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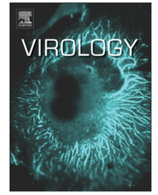

Gene duplication and phylogeography of North American members of the Hart Park serogroup of avian rhabdoviruses

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ABSTRACT

Flanders virus (FLAV) and Hart Park virus (HPV) are rhabdoviruses that circulate in mosquito–bird cycles in the eastern and western United States, respectively, and constitute the only two North American representatives of the Hart Park serogroup. Previously, it was suggested that FLAV is unique among the rhabdoviruses in that it contains two pseudogenes located between the P and M genes, while the cognate sequence for HPV has been lacking. Herein, we demonstrate that FLAV and HPV do not contain pseudogenes in this region, but encode three small functional proteins designated as U1–U3 that apparently arose by gene duplication. To further investigate the U1–U3 region, we conducted the first large-scale evolutionary analysis of a member of the Hart Park serogroup by analyzing over 100 spatially and temporally distinct FLAV isolates. Our phylogeographic analysis demonstrates that although FLAV appears to be slowly evolving, phylogenetically divergent lineages co-circulate sympatrically.

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Introduction

Flanders virus (FLAV) and Hart Park virus (HPV) are two closely-related members of the Hart Park serogroup of the family *Rhabdoviridae* that are maintained in mosquito–passerine bird transmission cycles in the eastern and western United States, respectively (Whitney, 1964; Johnson, 1965; Kokernot et al., 1969; Crane et al., 1970; Main et al., 1979; Main, 1981). Viruses in the Hart Park serogroup were initially classified together based on antigenic cross-reactivity in complement fixation, neutralization, immunodiffusion and/or immunofluorescence assays (Boyd, 1972; Frazier and Shope, 1979). In addition to FLAV and HPV, other members in earlier classifications of the serogroup included Mosqueiro virus (MQOV), a virus first isolated in Brazil, and two African viruses, Mossuril virus (MOSV) and Kamese virus (KAMV) (Tesh et al., 1983; Calisher et al., 1989). Besides their antigenic relatedness, these five geographically disparate viruses appear to share a similar mechanism of transmission, as virus isolation data indicated that they were predominately associated with birds and/or culicine (e.g., *Culex*, *Culiseta*) mosquitoes (Karabatsos, 1985).

More recently, *Wongabel virus* (WONV), Parry Creek virus (PCRV), and *Ngaingan virus* (NGAV) have also been provisionally included into the serogroup based on genetic and phylogenetic (rather than antigenic) relationships (Bourhy et al., 2005; Gubala et al., 2008, 2010). These three viruses were originally isolated in Australia, and besides the serological observation that the natural host range of NGAV may include macropods, they also appear to be predominately associated with birds and culicine mosquitoes or other hematophagous insects such as *Culicoides* biting midges (Humphery-Smith et al., 1991; Bourhy et al., 2008; Gubala et al., 2010). Additionally, two recently described (but historically isolated) Australian viruses recovered from *Culex annulirostris* – Holmes Jungle virus (HOJV) and Ord River virus (ORRV) – appear to be new members of the serogroup (Gubala, 2012), as do Bangoran virus (BGNV) and Porton's virus (PORV) (Dacheux et al., 2010). Whether these twelve potential Hart Park serogroup

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members will eventually be designated as a new genus within the *Rhabdoviridae* will likely entail a more comprehensive phylogenetic analysis (such as full genome studies) of these and other unclassified rhabdoviruses of the Dimarhabdovirus supergroup.

FLAV is unique among the rhabdoviruses in that it purportedly contains a 19 kDa protein gene flanked on either side by putative pseudogenes (GenBank accession AH012179). No comparative sequence for HPV has previously been available. These three consecutive genes, originally termed pseudogene 1, 19 kDa protein gene, and pseudogene 2, are located between the phosphoprotein (P) and matrix (M) genes, such that the FLAV genome is currently represented as 3′-nucleoprotein(N)-P-pseudogene1-19K-pseudogene2-M-glycoprotein(G)-polymerase(L)-5′ (Dietzgen et al., 2011). However, given the constraints on genome size that seem to characterize RNA viruses as a whole (Holmes, 2009), it is surprising that FLAV would apparently carry two sequences that have no functional role. As Australian Hart Park serogroup viruses (i.e., WONV and NGAV) contain three complete intact ORFs between their P and M genes (Gubala et al., 2008, 2010), we sought to analyze this region in the two North American members of the serogroup, FLAV and HPV, and clarify this apparent genomic complexity. Additionally, we investigated the potential encoding of a viroporin-like small hydrophobic (SH) protein located between the G and L proteins and undertook the first comprehensive evolutionary study of a Hart Park serogroup virus by analyzing more than 100 pseudogene region sequences of FLAV isolates collected over a 50-year period.

Results and discussion

Gene, mRNA, and protein analysis of the pseudogene region and SH ORF

Our genetic analysis of multiple FLAV isolates indicated that the two putative pseudogenes located between the P and M genes contained complete uninterrupted ORFs flanked by conserved transcriptional start (UCGUCMKUAG) and stop/polyadenylation (CU_7) sequences, suggesting that they in fact encode functional proteins (GenBank accessions KF028661–KF028670). The predicted proteins associated with pseudogene 1, the 19 kDa protein gene, and pseudogene 2 ORFs in FLAV were very similar in size, with lengths of 161, 165, and 160 amino acids, respectively. Similar results were found with HPV (GenBank accession KF028764), indicating both viruses had three complete ORFs between the P and M genes. Cloning of RT-PCR products generated from RNA extracted from FLAV-infected Vero cells demonstrated that polyadenylated transcripts of the two putative pseudogene sequences (as well as the 19 kDa protein gene) were being produced, again indicating that they are functional ORFs. Functionality was further supported as an analysis of the pseudogene 1, 19 kDa protein gene, and pseudogene 2 sequences of 10 FLAV isolates produced d_N/d_S ratios of 0.07, 0.02 and 0.09, respectively, indicative of strong selective (i.e., functional) constraints rather than the selective neutrality expected of pseudogenes (in which d_N/d_S ratios would tend to be a value of ~1.0). Similarly, a d_N/d_S of 0.07 was observed in 103 pseudogene 1 (U1) sequences (see below), again revealing strong selective constraints.

In addition to the predicted N, P, M, G, and L proteins, we detected three small viral protein bands when we probed FLAV-infected Vero cell lysates in a Western blot using FLAV-specific antisera (Fig. 1). Based on their respective molecular weights, the L (238.54 kDa), G (71.05 kDa), N (50.40 kDa), and M (25.83 kDa) proteins were identified by their approximate size in the immunoblot (Fig. 1). Although the predicted P protein (25.78 kDa) is very similar in size to the M protein, the former is known to migrate in

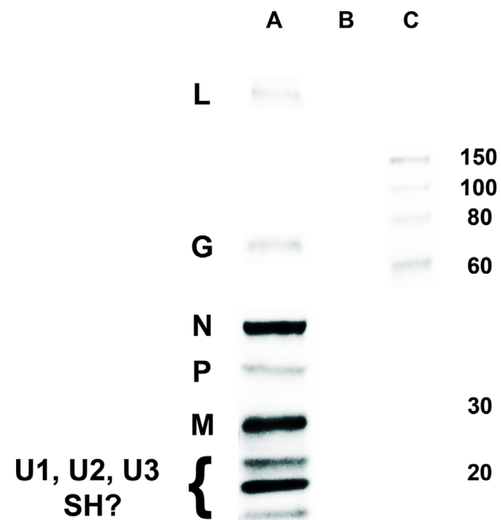

Fig. 1. SDS-PAGE and immunoblot analysis of FLAV-infected Vero cell lysates. The membrane was probed with FLAV-specific mouse hyperimmune ascites fluid and a goat anti-mouse IgG-HRP conjugate. The molecular weights (kDa) of the individual proteins in the ladder are indicated and the tentative FLAV protein designations of the immunoreactive bands are shown. See text for details. Individual lanes are as follows: (A) FLAV-infected Vero cells, day 3 post-infection; (B) mock-infected Vero cells; (C) protein ladder (20–150 kDa).

SDS-PAGE gels at between 40 and 50 kDa (Dietzgen et al., 2011), suggesting P is the band around 40 kDa (size known from additional blots) beneath N. As the predicted molecular weights of the products of pseudogene 1, the 19 kDa protein gene, and pseudogene 2 are essentially identical to one another (18.58, 18.98, and 18.93 kDa, respectively), this suggests that the band just beneath the 20 kDa marker (which is as immunoreactive as the N or M bands) might be the co-migration of the three protein products, provided that their migration is not affected by any post-translational modifications or physiochemical differences. Similarly, the slightly larger band of ~23 kDa might represent a modified form (e.g., phosphorylated) of one of the pseudogene region proteins or an in vivo cleavage product as suggested by Boyd and Whitaker-Dowling (1988). Finally, the lowest band could represent an additional cleavage product, a faster migrating form of one of the pseudogene region proteins (e.g., the acidic pseudogene 1), or the putative SH protein, a predicted 10.37 kDa viroporin-like protein lying between the G and L genes (see below).

To determine if the lower viral protein bands detected in the immunoblot were the pseudogene region products (and/or SH protein) or proteolytic truncated forms of the five major structural proteins, FLAV was purified by sucrose density gradient ultracentrifugation and select SDS-PAGE protein bands were further analyzed by nano-scale high performance liquid chromatography coupled to tandem mass spectrometry (nano HPLC-MS/MS). Although the same or similarly-sized viral bands seen in the infected cell lysates (Fig. 1) were also present (but at a lower intensity) in purified virions by immunoblotting, they were not clearly observed in the SYPRO Ruby-stained gels, suggesting that these proteins/peptides may be incorporated into virions at low concentrations, either selectively or randomly. However, a bright band(*s*) approximately 10–20 kDa was demonstrated to be abundantly present in purified viruses and was the only distinct band(*s*) present beneath the putative M protein in the fluorescent gel (not shown). In-gel tryptic digestion of this band followed by nano HPLC–MS/MS analysis identified peptides corresponding to both pseudogene 1 and pseudogene 2 products (Table 1), conclusively demonstrating that proteins of these reported pseudogenes are being expressed; whether they are normal structural components

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