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# Differential transcription of fathead minnow immune-related genes following infection with frog virus 3, an emerging pathogen of ectothermic vertebrates

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ABSTRACT

Frog virus 3 (FV3) and other ranaviruses are responsible for die-offs involving wild, farmed, and captive amphibians, fish, and reptiles. To ascertain which elements of the immune system respond to infection, we explored transcriptional responses following infection of fathead minnow cells with either wild type (wt) FV3 or a knock out (KO) mutant targeting the 18 kDa immediate early gene (18K). At 8 h post infection we observed marked upregulation of multiple transcripts encoding proteins affecting innate and acquired immunity. Sequences expressed 4-fold or higher in wt-infected cells included transcripts encoding interferon (IFN), IFN regulatory factors (IRFs), IFN stimulated genes (ISGs) such as Mx and MHC class I, and interleukins IL-1β, IL-8, IL-17C and IL-12. Cells infected with the 18K KO mutant (Δ18K) showed qualitative differences and lower levels of induction. Collectively, these results indicate that ranavirus infection induced expression of multiple cellular genes affecting both innate and acquired immunity.



## Introduction

During the past 30 years, ranaviruses have caused considerable morbidity and mortality among ectothermic vertebrates, i.e., amphibians, fish, and reptiles (Chinchar et al., 2009, 2011). Although infections with the chytrid fungus *Batracochytrium dendrobatidis* are responsible for the extinction of several amphibian species and have been viewed as the principal pathogen threatening amphibians, ranavirus infections are common and are the leading cause of localized die-offs among amphibians in North America (Gray et al., 2009; Green et al., 2002). Moreover, ranavirus infections affect multiple species throughout the world and it is thought that die-offs may push small populations with limited geographic ranges to extinction.

Frog Virus 3 is the best-characterized member and the type species of the genus *Ranavirus* (family *Iridoviridae*), a group of large, icosahedral, double-stranded DNA viruses (Chinchar, 2002; Chinchar et al., 2009). Within the family *Iridoviridae*, two genera (*Iridovirus* and *Chloriridovirus*) infect invertebrates, whereas three genera (*Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus*) target cold-blooded vertebrates (Jancovich et al., 2012). Among these five genera, ranaviruses and megalocytiviruses are currently viewed as emerging pathogens of fish and amphibians (Chinchar et al., 2009).

Ranaviruses are promiscuous pathogens and infect a wide range of species belonging to one or more taxonomic classes (Jancovich et al., 2012). For example, although originally isolated from leopard frogs, FV3 and FV3-like viruses have been detected in other frog species, as well as from salamanders, fish, and turtles (Chinchar and Waltzek, 2014). Previous studies (Gantress et al., 2003; Tweedell and Granoff, 1968) demonstrated that although immunocompetent adults confine ranavirus infection to the kidney and successfully recover, tadpoles fail to clear infection, develop systemic disease, and succumb to infection. The sensitivity of tadpoles to infection likely reflects the fact that tadpoles lack full expression of MHC class I molecules and are thought to be deficient in the development of T cell responses (Robert and Ohta, 2009). Moreover, metamorphosis imposes considerable metabolic

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costs on amphibians and heightens their susceptibility to severe ranavirus infections (Rollins-Smith, 1998). In contrast, adult frogs develop protective innate and acquired responses, the latter involving the induction of anti-viral antibodies and cytotoxic T cells (Maniero et al., 2006; Morales and Robert, 2007; Robert et al., 2005). While cellular genes play critical roles in protection and recovery from ranavirus infection, viral genes are thought to play important roles as possible immune antagonists. For example poxviruses encode a dozen or more genes whose function is to circumvent various aspects of the host immune response or enhance replication (Finlay and McFadden, 2006; Johnston et al., 2005; Johnston and McFadden, 2003; Seet et al., 2003; Wang et al., 2009). Ranaviruses likely encode functionally similar genes and, consistent with that view, a FV3 knock out mutant lacking the 18 kDa immediate early gene (Δ18K-FV3) displayed reduced lethality following infection of *Xenopus* tadpoles (Chen et al., 2011). Likewise, an Ambystoma tigrinum virus (ATV) mutant lacking the viral homolog of eukaryotic initiation factor 2 (vIF-2α), a putative virulence gene, was unable to prevent the phosphorylation and subsequent inactivation of eIF-2α and displayed slightly less virulence in vivo (Jancovich and Jacobs, 2011)

In this study we explored whether FV3 infection induced expression of immune-related genes in fathead minnow (*Pimephales promelas*, FHM) cells, a permissive epithelial line commonly used to study the replication of FV3 and other ranaviruses. FHM cells were infected with either *wt* FV3 or Δ18K-FV3 and assayed to determine whether immune-related genes were differentially expressed. Expression was monitored using a 60 K-feature FHM microarray and results of several key genes were validated by quantitative real time RT-PCR (qPCR). As discussed below, we observed induction of numerous immune-related genes in cells infected with *wt*- or Δ18K-FV3 virus suggesting that host immune responses likely play critical roles in combating ranavirus infection.

## Results

### *Induction of host immune-related transcripts*

Ranavirus infections result in the progressive inhibition of cellular RNA and protein synthesis (Raghow and Granoff, 1979; Tannenbaum et al., 1978, 1979). As a consequence, virus-induced host gene expression is likely a transient event that takes place within a window defined by virus-induced onset and virus-mediated inhibition of host protein and RNA synthesis. To determine the time at which cellular immune-related transcripts were present, we infected FHM cells at a multiplicity of infection sufficient to infect all cells (5 PFU/cell) and monitored expression of the viral major capsid protein (MCP) gene and cellular transcripts encoding Mx and β actin by RT-PCR. Expression of the MCP, a late viral gene product, serves as a marker of infection, Mx, a cellular anti-viral protein whose expression is induced by interferon (IFN), is a marker for virus-induced immune-related transcripts, and β actin is a constitutively expressed housekeeping gene that serves as an indicator for RNA integrity. As shown in Fig. 1, MCP transcripts were not detected in uninfected cells, marginally expressed at 4 h p.i., abundant at 8 h, and maximally present at 16 h. Reflective of a low level of constitutive synthesis, Mx transcripts were present at reduced levels in mock-infected cells, but were upregulated by 4 h p.i. and remained at high levels thereafter. As expected, β-actin levels were abundant and constant throughout infection. Based on these results, cellular gene expression was examined at 8 h p.i. as this time point provides an excellent opportunity for detecting cellular genes that were differentially regulated by virus infection.

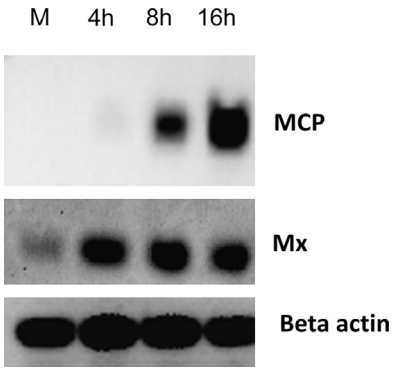


**Fig. 1.** Expression of a representative host immune-related gene, Mx, following FV3 infection. FHM cells were infected with *wt* FV3 at a MOI=5 PFU/cell, and at 4, 8, and 16 h after infection total RNA was isolated and subjected to RT-PCR analysis using primers specific for transcripts encoding viral MCP, FHM Mx, and FHM β actin. Total RNA from uninfected cells (M) served as a measure of constitutive gene expression.

### *Ranavirus-induced transcriptional changes*

To monitor global cellular gene expression, total RNA was isolated from replicate cultures of mock-, *wt*-, or Δ18K- infected FHM cells at 8 h p.i., and subject to microarray analysis. Prior to array hybridization, we confirmed a productive virus infection by monitoring viral protein and RNA synthesis in replicate cultures. Fig. 2A displays protein synthesis profiles in mock-, *wt*-, and Δ18K-infected cells at 8 h p.i.. Infection by *wt* virus led to a marked reduction of cellular protein synthesis and to the appearance of numerous virus-specific proteins, including the 48 kDa MCP and 18K immediate early protein. In contrast, infection with the Δ18K mutant did not suppress host protein synthesis as strongly as *wt* virus. Nonetheless, indicative of a productive infection, multiple viral proteins, including the MCP, were clearly detected in Δ18K-infected cells. As expected, the 18K protein was not detected due to the deletion of its cognate gene, and a prominent band of lower molecular weight, indicated by the asterisk, was also not detected. The presence of this latter band in *wt*-infected cells suggests that this unknown product might represent either a degradation product of the 18K protein or a polypeptide generated by internal initiation within the 18K coding region. Using a RT-PCR assay to monitor viral transcription (Fig. 2B) we confirmed the results of the protein screen and showed that *wt* virus induced expression of both MCP and 18K transcripts, whereas only MCP messages were present following infection with the KO virus.

Having confirmed a productive infection, total RNA from replicate cultures of mock-, *wt*-, and Δ18K-infected cells was subjected to microarray analysis using a 60K feature oligonucleotide array and validated by qPCR. Microarray analyses identified a total of 10,051 differentially expressed genes (DEGs) (fold change $>2$; $p$ value $<0.01$) in wt- infected cells of which 6681 were upregulated and 3370 down regulated (Supplementary Table S1). Within Δ18K-infected cells 5386 DEGs were detected with 3572 showing upregulation and 1814 downregulation (Supplementary Table S2). Note that because some genes are present at multiple sites on the array, the actual number of differentially expressed genes is likely lower than the values shown here. Surprisingly, despite the marked downturn in cellular protein synthesis depicted in Fig. 2A, approximately twice as many gene transcripts were upregulated than downregulated. This finding suggests that following viral infection cellular transcription is transiently upregulated prior to the onset of virus-induced transcriptional and translation shutdown. Moreover, although genes identified in *wt* virus-infected cells were generally also upregulated in Δ18K-infected cells, the response in the latter was not as