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No evidence for translation of *pog*, a predicted overlapping gene of Solenopsis invicta virus 1

Steven M. Valles · Niv Sabath

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Abstract An overlapping open reading frame (ORF) with a potential to encode a functional protein has been identified within the 3′-proximal ORF of Solenopsis invicta virus 1 (SINV-1) and three bee viruses. This ORF has been referred to as *predicted overlapping gene* (*pog*). Protein motif searches of POG revealed weak relationships precluding assignment of a potential function. Neither a transcript nor a protein encoded by the *pog* ORF has been detected. However, recently, a protein encoded by the corresponding +1 overlapping ORF (termed ORFx) in the Israeli acute paralysis virus (IAPV) was demonstrated by recombinant means as well as in IAPV-infected honey bees. The objective of our study was to attempt to provide empirical evidence for the presence of a *pog*-derived protein from SINV-1-infected fire ants. A number of different laboratory and field SINV-1-infected *Solenopsis invicta* preparations were examined by western blotting for the presence of a POG protein sequence. In every case, these preparations failed to yield any detectable bands when probed with a polyclonal antibody preparation raised to a portion of the *pog* predicted protein sequence. Although impossible to prove a negative result, proper controls used in these studies suggested that the *pog* ORF is not translated into a functional protein in SINV-1.

S. M. Valles (✉)
Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, 1600 SW 23rd Drive, Gainesville, FL 32608, USA
e-mail: steven.valles@ars.usda.gov

N. Sabath
Institute of Evolutionary Biology and Environmental Studies, University of Zurich, 8057 Zurich, Switzerland

N. Sabath
The Swiss Institute of Bioinformatics, Basel, Switzerland

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Introduction

Solenopsis invicta virus 1 (SINV-1) is the first virus discovered in an ant species. SINV-1 is a positive sense, single stranded RNA virus which has been placed taxonomically in the Dicistroviridae family, *Aparavirus* genus [1, 2]. The 8,026 nucleotide genome of SINV-1 (Fig. 1) is composed of two large open reading frames (ORFs) in the sense orientation interrupted by an untranslated region containing an active internal ribosome entry site [3, 4]. The 5′-proximal ORF (genome position 28–4,218) encodes the non-structural viral proteins (helicase, protease, and RNA-dependent RNA polymerase) while the 3′-proximal ORF (genome position 4,423–7,803) encodes the structural (or capsid) proteins. The 3′-proximal ORF of SINV-1 is well characterized. Western analysis conducted with polyclonal antibodies developed from a peptide synthesized from the predicted amino acid sequence of VP3 (SRGGYRYKFF-ADDN) confirmed its position and synthesis [5]. N-terminal analyses of purified SINV-1 particles established the margins of each capsid protein within the structural polyprotein [6]. The empirically-determined and predicted molecular mass of VP0 (VP2 + VP4) (60.6 kDa), VP1 (41.8 kDa), and VP3 (24.0 kDa) were in agreement [6].

A third, overlapping ORF (Fig. 1) has been identified at the 5′ end of the 3′-proximal ORF in the +1 reading frame [7]. The ORF, which has been provisionally named predicted overlapping gene (*pog*), was identified in SINV-1 and three bee viruses, Israeli acute paralysis virus (IAPV), acute bee paralysis virus (ABPV), and Kashmir bee virus (KBV) [7, 8], all hymenopteran-infecting dicistroviruses in

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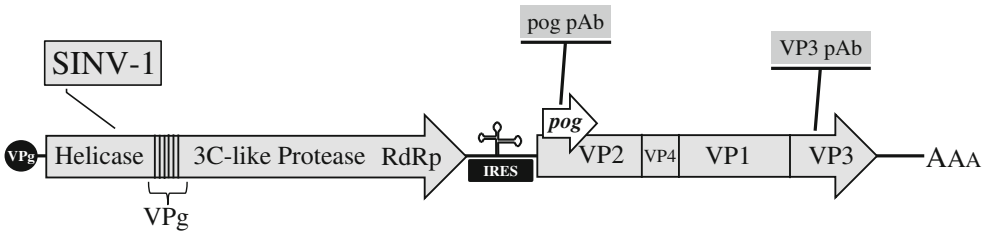

Fig. 1 Genome architecture of SINV-1; orientation is from 5′ to 3′ (*left* to *right*) and open reading frames are represented by *block arrows*. The genome is monopartite, dicistronic, and possesses six copies of the viral protein genome (VPg) peptide [17], an intergenic IRES [4, 18], a *predicted overlapping gene* (*pog*) within ORF 2 [7], and well-characterized capsid proteins (VPs) of known mass [6]

the *Aparavirus* genus. The *pog* ORF was predicted to encode a functional protein using a computational method that detected the signature of purifying selection [7]. The *pog* ORF is absent from the genomes of other related dicistroviruses, suggesting that (if functional) the gene originated de novo through overprinting, a process in which a mutation within a protein-coding reading frame leads to the expression of a second reading frame in addition to the first [9–11]. Similar to most genes that originated de novo, protein motif searches of POG revealed weak matches precluding assignment of a known function. At the time of preparing this manuscript, neither a transcript nor a protein encoded by the *pog* ORF had been detected. However, just prior to submission, Ren et al. [12] detected a protein encoded by the corresponding +1 overlapping ORF (termed ORFx) in the IAPV. The IAPV ORFx protein synthesis was shown to be directed by the intergenic IRES. The objective of our study was to attempt to provide empirical evidence for the presence of a *pog*-encoded protein and, upon verification, characterize its function. Polyclonal antibodies were raised to a portion of the *pog* translated region and used to probe SINV-1-infected *S. invicta* for the presence of a POG protein.

Materials and methods

Ants

Worker ants were collected by plunging a 20 ml glass scintillation vial into an established *S. invicta* nest. After sufficient numbers of ants (>30) had fallen into the vial they were returned to the laboratory for SINV-1 detection by RT-PCR [13] and/or protein separation and western blotting. When larval and adult stages were required, SINV-1-positive colonies were excavated from the field and maintained in the laboratory until used.

Polyclonal antibodies

Polyclonal antibodies to a portion of the SINV-1 POG protein (RNLARRPQKIPLPDK) and SINV-1 VP3 (SRGGYRYKFFADDN) were raised in a rabbit host by ProMab Biotechnologies, Inc. (Richmond, CA), and GenScript USA, Inc. (Piscataway, NJ), respectively, according to each company's standard protocols.

Construction of the *pog* fusion protein

In order to provide a positive control for the POG antibody, the *pog* ORF was cloned in entirety and expressed as a fusion protein using the pMAL Protein Fusion and Purification System (New England Biolabs, Inc., Ipswich, MA). cDNA was synthesized from SINV-1 RNA prepared from virus-infected *S. invicta* collected from Gainesville, FL. RNA (50–100 ng) was mixed with 10 mM dNTPs and 1 μM reverse oligonucleotide primer, p1018 (5′TAAAG TATTGATGGTTATTTGCCTTATCTA) and heated to 65 °C for 5 min and then placed on ice for at least 1 min. First strand buffer and Superscript reverse transcriptase (RT, Invitrogen, Carlsbad, CA) were added and the reaction incubated at 55 °C for 30 min before inactivating the RT reaction at 70 °C for 15 min. cDNA was used as template for PCR using oligonucleotide primers appended with restriction sequences to facilitate ligation into the pMAL-c5X vector. Oligonucleotide primer p1288 (5′**GTCGAC**ATGC GAATACTTTTGAGACGAAAACGGCA) was appended with a *Sal*I restriction site (bold font) and primer p1293 (5′**GAATTC**CTAACTTGCCACCCAGCTTAAGAATGT) appended with an *Eco*RI restriction site (bold font). PCR was conducted using the following temperature regime: 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 60 °C for 15 s; 68 °C for 30 s and a final 68 °C step for 5 min in a PTC 100 thermal cycler (MJ Research, Waltham, MA). The amplicon and pMAL-c5X vector were double digested with *Sal*I and *Eco*RI according to the manufacturer's protocol (New England Biolabs) and agarose gel-purified. Plasmid vector and insert were ligated with Quick T4 DNA Ligase (New England Biolabs). Express Competent cells (New England Biolabs) were transformed with the ligated plasmid. Insert-positive clones were selected for induction and production of the POG protein and sequenced by the University of Florida, Interdisciplinary Center for Biotechnology Research (Gainesville, FL).

Quantitative PCR

Quantitative PCR (QPCR) was conducted as described previously [14]. In brief, cDNA was synthesized from 50 ng of total RNA isolated from SINV-1-infected ants with oligonucleotide primer p523 (5′CCTCATTGAAGAT AAATCCTCTCTTGAGAAA). QPCR assays were performed on an ABI PRISM 7000 Sequence Detection System (SDS) interfaced to the ABI prism 7000 SDS software (Applied Biosystems, Foster City, CA) in a 25 µl reaction volume. The reaction contained 12.5 µl of SYBR Green SuperMix (Invitrogen), 0.4 µl each of 10 mM oligonucleotide primers, p517 (5′CAATAGGCACCAACGTATA TAGTAGAGATTGGA) and p519 (5′GGAATGGGTCAT CATATAGAAGAATTG), 1 µl of the cDNA synthesis reaction, and 10.7 µl of H_2O. The thermal conditions were as follows: one cycle of 50 °C for 2 min; 95 °C for 2 min followed by 35 cycles of 95 °C for 15 s, 56 °C for 15 s; 72 °C for 1 min. Dissociation analysis was conducted after all amplifications to verify amplicon size. For every QPCR run, non-template control reactions were included as negative controls.

Strand-specific detection of SINV-1

Detection of the SINV-1 minus (replicative) and plus strands was accomplished by the modified method of Craggs et al. [15]. This method permits discrimination of each genome strand without carryover effects causing false positive detection of either strand. Two-step RT-PCR was employed to amplify a portion of the genome strand (plus or minus) of SINV-1. First, 1 µl (50 ng) of total RNA was mixed with 10 mM dNTPs, 1 µM of either tagged oligonucleotide primer p1034T (5′GGCCGTCATGGTGG CGAATAACAATTCTTCTATATGATGACCCATTC) for minus strand detection, or tagged oligonucleotide primer p1033T (5′GGCCGTCATGGTGGCGAATAACCAAATTT CTTCATAAGTTCCAT) for plus strand detection, heated to 65 °C for 5 min, and then placed on ice for at least 1 min. First strand buffer and Superscript reverse transcriptase (RT, Invitrogen) were then added and the reaction mixture was incubated at 55 °C for 30 min before inactivating the RT at 70 °C for 15 min. Unincorporated oligonucleotides were digested with 10 U of Exonuclease I (New England Biolabs) at 37 °C for 1 h. The reaction was terminated by heating to 80 °C for 20 min.

PCR was subsequently conducted with the plus and minus strand-specific cDNAs as template. The reaction was conducted in a 25 µl volume containing 2 mM $MgCl_2$, 200 µM dNTP mix, 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen), 0.2 µM of each oligonucleotide primer, and 5 µl of the cDNA preparation. Oligonucleotide primers used for plus strand amplification included p1040 (5′ TGGAAAATCGAATAGATAATGAAGTAGCAATA GG) and TAG (5′GGCCGTCATGGTGGCGAATAA). Minus strand amplification was completed with p1038 (5′ TCCATCCGTGTTTTACAGCCAAATTTCTTC) and TAG. PCR products were separated on a 1 % agarose gel and visualized by SYBR-safe (Invitrogen) staining. Positive controls for each SINV-1 genome strand and negative template controls were included in every evaluation.

SDS-PAGE and western blotting

Solenopsis invicta proteins were separated by SDS-PAGE (4–20 % gradient) and subsequently electroblotted onto a polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, CA) for 2 h at 350 mA. Western analysis was conducted with SINV-1 POG polyclonal antibodies or SINV-1 VP3 polyclonal antibodies at 500- to 10,000-fold dilutions. In brief, the electroblotted PVDF membrane was blocked in TBS (tris buffered saline; 20 mM Tris–HCl, 500 mM NaCl, pH 7.5) + 1 % BSA (bovine serum albumin) for 1 h. Primary antibody (POG or VP3) was added to the TBS + 1 % BSA solution for 2 h at room temperature with shaking (40 rpm). The membrane was rinsed twice with TTBS (TBS + 0.05 % Tween 20), probed with secondary antibody (10,000-fold dilution), goat anti-rabbit conjugated with alkaline phosphatase (Sigma, St. Louis, MO) for 1 h, and rinsed twice with TTBS. The membrane was incubated for several minutes with BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitro blue tetrazolium) for the colorimetric detection of alkaline phosphatase activity. Once bands were detected on the blot, the reaction was terminated by rinsing the membrane with deionized water three times.

Detection limits of the pog antibody

Detection limits of the POG polyclonal antibody were determined empirically by slot blotting. The MBP–POG fusion protein was affinity purified on a bed of amylose resin according to the manufacturer's protocol (New England Biolabs, Ipswich, MA). Bradford assay was performed to quantify the concentration of the affinity-purified MBP–POG fusion protein. Precise quantities of the fusion protein [0–3.2 µg/slot (56.5 pmol)] were applied to a vacuum-assisted Minifold I slot blot apparatus (Schleicher and Schuell, Keene, NH) holding a TBS-pre-wetted BA85 nitrocellulose membrane. Samples were drawn to the membrane by vacuum for 10 min. The membrane was allowed to air dry and then blocked in TBS + 1 % BSA for 30 min. Primary antibody (POG) at 500-fold dilution was added and allowed to react for 2 h at room temperature with shaking (40 rpm). The membrane was rinsed twice with TTBS, probed with goat anti-rabbit conjugated

with alkaline phosphatase for 1 h, and rinsed twice with TTBS. The membrane was incubated for several minutes with BCIP and NBT for the colorimetric detection of alkaline phosphatase activity. Once bands were detected on the blot, the reaction was terminated by rinsing the membrane with deionized water three times.

Results and discussion

Preliminary experiments failed to detect a POG protein in field and laboratory samples of SINV-1-infected *S. invicta* with POG polyclonal antibodies. As the objective of the research was to establish whether *pog* produced a functional protein product, a positive control was necessary to verify that the POG antibody preparation was capable of binding and detecting a POG protein sequence. This task was accomplished by cloning the entire *pog* ORF into the pMAL-c5X expression vector (Fig. 2a) and inducing production of a fusion protein with isopropyl β-D-1-thiogalactopyranoside (IPTG). The *pog* ORF was inserted downstream from, and in-frame with, the *malE* gene of *E. coli*, which encodes maltose-binding protein (MBP). Induction with IPTG resulted in expression of an MBP–POG fusion protein (Fig. 2b). Sanger sequencing verified that the *pog* ORF insert sequence was correct and the translated protein from this sequence contained the peptide sequence used as antigen for antibody production. The pMAL-c5X vector carries the *lacI*^{q} gene which encodes for the Lac repressor (New England Biolabs pamphlet #E8200S) which keeps expression from the P_{tac} promoter low in the absence of IPTG. Indeed, a POG protein sequence was not detected in uninduced *E. coli* containing the pMAL-c5X_*pog* construct by western blotting (Fig. 2b, lane 2). Detection limits of the POG polyclonal antibody were determined to be between 0.1 and 0.2 µg, or 1.8 and 3.5 pmol of MBP–POG protein (Fig. 2c). The band at 0.1 µg was very faint.

A number of different *S. invicta* preparations were examined by western blotting for the presence of a POG protein sequence. Worker ants and larvae (mixed instars) from three SINV-1-infected *S. invicta* colonies were examined initially. These colonies (C1, C2, and C3) contained approximately 10^5–10^6 SINV-1 particles per ant (based on genome equivalents; Fig. 3a) and 3.75 ant equivalents were separated by SDS-PAGE and examined by western blotting. All of these preparations (Fig. 3b) failed to yield any detectable bands when using the POG antibody (at 10,000- and 500-fold dilutions). However, corresponding samples of recombinant POG protein used as a positive control produced a single band of anticipated size (Fig. 3b). In addition, all the ant samples produced a band at 24 kDa when probed with SINV-1 VP3 antibody preparation (Fig. 3c). Therefore, detection of the VP3 capsid protein indicated that SINV-1 was present in the preparations and that the 3′-proximal ORF was being translated. The presence of minus genome strand of

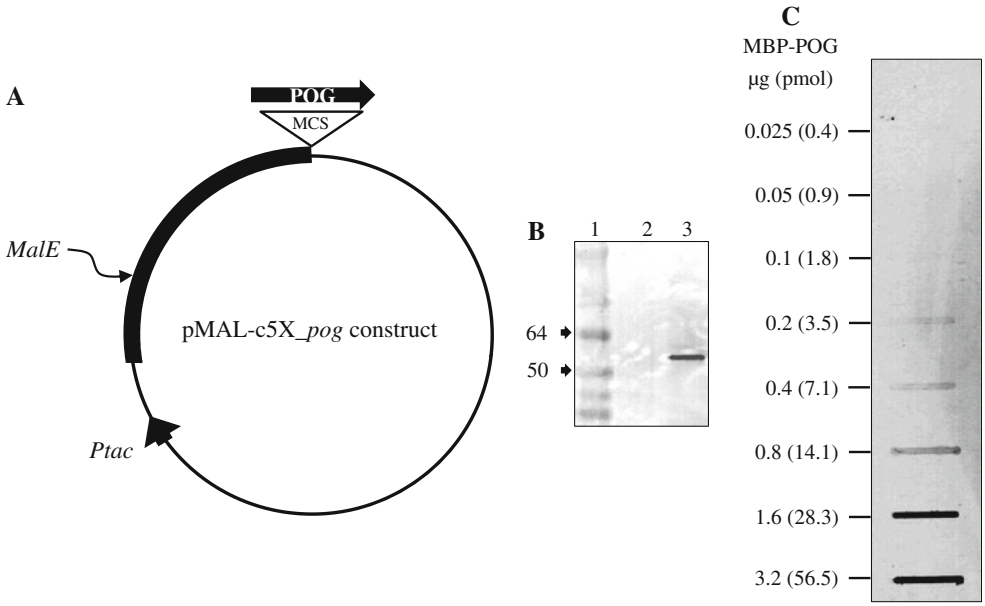

Fig. 2 **a** Expression of the MBP–POG fusion protein was induced by addition of IPTG under direction of the Ptac promoter. **b** Western blot of preparations of IPTG-uninduced (*lane 2*) and -induced (*lane 3*) pMAL-c5X_*pog*-transformed *E. coli* probed with POG polyclonal antibodies. Molecular weight marker (*lane 1*) units are expressed in kiloDaltons. **c** Empirical evaluation to determine the detection limits of the POG polyclonal antibody preparation. Known quantities [0.025–3.2 µg (0.4–56.5 pmol)] of the affinity-purified MBP–POG fusion protein were probed with the POG antibody preparation

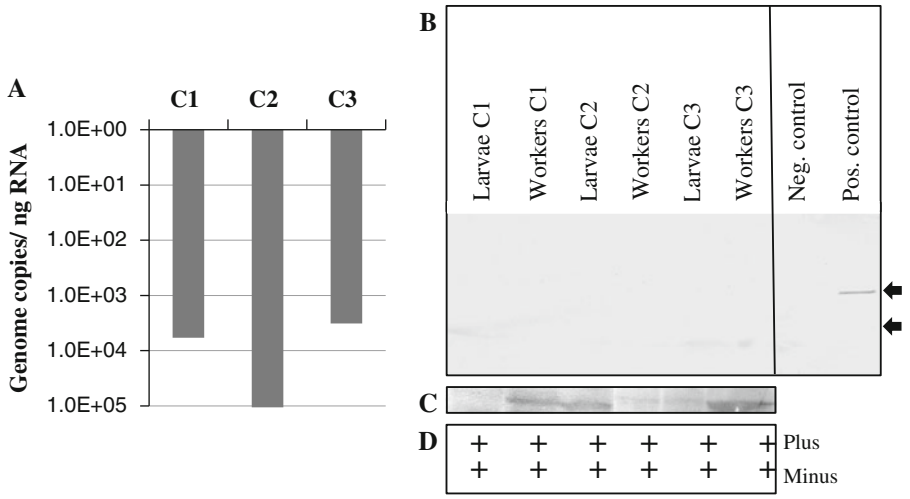

Fig. 3 **a** Quantitative PCR data analysis summarizing the number of SINV-1 genome copies detected per ng of total RNA extracted from ants from each *S. invicta* colony (C1, C2, and C3). **b** Western blot of SDS-PAGE-separated proteins (4–20 %) of *S. invicta* larvae and worker ant stages from the three different colonies (C1, C2, and C3) infected with SINV-1. The blot was probed with a 500-fold dilution of the POG polyclonal preparation. The negative control was a preparation of IPTG-uninduced pMAL-c5X_*pog*-transformed *E. coli* and the positive control was a preparation of induced pMAL-c5X_*pog*-transformed *E. coli*. *Arrows* indicate the MPB-POG fusion protein (*upper arrow*, 55.9 kDa) and the anticipated position (*lower arrow*, 14,135 Da) of a protein predicted from the *pog* ORF. **c** Corresponding western blot of each ant sample probed with the SINV-1 VP3 antibody preparation. **d** Presence (+) or absence (−) of the plus and minus genome strands of SINV-1 detected in each of the corresponding preparations

SINV-1 (Fig. 3d) further indicated that SINV-1 was actively replicating in the ant samples examined.

In an effort to increase the chance of detection of a POG protein sequence, 28 *S. invicta* field-collected SINV-1-infected samples were examined by western analysis. Corresponding RT-PCR evaluations were conducted to verify the presence of SINV-1. In all cases, POG polyclonal antibody preparations failed to yield a discernable band (except for the recombinant MBP-POG fusion protein serving as positive control). Corresponding analyses with SINV-1 VP3 antibodies corroborated the molecular assays indicating that SINV-1 viral coat proteins were present in the samples.

Although impossible to prove a negative result, proper controls used in our studies suggested that the *pog* ORF is not a functional gene translated into a protein in SINV-1. Sabath et al. [7] predicted that *pog* encoded a functional protein based on the signature of purifying selection and the sequence conservation of *pog* across the long evolutionary history since the divergence of SINV-1 and the bee viruses (IAPV, ABPV, and KBV). The authors conceded the absence of Kozak consensus sequences [16] upstream of the potential initiation sites. However, lack of Kozak sequences does not necessarily preclude translation of *pog*. Indeed, reporter constructs containing the IAPV IRES and 3′-proximal ORF components in wild-type configurations were shown to direct translation of the 3′-proximal ORF and +1 overlapping ORFx [12]. The ORFx protein was detected by western analysis in recombinant constructs but not in IAPV-infected honey bees [12]. However, an ORFx protein sequence was detected at very low levels in IAPV-infected honey bees (single bee equivalents) using the very sensitive multiple reaction monitoring mass spectroscopy. Ren et al. [12] concluded that expression of ORFx protein may be limited to a narrow window during the infection process and that it appears to be a rather labile protein. While failure to detect a POG protein by western analysis in SINV-1-infected *S. invicta* suggests that this ORF is non-functioning, the apparent transitory and labile characteristics attributed to IAPV ORFx may well explain our failure to detect POG in ants. Furthermore, we acknowledge the limitations of the antibody detection method and recognize that optimal expression may be required for successful detection. Indeed, detection of the MBP–POG fusion protein was capable only down to the 0.1–0.2 μg range. While every attempt to examine samples with the highest probability for detection of a POG protein (i.e., high SINV-1 titer and active viral replication), the gene may well be expressed only during certain periods of the SINV-1 infection process or at levels below the detection ability of the POG antibody preparation. Despite these potential shortcomings, this study provides the first empirical attempt to establish the existence and functionality of the *pog* gene.

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