin* genes from other IV genomes but are absent in genes predicted from BV genomes. Differences in the zinc-binding motif could indicate that vankyrins possess variations in their ability to bind zinc (or potentially other divalent cations) and may also indicate divergences of protein function both within and between PDV genera. Investigations of the zinc-binding and proteolytic capacities of

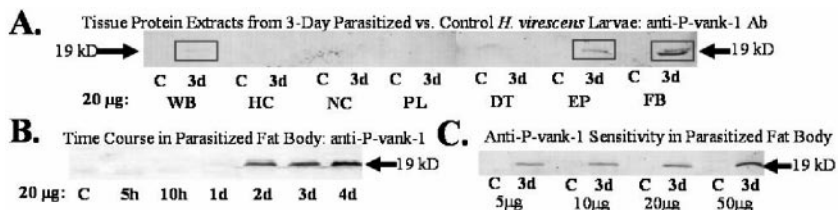


FIG. 6. Western blots. A. Tissue specificity of the anti-P-vank-1 Ab in 3-day-p.p. insects. Crude protein extracts were prepared from nonparasitized control (C) and 3-day-parasitized (3-day) *H. virescens* whole-body tissue (WB), hemocytes (HC), nerve cord (NC), hemolymph plasma (PL), digestive tract (DT), epidermal layer (EP), and fat body tissue (FB). A 19-kDa protein of the predicted size from parasitized larval whole-body, epidermal layer, and fat-body extracts reacted specifically with the anti-P-vank-1 Ab. The signal appeared by 1 day p.p. in the fat body (B), and the Ab was sensitive to as little as 5  $\mu$ g (C) of extract from the 3-day-p.p. fat body.

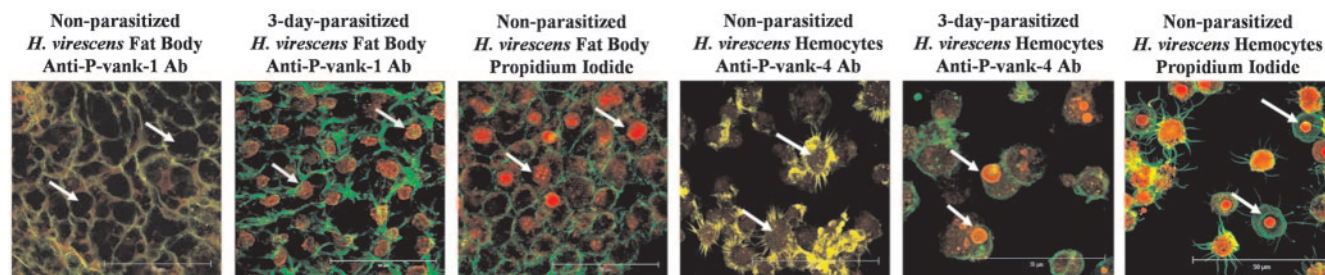


FIG. 7. Immunofluorescence assays using anti-P-vank-1 and anti-P-vank-4 Abs. The anti-P-vank-1 (red) antibody localized to the 3-day-p.p. fat-body nucleus (arrows). Nonparasitized *H. virescens* fat-body tissue showed no specific nuclear staining (arrows). Propidium iodide staining of fat-body tissue verified the subcellular localization of cell nuclei (arrows). The anti-P-vank-4 antibody (red) localized to the 3-day-p.p. hemocyte nucleus (arrows). There was no specific staining in nonparasitized *H. virescens* hemocytes (arrows). Propidium iodide staining of hemocytes verified the subcellular localization of cell nuclei (arrows). All cells were counterstained with FITC-Phalloidin (green) to visualize F-actin. Magnification,  $\times 100$ . Scale bar, 50  $\mu\text{m}$ .

individual vankyrin variants may reveal additional insights into physiological protein functions.

The vankyrins of *C. sonorensis* IV and those predicted from other PDVs encode a series of four tandem ankyrin repeat domains (Fig. 1B). Ankyrin repeats consist of a 33-amino-acid motif centered around a consensus core protein sequence of TPLHLA and are found in a diverse variety of proteins, including  $\text{I}\kappa\text{B}\beta$ s, cytoskeletal proteins (ankyrins), cell cycle regulators (Swi6), developmental regulators (Notch), and toxins ( $\alpha$ -latrotoxin) (1, 8, 28, 56, 59). Many of these peptides play important roles in protein-protein interactions involved with cellular signaling cascades and/or scaffolding of cytoskeletal networks (1, 59). Interestingly, cytoskeletal breakdown is a predominant phenotype following *C. sonorensis* IV infection of *H. virescens* tissues (Fig. 7) (81, 82, 84). The nuclear localization of vankyrins is not consistent with direct disruption of cytoplasmic cytoskeletal networks, but current data do not exclude an indirect role for the proteins in producing this phenotype.

The ankyrin repeat domains of the *C. sonorensis* IV vankyrins resemble most closely those of  $\text{I}\kappa\text{B}\beta$ s (Blastx  $10^{-5}$  to  $10^{-8}$ ), making their activity as  $\text{I}\kappa\text{B}\beta$ s the most conservative hypothesis for protein function. Targeted tissues (fat body and hemocytes) and pathological alterations apparent in parasitized hosts are also consistent with those expected if NF- $\kappa\text{B}$  signal transduction cascades were a target for *C. sonorensis* IV disruption. *C. sonorensis* IV infection of *H. virescens* larvae causes an increased susceptibility to pathogenic attack as well as developmental arrest and hemocyte immunosuppression in parasitized hosts (13, 38, 41, 62, 63, 64, 84). NF- $\kappa\text{B}$ -dependent responses have been implicated in all of the aforementioned systems affected by host parasitization (7, 24, 25, 80). Consensus  $\kappa\text{B}$  binding sites occur in the promoters and enhancers of several insect antimicrobial genes (e.g., *lysozyme*, *cecropins*, *attacins*, *defensin*, *diptericin*, and *drosomycin*) and genes involved in development and immunity (e.g., *zerknult*, *twist*, *snail*, and *rhomboid* from *Drosophila*; IL-1, IL-6, TNF- $\alpha$  in mammals) (7, 18, 20, 24, 25, 33, 36, 45, 47, 76, 80). A decrease in NF- $\kappa\text{B}$ -induced expression of any one of these genes may contribute to the observed increase in susceptibility to pathogenic diseases and developmental arrest in hosts following parasitization.

$\text{I}\kappa\text{B}$  ankyrin repeats are specifically involved in inhibitory interactions with NF- $\kappa\text{B}$  dimers.  $\text{I}\kappa\text{B}$  proteins possess the ability to directly release NF- $\kappa\text{B}$  dimers bound to DNA in the nucleus and show different specificities for binding of individ-

ual NF- $\kappa\text{B}$  variants (25, 27, 28, 40, 48, 53, 56, 91). The N-terminal domains of typical  $\text{I}\kappa\text{B}$  proteins contain two highly conserved serine residues in the motif (*DSG\P X S*), where  $\Psi$  represents a hydrophobic amino acid and X represents any amino acid (Fig. 1B) (34, 67). In response to immune challenge or developmental signals, the serine residues become phosphorylated, leading to polyubiquitination and degradation of  $\text{I}\kappa\text{B}\beta$ s and the release of NF- $\kappa\text{B}$  transcription factors (11, 35, 39, 42, 49, 61, 34, 67). The C-terminal acidic PEST domains (Fig. 1B) of  $\text{I}\kappa\text{B}$  proteins also contain target residues that become phosphorylated by intracellular kinase enzymes and are implicated in basal level/signal-independent protein degradation in the cell (4, 25, 43).

Vankyrin proteins in *C. sonorensis* IV and other PDV genomes lack these two regulatory domains implicated in the signal-induced degradation and basal turnover of typical  $\text{I}\kappa\text{B}\beta$ s (Fig. 1B). This gene structure suggests the hypothesis that vankyrin activity mediates inhibition of NF- $\kappa\text{B}$  signaling via the irreversible sequestration or destruction of NF- $\kappa\text{B}$  dimers in parasitized lepidopteran hosts. A similar potent inhibitory activity has been implicated for an  $\text{I}\kappa\text{B}$ -like protein A238L/5EL in African swine fever virus (ASFV), whose protein structure resembles that of the *C. sonorensis* IV vankyrins (Fig. 1B) (51, 54, 57, 77). A238L has identifiable ankyrin repeats, lacks N- and C-terminal regulatory motifs involved in  $\text{I}\kappa\text{B}$  proteolysis, and is known to function as a potent suppressor of NF- $\kappa\text{B}$  DNA binding activity in ASFV-infected cells (51, 54, 57, 77).

Nuclear localization of vankyrin proteins would not be predicted if they act as typical  $\text{I}\kappa\text{B}\beta$ s, binding to host NF- $\kappa\text{B}$  in the cytoplasm to prevent its nuclear translocation. The two N-terminal ankyrin repeats of  $\text{I}\kappa\text{B}\beta$ s are required for binding and masking of nuclear localization signals (NLS) in NF- $\kappa\text{B}$  dimers (31, 32, 39, 40). Interestingly, the vankyrin proteins in *C. sonorensis* IV lack the N-terminal repeats involved with these particular interactions in typical  $\text{I}\kappa\text{B}$  proteins (Fig. 1B). Thus, our immunofluorescence results may indicate that the proteins incompletely mask NF- $\kappa\text{B}$  NLS protein sequences. If so, vankyrins in *C. sonorensis* IV may function biologically through inhibition and active sequestration of NF- $\kappa\text{B}$  complexes in the host nucleus, effectively reducing cytoplasmic pools of NF- $\kappa\text{B}$  available for immune responses against the developing endoparasitoid.

PDV genome sequencing has revealed that  $\text{I}\kappa\text{B}$ -related ORFs exist in several IVs and BVs and that the identified sequences share similar structures (Fig. 1B) (21, 38, 44, and Webb et al.,

unpublished data; D. B. Stoltz et al., unpublished data; M. Cusson et al., unpublished data; D. Gundersen-Rindal, personal communication; F. Pennacchio, personal communication). The presence of related *vankyrin* genes in both IVs and BVs suggests convergent evolution has occurred in the two PDV genera and may indicate an essential role for these genes in the overall success of parasitization in both lineages of parasitoid wasps. Though evolutionarily distinct, both braconid and ichneumonid parasitoids can successfully parasitize identical hosts (e.g., *C. sonorensis*, *Hyposoter didymator*, *Microplitis demolitor*, and *Toxoneuron nigriceps* all parasitize *H. virescens* larvae), and it is likely that they have developed similar evolutionary strategies to evade or disrupt host immunity and growth. Begun and Whitley (5) suggested that adaptive evolution occurring in the I $\kappa$ B domain of the *Drosophila simulans* relish NF- $\kappa$ B protein may be the consequence of an evolutionary "arms race" between the flies and their microbial pathogens. Adaptive evolution through duplication and divergence of *vankyrin* genes in PDVs could allow parasitoid wasps to "keep up" evolutionarily with host adaptations that evolve to prevent successful parasitizations. Future comparisons between the activities of *vankyrin* genes will aid in revealing whether tissue specific patterns and gene activities are conserved between the PDV genera and whether variants of gene families play independent roles in establishing parasitoid host ranges.

Research into the functions associated with vankyrin variants in *C. sonorensis* IV will expand understanding of how this virus has evolved and how it aids in the survival of the endoparasitic larvae during development. Resolving the specific activities of individual vankyrin proteins will be essential to our understanding of multigene families in PDVs and assist in elucidating how gene family variants may be evolving for specialized functions in parasitized insects. Although their role as specific inhibitors of NF- $\kappa$ B remains unclear, it is apparent that *C. sonorensis* IV *vankyrin* genes are evolving to play unique roles during parasitization, as evidenced by their divergent protein properties and tissue specificities during expression within the parasitized lepidopteran host.

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