AN ABSTRACT OF THE DISSERTATION OF

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Title: The Conserved Biology of Herpesvirus Latency: A Study in Cyprinid Herpesvirus 3

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Cyprinid herpesvirus 3 (CyHV-3), commonly known as koi herpesvirus (KHV), is a member of the *Alloherpesviridae* and is a deadly pathogen for koi and common carp, *Cyprinus carpio*. It causes severe gill necrosis and nephritis, dermal ulceration and hemorrhage, and mass mortality of up to 100% of affected fish. Fish that survive KHV infection are latently infected lifelong carriers. Latency is a conserved mechanism among known herpesviruses and is under the control, in part, of viral gene and protein expression.

Our previous study demonstrated that KHV becomes latent in peripheral white blood cells (WBC) of koi. In this study, KHV latency was further investigated in IgM^+ WBC. The presence of the KHV genome in IgM^+ WBC was about 20-fold more abundant than in IgM^- WBC. To determine if KHV expressed genes during latency, transcription from all 8 open reading frames (ORFs) in the terminal repeat was investigated in IgM^+ WBC from koi with latent KHV infection. Only a spliced ORF6 was found to be abundantly expressed in IgM^+ WBC from KHV latently infected koi. The spliced ORF6 transcript was also detected *in vitro* during productive infection as early as 1 day post-infection. The ORF6 transcript from *in vitro* infection begins -127 bp upstream of the ATG and ends +188 bp downstream of the stop codon, +20 bp downstream of the polyadenylation signal. The hypothetical protein of ORF6 contains a consensus sequence with homology to a conserved domain of EBNA-3B and ICP4 from Epstein Barr virus and herpes simplex virus 1, respectively and both members of

the *Herpesviridae*. This is the first report of latent KHV in B cells and identification of gene transcription during latency for a member of the *Alloherpesviridae*.

To identify and collect an enriched population of KHV^+ latently infected cells, a nanoflare probe was generated specific to ORF6 RNA and used to separate live KHV latently infected cells from total peripheral white blood cells. Using the nanoflare ORF6 probe, about 1% of peripheral WBC from latently infected koi were identified and collected by their expression of ORF6. When this enriched population of KHV^+ latently infected cells was examined by RNA-seq, the ORF6 transcript was found to be the only viral transcript that consistently mapped to the KHV reference genome. This study demonstrated that a nanoflare RNA probe could be used to enrich latently infected cells, which can subsequently be used to characterize gene expression during KHV latency.

Little is known about the molecular mechanisms and control of latency for KHV. In this study, the expression of viral protein from ORF6 mRNA was investigated by a polyclonal antibody specific to a synthetic peptide derived from predicted ORF6 protein (anti-ORF6). Using an immunofluorescence assay (IFA), positive staining to the anti-ORF6 was observed in both KHV-infected common carp brain (CCB) cells *in vitro* and IgM^+ B cells from koi latently infected with KHV. No IFA staining was observed in uninfected CCB cells nor from IgM^- B cells from KHV^+ latently infected koi. The ORF6 protein expressed during productive infection was detected around 140 kDa, which is bigger than the ~80 kDa predicted protein. ORF6 protein at a similar size as the predicted protein was identified from cloned ORF6 protein in an expression vector pet6XHN transformed in *E. coli*. Based on an analysis using software GPS-SUMO, 5 potential sumoylation sites were identified in the ORF6 protein sequence. This study demonstrated that ORF6 protein is expressed during KHV latency in koi and may be sumoylated in infected cells.

These works have unveiled molecular strategies of herpesvirus latency for KHV; the identification of a latency associated transcript as well as viral protein expression during latency, which demonstrate conserved mechanisms as other herpesvirus latency programs. Through these discoveries, it is possible to further

investigate the conserved biology of herpesvirus latent infections by using the koi and KHV model for human herpesvirus associated diseases and therapies.

The Conserved Biology of Herpesvirus Latency: A Study in Cyprinid Herpesvirus 3

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Major Professor, representing Microbiology

Chair of the Department of Microbiology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Aimee N. Reed, Author

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Dr. Brian Dolan assisted with flow cytometry of koi B cells. Dr. Scott LaPatra provided materials for cell culture. Dr. Michael Kent advised throughout the project. Dr. Timothy Miller-Morgan assisted in koi procurement as well as advising for manuscripts and KHV testing. Ms. Satoko Izume and Ms. Jing Dong assisted in the RACE experiments. Mr. Christopher Sullivan advised for bioinformatics analysis of the latent transcriptome. Mr. Timothy Putman assisted in latent transcriptome analysis and provided programming scripts. Ms. Claire Ostertag-Hill contributed lab support in western blots for protein experiments.

TABLE OF CONTENTS

Page

Chapter 1. Herpesvirus Background and KHV Literature Review....1
Herpesvirus Background....2
Introduction....2
Genome Structure....3
Disease Associated with Herpesvirus Infection....4
Latency....6
Latency of Alphaherpesviruses....7
Latency of Betaherpesviruses....11
Latency of Gammaherpesviruses....12
Conclusion....16
Koi Herpesvirus Literature Review....17
Susceptible Species....17
Distribution....18
Clinical Disease During Productive Infection....18
Transmission....20
Diagnosis....22
Virion Structure....24
Classification....25
Genome....27
Host Response and Interaction....29
Vaccine Development....31
Latency of KHV....32
Conclusion....34
Scope of Dissertation....35
References....36

TABLE OF CONTENTS (Continued)

Page

Chapter 2. Identification of B Cells as a Major Site for Cyprinid Herpesvirus 3 (CyHV-3) Latency 59

Introduction 61

Materials and Methods 63

Results 69

Discussion 73

References 79

Chapter 3. Transcriptome analysis of latent KHV from koi cells obtained by a nanoflare probe specific for KHV ORF6 RNA 100

Introduction 102

Materials and Methods 103

Results 107

Discussion 109

References 114

Chapter 4. Detection of the ORF6 protein during latent KHV infection 125

Introduction 127

Materials and Methods 128

Results 133

Discussion 135

References 140

Chapter 5. Closing Remarks 151

Bibliography 155

LIST OF FIGURES

Figure Page

Figure 1.1 Herpesvirus morphology 53

Figure 1.2 Examples of genome structure of different herpesviruses 54

Figure 1.3 Epstein Barr virus latency classes 55

Figure 1.4 Clinical signs of koi herpesvirus disease.....56

Figure 1.5 Transmission electron microscopy of KHV 57

Figure 1.6 KHV genome structure.....58

Figure 2.1 Schematic of CyHV-3 genome and location of primers and DNA probes 87

Figure 2.2 Fluorescence-activated cell sorting (FACS) of koi peripheral WBC.....88

Figure 2.3 Confocal micrographs of koi peripheral WBC.....89

Figure 2.4 Real-time PCR of koi WBC DNA.....90

Figure 2.5 Autoradiogram of CyHV-3 ORF6 RT-PCR amplicons 91

Figure 2.6 Temporal expression of ORF6 during CyHV-3 infection in CCB cells ...92

Figure 2.7 Alignment of CyHV-3 lytic and latent ORF6 transcripts with CyHV-3 genome.....93

Figure 2.8 Alignment of ORF6 conserved domain with consensus sequences from the Conserved Domain database.....94

Figure 2.9 ORF6 RACE products generated by using RNA from CyHV-3 infected CCB cells at 13 d p.i. 95

Figure 2.10 Sequence alignment of the ORF6 transcript ends with CyHV-3 Genome at ORF6.....96

Figure 3.1 SmartFlare RNA probe.....117

LIST OF FIGURES (Continued)

Figure Page

Figure 3.2 Confocal micrographs of live WBC incubated with nanoflare probes....118

Figure 3.3 Flow cytometry analysis for WBC incubated with nanoflare probes.....119

Figure 3.4 Quantitative Taqman PCR of KHV genome copies in cells collected from each gate sorted by flow cytometry by ORF6-Cy5 probe 120

Figure 3.5 Bioinformatics analysis of Illumina reads flowchart.....121

Figure 4.1 Schematic of KHV genome and location of primers.....145

Figure 4.2 Detection of full-length ORF6 transcript 146

Figure 4.3 Indirect immunofluorescence assay for CCB cells 147

Figure 4.4 Indirect immunofluorescence assay for koi peripheral WBC 148

Figure 4.5 Detection of ORF6 protein by western blot 149

LIST OF TABLES

Table Page

Table 2.1 Primer pairs used to detect KHV gene transcription and mapping.....98

Table 2.2 Predicted binding sites in the putative promoter of ORF6 as labeled in Fig. 2.10.....99

Table 3.1 Quantitative Taqman PCR results for WBC in each gate by ORF6-Cy5 probe 122

Table 3.2 Post-run data from 100 base reads from Illumina HiSeq 2000.....123

Table S3.1 RNA and cDNA pre-run quality analysis.....124

Table 4.1 Predicted sumoylation sites for the 725 aa ORF6 protein from GPS-SUMO prediction software.....150

Chapter 1. Herpesvirus Background and KHV Literature Review

Aimee N. Reed

Herpesvirus Background

Introduction

Herpesviruses are an ancient group of viruses that infect almost every type of animal investigated, from invertebrates to humans. All herpesviruses belong in the order *Herpesvirales* (Davison et al., 2009). This order contains over 200 viruses, with at least 100 classified viruses, and many more unclassified characterized or uncharacterized viruses, and will be the home for many more undiscovered herpesviruses. According to the Baltimore systems of virus classification, which distinguishes groups of viruses based on their method of viral mRNA production, herpesviruses belong to Group I: the double stranded DNA viruses. This successful group of virus has co-evolved with its host over time and has a phylogeny that resembles its host's, subsequently leading to a narrow susceptible host range and a high level of specialty. The taxonomy of herpesviruses has recently been reorganized to accommodate newly discovered viruses and to reflect further characterization of viral genomes and their phylogeny. Under one order, *Herpesvirales,* herpesviruses are divided into three families: *Herpesviridae*, which includes herpesviruses of mammals, birds, and reptiles; *Alloherpesviridae*, which includes herpesviruses which infect fish and amphibians; and *Malacoherpesviridae*, which includes herpesviruses which infect invertebrates. The family *Herpesviridae* is further grouped into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. Each family or subfamily consists of genera, which typically group viruses together based on the phylogeny of their host and disease presentation.

The herpesvirus virion consists of an icosahedral capsid of 100-200 nm in diameter that is assembled from 162 capsomeres which houses a linear double-stranded DNA genome. The capsid is surrounded by the tegument that varies in thickness and consists of viral proteins and enzymes that are needed prior to initiation of viral replication (Fig. 1.1). The core, capsid, and tegument are assembled inside the nucleus of a host cell, and upon its egress from the nucleus the capsid gains a primary envelope as it exits the nucleus by budding. This primary protein envelope fuses with

the cell plasma membrane upon exit from the cell, at which time the virion gains an envelope from the cell's plasma membrane that surrounds the tegument and capsid (Mettenleiter, 2002). Virally encoded glycoproteins are expressed on the plasma membrane near where budding occurs to complete the assembly of the enveloped virion. Many envelope glycoproteins are required for infection but the envelope is also sensitive to desiccation; therefore herpesviruses are generally transmitted to a naïve host through mucosal contact from an infected host or close airborne droplet transmission and lose their infective integrity if exposed to the elements for an extended period of time. The major glycoproteins involved in entry for alphaherpesviruses are gB, gD, gH, gL, and gC; gD is considered the ligand for viral entry receptors (Spear and Longnecker, 2003).

Genome Structure

The herpesvirus genome has one segment of linear double-stranded DNA that ranges from 120 – 300 kbp in length. The genome organization is varied but typically remains similar in each family of herpesviruses. The genome structures exhibit quite a bit of variance between species of virus but are characterized by at least one unique region (UR) that is usually flanked by highly repeatable elements that are repeated on the terminal ends of the genome, the terminal repeats (TR) (Fig. 1.2). Some herpesvirus genomes have more than one unique region, identified as a short unique region (US) and a long repeat region (UL). Each unique region, typically, is bracketed by its own pair of repeats, designated as repeat long or repeat short and labeled as either terminal or internal repeat (designated by TR_L, IR_L, IR_S, and TR_S). These repeats become important during circularization of the genome during latency when the genome is maintained as an episome as well as during viral genome replication. The unique regions of the genome can invert upon replication leading to the existence of either two or four equimolar isomers of the genome at any given time.

Upon primary or reactivated productive infection, herpesvirus genes are expressed in cascades that can be grouped by their temporal expression as immediate-early genes, early genes, and late genes. Immediate early genes (IE or α genes) do not

require prior viral protein synthesis and can be expressed in the presence of host protein synthesis inhibitors. These genes are typically involved in viral gene regulation and can be important for initiation of productive infection and switching between latent and lytic infection patterns. Early genes (E or β genes) generally encode for enzymes required for genome replication (DNA polymerase) and nucleotide sequestration (thymidine kinase). Late genes (L or γ) encode for structural proteins needed for virion synthesis and assembly. Not all viral gene expression falls into these three categories, however. There are a number of non-coding RNA products important for herpesvirus biology that are not classified as IE, E, or L genes, such as latency associated transcripts (LAT; see sections below on Latency) and microRNAs. MicroRNAs (miRNA) are short sequences that derive from a hairpin-type structure in a transcript and are then cleaved by endonucleases to 19-23 nucleotide long fragments of RNA. The roles of miRNAs are to regulate gene expression and target mRNA degradation by their incorporation into the RNA-induced silencing complex (RISC) (Filipowicz et al., 2008); during herpesvirus infections they prevent cell apoptosis and prolong infected cell life, evade host immune-responses, and switch from latent to lytic infection (Kincaid and Sullivan, 2012). Viral encoded miRNAs have been identified in all eight human herpesviruses with a various frequency of 3-25 per genome, and they are implicated in both lytic and latent infections (Boss et al., 2009; Murphy et al., 2008).

Disease Associated with Herpesvirus Infection

When viral transcription continues past IE gene expression into E and L gene expression, the production of virus particles ensues; this pattern of infection is referred to as a productive or lytic infection and can be accompanied by viral transmission of newly assembled virions to novel hosts and/or clinical disease presentation. Although not all herpesvirus infections lead to disease, herpesviruses are involved in a broad palette of clinical diseases that are resultant in a large proportion to the host immune response to the virus. Ulcerative dermatitis, commonly referred to as herpetic dermatitis, is a disease presentation of alphaherpesviruses such as HSV-1 and HSV-2,

and VZV in humans, as well as many examples from the veterinary realm such as feline herpesvirus 1 (FeHV-1) (Gaskell et al., 2007) and mustelid herpesvirus 2 (Tseng et al., 2012). Ophthalmic disease is a common clinical presentation such as infection of HSV-1 causing keratitis (Liesegang, 2001), corneal ulceration and sequestrum formation due to FeHV-1 (Maggs, 2005), and ulcerative keratitis and conjunctivitis in dogs due to canine herpesvirus 1 (Ledbetter et al., 2009). Respiratory disease is another common presentation such as phocid herpesvirus 1 in adult harbor seals (Osterhaus et al., 1985) and leporid herpesvirus 4 infection in rabbits (Jin et al., 2008). Neurologic disease is a serious and deadly presentation of productive herpesvirus infection and is often accompanies other presentations of clinical disease. Equine herpesvirus type 1 causes significant neurologic disease known as equine herpesvirus myeloencephalopathy and can be fatal. This virus is also responsible for abortions as well as upper respiratory disease in horses (Lunn et al., 2009). Similarly, clinical signs of cattle infected with bovine herpesvirus 1 are seen in the respiratory, reproductive, and neurologic systems (Tikoo et al., 1995).

Often a herpesvirus can play a part in a disease complex, acting with other pathogens in a host contributing to syndromes that span multiple organ systems such as shipping fever of cattle wherein BoHV-1 interacts with *Pasteuralla spp., Mannheimia haemolytica,* or *Histophilus somni* leading to the development of rhinotracheitis (Yates, 1982). Another critical example of herpesvirus infections as a disease complex as well as infecting multiple species is that of malignant catarrhal fever (MCH), caused by ovine herpesvirus 2 and alcelaphine herpesvirus 1. These viruses do not cause apparent disease in their reservoir hosts, sheep and wildebeest respectively, but can cause fatal lymphoproliferative disease when spread to other susceptible hosts, which include the pig, cattle, deer, and water buffalo (Russell et al., 2009). Although herpesvirus infections are not commonly fatal, they can cause significant morbidity or mortality if they reactivate in an immunocompromised individual or if a particular herpesvirus jumps species to infect a novel host. Generally herpesviruses are not “zoonotic”; however there have been reports of the ability of herpesviruses to jump species causing significant morbidity and mortality most

commonly between human and non-human primates, the most deadly example being macacine herpesvirus, commonly referred to as monkey B virus (formerly Cercopithecine herpesvirus 1) (Tischer and Osterrieder, 2010). Herpesviruses can also cause disease if they infect another mammalian species that is different than its usual host, such as equine herpesviruses type 9 which can cause neurologic disease in polar bears (Donovan et al., 2009), or suid herpesvirus 1 which can cause clinical disease in other mammals leading to death (Glass et al., 1994; Schmidt et al., 1987). Herpesviruses of Asian and African elephants cause severe hemorrhagic disease and abortions and can be fatal if contracted from one species to another, which is often the case in captive situations that house both Asian and African elephants (Richman, 1999).

Proliferative or oncogenic diseases are also associated with herpesvirus infections. There are more than a few examples of herpesviruses associated with papillomatosis with intranuclear inclusion bodies observed in affected cells. A herpesvirus of marine turtles was found to be associated with the development of fibropapillomas, a common condition of both wild and captive turtles (Jacobson et al.). Carp pox describes the formation of dynamic epithelial plaques present on the fins and skin of common carp and is associated with cyprinid herpesvirus type 1 (Calle et al., 1999). Bottlenose dolphin herpesvirus type 1, a gammaherpesvirus, has been associated with epidermal plaques on genital tissues of both male and female bottlenose dolphins (van Elk et al., 2009). Psittacid herpesvirus has been associated with cloacal and oropharyngeal or esophageal papillomas (Styles et al., 2004). There are many oncogenic diseases that are associated with latent herpesvirus infections and will be discussed at length in the following paragraphs.

Latency

A hallmark characteristic of *Herpesviridae* is latency. Latency is a pattern of infection in which the viral genome persists inside a host cell without the presence or production of virion, during which viral gene expression is limited. A latent herpesvirus genome can reactivate or animate, to initiate IE gene expression, leading

to a productive and transmissible infection pattern, with the ability to return into a latent state. These cycles of latency and reactivation continue throughout the lifespan of the host and make a true clearance of herpesvirus infections seemingly unattainable. The latent viral genome persists in a preferred cell type as extrachromosomal circular DNA; however some oncogenic herpesviruses have some level of genome integration, either whole or fractured linear genome, into the host chromosome and have been reported for HHV-6, Marek's Disease virus, and Epstein-Barr virus (Morissette and Flamand, 2010). There are many strategies employed by herpesviruses to establish and maintain latency, but three main objectives persist in the latent herpesvirus program: host immune modulation and evasion, viral and host transcription regulation, and prevention of apoptosis. These themes are reflected in gene expression profiles as well as sites of latency establishment for varying herpesvirus latency programs.

Latency of Alphaherpesviruses

Alphaherpesviruses are neurotropic; after initial infection into epithelial and nerve cells, alphaherpesvirus particles are transported retrograde through axons into sensory nerve cells and establish latency in neuronal ganglia, a terminally differentiated cell lineage. In contrast to cells in which a lytic infection is established, it is believed that IE gene transcription is not initiated in the establishment of a latent alphaherpesvirus infection. Instead, latency can be thought of as a failure to establish IE gene expression and is governed by defunct viral proteins, the most important of these is VP16, a Late gene protein of HSV-1 that is packaged into the tegument of alphaherpesvirus virions. During lytic infection of a host cell, VP16 is released into the cytoplasm where it binds with cellular protein host cell factor 1 (HCF-1) and translocates to the nucleus. Another cellular protein, the POU homeodomain protein Oct-1, binds to IE promoter motifs of TAAGARAT, and when coupled to the VP16/HCF-1/Oct-1 complex, IE gene transcription is initiated (Wysocka and Herr, 2003) and productive infection ensues. Without this complex, IE viral transcription is not initiated, which in turn cannot initiate transcription of E and L genes or production of virions (Penkert et al., 2011). Although HCF-1 is expressed in neurons, it is located only in the cytoplasm and transported to the nucleus only under times of physiologic

stressors (Kristie et al., 1999; Roizman and Whitley, 2013); therefore without translocation of transcription initiation complex, the majority of viral gene transcription remains quiescent and a latent viral genome resides inside the nucleus.

Although there is very little gene transcription during latency, not all viral genes are silenced. For HSV-1, there is one latent transcript expressed, the latency associated transcript (LAT); it is encoded on the antisense strand of IE gene ICP0 (Rock et al., 1987) within the internal repeat (IR) region of the genome. Comparatively, the LAT of Marek's Disease virus maps to the antisense of the ICP4 gene, another IE gene important for reactivation of latency (Cantello et al., 1994). LAT is a non-polyadenylated transcript initially of 8.3 kb, which is spliced producing two exons of 1.5 kb and 2.0 kb (Wagner et al., 1988) and localizes to the nuclei; however LAT does not translate to any protein during latent infection. LAT transcripts have been shown to block HSV-1 induced apoptosis in infected neurons (Perng et al., 2000). Mutant recombinant viruses deficient in LAT have been shown to spontaneously reactivate at low levels, indicating that LAT is required for reactivation (Perng et al., 1994). There are several studies which observe LAT-null mutant viruses which can still establish, maintain, and reactivate from latency (Ho and Mocarski, 1989; Leib et al., 1989; Thompson and Sawtell, 1997).

HSV-1 miRNAs have been described deriving from LAT. Recently, there has been an increased effort investigating these miRNA derived from LAT and their anti-apoptosis abilities (Gupta et al., 2006), although the replication of this experimentation leads to questionable results. A decreased expression of ICP0, an IE gene capable of reactivating lytic infection, was observed by the miRNA encoded antisense to ICP0, although its function was not completely blocked. Other miRNAs which are antisense to ICP34.5 as well as ICP4 have also been identified; their involvement in the maintenance of the latent state by blocking the actions of their complimentary transcripts has been postulated (Umbach et al., 2008). The functions of virally encoded miRNAs has become a central investigation of alphaherpesvirus biology

(Jurak et al., 2011) and it will be interesting to identify how these miRNAs might lead to implications for anti-viral therapy (Flores et al., 2013).

Epigenetic modulation has implications for viral latency as well. Latent viral DNA is closely associated with histones (Knipe and Cliffe, 2008). Additionally, the VP16/HCF/Oct-1 complex has implications for modulating histone occupancy, positioning, and modifications at IE promoter regions. Without the demethylation of the histones, viral transcription cannot initiate. VP16 is also known to interact with histone acetyltransferases, leading to increased acetylation of IE promoter regions (Memedula and Belmont, 2003; Penkert et al., 2011). Epigenetic modulation is still a hot topic in viral latency and reactivation and is just beginning to be understood.

Although HSV-1 or HSV-2 do not express protein during latency, this theme is not conserved throughout the alphaherpesviruses, as there are well-studied examples of alphaherpesviruses that do in fact encode protein during latency (Croen et al., 1988). The best example of this is VZV, for which polyadenylated mRNA has been described, and at least 9 genes from three different genome locations were found to be expressed during latency in the trigeminal ganglia (Croen et al., 1988; Kennedy et al., 2000). Researchers have detected the presence of an IE protein ORF63, a homologue of HSV ICP22, from the cotton rat model to be critical for the establishment of latent VZV (Cohen et al., 2004). The detection of an additional protein ORF29, an HSV-1 ICP8 homologue, has also been described recently during VZV latency which has been shown to be critical in the rat model (Cohen et al., 2007).

Reactivation of herpesviruses from latent state often leads to serious diseases, and its prevention is the aim of many therapeutic interventions. Although some would say that initial infection of alphaherpesiruses is somewhat uneventful, reactivation from latency is correlated with significant clinical disease, examples of which are ocular ulceration and keratitis, cold sores, genital ulcers, and shingles. Reactivation is typically brought on by physiologic stressors such as immune suppression, local trauma such as surgery, mental stress, or heat stress, with UV light being the ultimate stressor for triggering the reactivation of simplexviruses (Laycock et al., 1991; Padgett

et al., 1998). VZV reactivation has been linked to the declination of protective adapted immunity to the virus; therefore, the risk of VZV reactivation increases as a person ages (Nagel and Gilden, 2013).

ICP0 is one of the five IE genes of HSV-1 and is a transcriptional transactivator that is translated within hours of cell infection. ICP0 has been considered the inducer of reactivation from latent HSV-1 infections; ICP0-defective mutant viruses fail to reactivate from animal model experiments (Halford and Schaffer, 2001). It is capable of activating promoters of IE, E, and L genes of HSV-1 through its N-terminal C3HC4 zinc-binding RING-finger domain, a structural motif that is conserved in all alphaherpesvirus ICP0 orthologs. ICP0 has been implicated in chromatin modulation, DNA damage response, regulation of protein stability, the interferon response, and a number of other pathways (Boutell and Everett, 2013). ICP0 plays a role in degrading proteins involved in the cell's defense against viral infection. The replication of viral DNA occurs in complexes called nuclear domains (ND10) or promyelocytic leukemia (PML) protein associated bodies, which are important for cell growth and DNA synthesis and are associated with SUMO-1, small ubiquitin modifier protein. ICP0 inhibits these ND10-related proteins paving the way for viral replication to ensue (Boutell and Everett, 2013; Chelbi-Alix and de The, 1999).

Host cell interactions also have implications for reactivation from latency. Although there is considerable amount of immune modulation on behalf of the herpesvirus a latent infection does not go undetected in the host. CD8+ T cells have been found to be associated with latently infected neurons and trigeminal ganglia (TG) by blocking the production of L genes and virus particles (Liu et al., 2000). Interestingly, high levels of mental stress compromise the ability of CD8+ T cells to regulate latency, thereby exhibiting a link of HSV-1 reactivated disease with the sympathetic nervous system (Freeman et al., 2007; Padgett et al., 1998). IFN-γ has also been implemented through CD8+ T cells to prevent the complete reactivation from latency (Liu et al., 2001). This highlights efforts to postulate novel vocabulary to

discriminate the particular stages of reactivation to two phases: animation, which would apply to the initiation of IE genes and a switch into lytic replication, and reactivation, which is determined by the complete gene cascade expression and production of virions (Penkert et al., 2011). The role of IFN- γ in the prevention of reactivation but not animation is an example of the need to differentiate these stages.

Latency of Betaherpesviruses

The best-studied betaherpesvirus, human cytomegalovirus (HCMV; HHV -5), is almost ubiquitous in its distribution, as 50-90% of people surveyed are seropositive with no clinical signs of disease. In the immunocompromised person, however, HCMV can cause severe morbidity or mortality as it reactivates from latency (Onorato et al., 1985). HCMV establishes latent infection in CD34+ progenitor cells, from which all lymphoid and myeloid cells derive; however, latent HCMV has not been found in all derivations of the CD34+ lineage. Latent HCMV DNA has not been identified in B cells, T cells, or PMNL. It has, however, been identified in the monocytes derived from CD34+ progenitor cells, which carry the latent genome as an episome. Epigenetic control seems to have the most prevailing control over establishment and maintenance of HCMV latency. The major immediate early promoter, MIEP, is repressed in the CD34+ progenitor cell population through histone-modification enzymes (Mendelson et al., 1996).

The identification of latent HCMV genome in the healthy individual does not confer to any yet detectable disease and no lytic infection, with little or no IE gene expression (Sinclair and Sissons, 2006). It remains increasingly challenging to obtain latently infected cells to investigate latent gene expression profiles with these extremely infrequent latent cells in the natural host, and this remains an obstacle in this field.

Although several latency-associated transcripts have been identified for HCMV, deletion of putative ORFs encoding these genes showed little effect on latency *in vitro*. ORF94 deletion mutant failed to infer any change in latency or reactivation phenotype (White et al., 2000). Cytomegalovirus latency-specific

transcripts, CLTs, have been identified from spliced and unspliced regions of IE genes and have been detected in healthy and unhealthy individuals. Their function during latency remains unknown, but in productive infection they regulate the anti-viral gene ORF94. Deletion mutants of ORF94, however, appear to confer no observable difference for HCMV latency (White et al., 2000). Genes that have been confirmed in a natural infection during latency include UL138, UL81-82ast (LUNA), and a spliced variant of UL111A, which encodes for the viral IL-10 gene termed LAcmvIL-10 (Bego et al., 2011). UL81-82ast promotes expression of UL138 during latency. UL138 is a regulator of TNFRI as well as MRP1 and potential repression of the MIEP. LAcmvIL-10 down-regulates MHC class II expression to aid in immune evasion and is also expressed during lytic infection. Lnc4.9 function during productive infection is unknown, but during latency binds polycomb repressor complex 2 as well as silences the MIEP (Jenkins et al., 2008). UL84 maintains the latent genome, but during productive infection is responsible for genome replication, UTPase activity, and transcriptional regulation. US28 and UL144 have also been detected during latency but their functions remain unknown (Hansen et al., 2003). Instead, reactivation of HCMV from latency seems to be more affected by the differentiation of the latently infected progenitor cell (Sinclair and Reeves, 2013). It is not to say that the transcriptional profile during latency has no function; but that their function remains mysterious in light of the healthy HCMV-infected individual.

Latency of Gammaherpesviruses

Gammaherpesviruses are lymphotropic herpesviruses that establish latency predominately in B lymphocytes, with a major exception being herpesvirus saimiri, a T-lymphotropic herpesvirus that causes lymphoproliferative disease in squirrel monkeys and rabbits, and has the ability to immortalize human T cells (Biesinger et al., 1992). Gammaherpesviruses have been known to cause lymphoproliferative diseases and cancers, and they have been associated with autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (Posnett, 2008). Epstein-Barr virus and Kaposi's Sarcoma-associated virus (HHV-4 and HHV-8,

respectively) are the best studied gammaherpesviruses, and murine gammaherpesvirus 68 in the mouse has currently been the most functional animal model for gammaherpesviruses (Simas and Efstathiou, 1998).

EBV employs intricate programs of latency, which can be distinguished by their infected cell type, protein expression pattern, and phenotypic disease presentation. Primary infections with EBV can be innocuous if the patient is exposed as a baby or young child, but can cause significant disease if exposed in late adolescence or adulthood which can lead in some cases to infectious mononucleosis (Papesch and Watkins, 2001). Latent EBV infection is associated with two main cancers of the blood, Hodgkin's disease and Burkitt's lymphoma, as well as nasopharyngeal carcinomas, AIDS-associated lymphomas, and immunoblastic lymphomas (Amon and Farrell, 2005).

Up to nine virally-encoded proteins are expressed during latency as different combinations, leading to three EBV latency programs, Latency I, Latency II, and Latency III; Latency 0 has also been described as the absence of virally encoded proteins (Thorley-Lawson and Gross, 2004). Latency III has all nine virally encoded latent proteins expressed: EBV nuclear antigens (EBNA-1, -2, and -3A, -3B, and -3C, and -LP), and latent membrane proteins (LMP1, LMP2A, and LMP2B). Type III latency is also called "Cp-on" latency in which the B lymphoid cell-specific C-promoter, Cp, is switched on and promotes expression of the six EBNA proteins which all derive from one transcript. In type I latency, where EBNA-1 is the only protein expressed, and in type II EBV latency, which has EBNA-1 as well as LMP1 and LMP2A and -2B expressed, the Cp is off and all EBNA-1 expression is regulated via the Q-promoter, Qp, which is inactive during type III latency (Fig. 1.3) (Niller et al., 2004).

After the infection of a primary naïve B cell, a brief abortive lytic state progresses in the B cell during which a few lytic genes are expressed-- then silenced-- by chromatinization of the genome (Murata, 2014). Genes expressed in latency III activate the B cell during a growth program, overcoming cell cycle checkpoints and

regulating viral gene expression and their interaction with host proteins, such as EBNA-2A interacting with RBP-jk (Amon and Farrell, 2005). ENBA-3A and -3C have been shown to be essential for the immortalization of infected cells (Tomkinson et al., 1993); conversely, EBNA-3B was shown to be non-essential for primary infection, cell transformation, or lytic virus infection in vitro; it is thought, however, that there may be a function during latency in vivo for EBNA-3B (Tomkinson and Kieff, 1992). LMP functions to block apoptosis and to inhibit BCR signaling, providing survival signals and also blocking tyrosine kinase activity to keep the viral replication out of lytic production. EBNA-1, which is expressed in all three latency types, is necessary to replicate viral DNA in dividing memory B cells (Thorley-Lawson and Gross, 2004). Type III latency has been implicated in immunoblastoid lymphomas; type II latency is observed in Hodgkin's disease, and type I latency is observed in Burkitt's lymphoma. EBV encoded miRNAs and BamHI A rightwards transcripts (BARTs) are highly expressed in nasopharyngeal carcinomas and are detected in all three EBV latency programs (Marquitz and Raab-Traub, 2012).

Lytic reactivation of EBV leads to virion production and transmission of virus to naïve hosts. In B cells and epithelial cells, a common trigger for reactivation is cell differentiation, leading to the transcription of IE genes, and it has been implicated in clinical disease such as oral hairy leukoplakia of AIDS patients. The viral IE gene BZLF1 and its promoter, Zp, are identified as inducers of reactivation for EBV. A major protein for the switch to lytic infection is RTA, the master regulator protein, encoded by the IE gene BRLF1. RTA usually interacts with RTA-response element (RRE) promoter regions, however it has also been shown to interact with SP-1 promoter regions (Chang, 2005), providing momentum for the lytic gene expression pattern. Ammon and Farrell (2005) have discussed implications of therapeutically reactivating EBV, turning cell cycles on and thereby identifying replicating targets for cancer therapies to encourage apoptosis or targeted destruction of EBV-infected cancer cells.

Kaposi's sarcoma associated herpesvirus (KSHV), human herpesvirus 8, is associated with three major clinical presentations during latency: Kaposi's sarcoma, a cancer of epithelial cells, and multicentric Castleman's Disease and primary effusion lymphoma (PEL), neoplastic diseases of B cells, making KSHV a primary lymphotropic herpesvirus. The preferred cell for establishing latency is the resting memory B cell. It has been observed that KSHV can only infect *in vitro* B cells if they are first activated by IL4 and CD40 ligand, or primed B cells that are already activated from the tonsils (Rappocciolo et al., 2008). Although the CD19+ B cell has been identified as the primary target for KSHV, there has recently been evidence that KSHV frequently infects populations of T cells which the virus quickly aborts and does not carry the infection to completed virion production (Myoung and Ganem, 2011). Additionally, there is evidence of infection of monocyte-derived dendritic cells, as well as dermal dendritic cells and Langerhans dendritic cells associated with Kaposi's sarcoma (Rappocciolo et al., 2010). It has also been proposed that KSHV-infected dendritic cells play an important role in CD4+ T lymphocyte suppression and promote *trans*-infections with HIV, highlighting the immunosuppressive action of KSHV and its accompanying complexities (Liu et al., 2013).

KSHV lacks the ability to immortalize cells during latency, which indicates it has no similar mechanism to the ENBA (-2A through -LP) expression of the EBV latency III program. It does have one major latency associated protein, latency-associated nuclear antigen (LANA) that has similar action as ENBA-1. Expression of LANA1 comes from the major latency locus of the genome at ORF73, which also encodes v-cyclin from ORF72, a homologue of cellular cyclin D, and vFLIP, which activates NF-κB, initiating a proinflammatory and anti-apoptotic program in many cell types (Speck and Ganem, 2010). LANA1 is necessary to maintain nuclear association of latent genomes during replication, as it tethers the viral episome to the chromosome of the daughter cell, thereby ensuring its persistence in replicating cells and can be thought of as the ENBA-1 equivalent (Ballestas and Kaye, 2011; Barbera et al., 2003). LANA1 has been implicated in the promotion of the S-phase entry via β-catenin upregulation (Fujimuro et al., 2003). LANA2 is a latent protein found in B cells of

Castleman's Disease and PEL, but not in epithelial cells of Kaposi's sarcoma, which belongs to the family of interferon regulatory factor (IRF) and has been shown to be a potent inhibitor of p53 (Rivas et al., 2001).

There are twelve microRNAs expressed during latency from a different genome locus called the Kaposin locus (Samols et al., 2005). The functions of these 12 miRNAs are still under investigation, but one group identified 81 cellular genes that changed their expression dynamic in the presence of these KHSV miRNAs, with the most dramatic change being a >10 fold decrease in cellular thrombospondin-1, an anti-tumor and anti-angiogenic factor that was previously identified to be diminished in Kaposi's sarcoma lesions (Samols et al., 2007).

Much like BRLF-1 of EBV, the KSHV reactivation is controlled by the virally-encoded RTA, the conserved master regulator protein and a transcriptional activator, which is encoded by ORF50. RTA is necessary for reactivation from the latent state as RTA-null mutants cannot reactivate from latency (Xu et al., 2005). An important and well-studied interaction for the reactivation from latency is RTA with RBP-Jκ, the main effector of the Notch signaling pathway, and through this pathway RTA mimics Notch signaling thereby cooperatively trans-activating promoters of KSHV lytic genes (Liang, 2002).

Conclusion

Although there are many different programs of latency among the herpesviruses, the latent pattern of infection is conserved among every subfamily. General conserved ideas pervade throughout each program, which include immune system evasion, apoptosis prevention, and quiescence in host cells. The ability to reactivate from latency is another conserved pattern among herpesviruses. Although the complexity of immune systems have evolved over time and display tremendous diversity among taxa, the herpesviruses have persisted and co-evolved within the host. Understanding the mechanisms of herpesvirus latency throughout the taxa will lead to an unveiling of conserved herpesvirus biology we see today, and therein lies potential

to mitigate clinical disease and mortality due to latent and reactivated herpesvirus infections.

KHV Literature Review

Susceptible species

Cyprinid herpesvirus type 3 (CyHV-3), more commonly known as koi herpesvirus (KHV), is a recently emerged disease that causes clinical disease and mortality among its primary host, the common carp (*Cyprinus carpio carpio)* and ornamental koi carp (*Cyprinus carpio koi)*. Other breeds of carp such as grass carp or Crucian carp are not affected; however hybrid crosses of common carp × Crucian carp (*Carassius carassius*), as well as koi × goldfish (*Carassius auratus*) succumb to fatal infection when exposed to KHV (Bergmann et al., 2010a). A difference of susceptibility of wild Amur mirror carp strains to KHV has been demonstrated by observing only 33% mortality, compared to 100% mortality of non-crosses, when exposed to KHV into progeny (Dixon et al., 2009). Ropsha scaly carp and its crosses, and a Hungarian common carp strain (Tat and HAKI15) also seem to show some resistance (Piačková et al., 2013). The susceptibility of indigenous Japanese carp compared to domestic (European) common carp was investigated after an outbreak that affected both breeds. It was observed that viral genome levels in the fish tissues peaked earlier and at higher levels in the indigenous koi carp compared to the domesticated carp and suggests that higher cortisol levels of the indigenous carp may lend to a faster and more robust response from the virus (Ito et al., 2014).

Goldfish, another species of cyprinid, appear to be susceptible to infection by KHV, as DNA has been detected in goldfish which cohabitate with KHV-infected koi by real-time PCR; it is important to note that observable viremia did not correlate to any clinical signs of KHV infection in the goldfish (El-Matbouli et al., 2007a; Sadler et al., 2008). Therefore, although they do not succumb to disease, infected goldfish can act as a carrier for KHV, transmitting the virus to naïve common carp or koi by cohabitation (El-Matbouli and Soliman, 2011). This has broad implications for those who keep both goldfish and koi in the same pond, a common practice among koi

hobbyists, as well as disease transmission at ornamental koi and goldfish competitive breed shows and distributors.

Distribution

KHV disease was first observed in Germany in 1997, then again in 1998 in Israel and the United States (Hedrick et al., 2000). Since then, KHV has been implicated in mass mortality events around the world of all ages and sizes of carp. A mass die off of 3500 metric tons of aquacultured carp was reported in Korea in 1998 which was associated with KHV (Choi et al., 2004). In 2004, KHV was isolated from a die-off of wild common carp in the Chadakoin River in New York (Grimmett, 2006). In 2007 and 2008, KHV caused significant mortality of wild common carp in several ponds in Canada (Garver et al., 2010). KHV has a broad distribution in the United Kingdom; one survey detected KHV in 79 out of 82 farms, highlighting the serious prevalence of the virus on the English carp aquaculture trade (Taylor et al., 2010). Major outbreaks of KHV have also occurred in Austria in 2007 (Marek et al., 2010). Many Asian countries, too, have experienced economic losses due to KHV, including Indonesia, Taiwan, China, and Japan (Avarre et al., 2012; Bondad-Reantaso et al., 2005; Cheng et al., 2011; Dong et al., 2013; Sunarto et al., 2005). As of 2012, KHV has not been detected from aquaculture facilities in India, although the authors acknowledge the lack of sensitive testing for non-replicating virus (Rathore et al., 2012). KHV disease is an OIE listed disease (2014).

Clinical disease during productive infection

Disease caused by KHV has devastated the carp aquaculture population around the world, in addition to severely affecting the show koi trade costing hundreds of millions of dollars in damage and loss. Because of the character of clinical signs involved, KHV is also known in some countries as interstitial nephritis and gill necrosis virus of carp (CNGV) (Pikarsky et al., 2004). Clinical signs of disease and acute death have been seen as early as day two, with mortalities peaking on days 7-11 in most experimental infections with some infected fish lingering until three weeks post exposure before succumbing (Gilad et al., 2002). Most KHV-infected fish exhibit

lethargy and some exhibit neurologic signs such as erratic swimming, spiraling, or twitching. They exhibit aspects of respiratory distress such as piping at the water surface or display rapid opercular movements due to ineffective oxygen transport across necrotic gill tissues, which appear discolored, mottled, eroded, and edematous and are covered in excess mucus. Severe skin damage is also observed as necrotic dermal ulceration and increased mucus production. Hemorrhaging of the scales, fins or bases of fins, perianal region, and ventrum are also observed, sometimes with petechiation (Grimmett, 2006) (Fig. 1.4). Enophthalmia is another common finding with acute KHV infection (although exophthalmia, which is more commonly associated with bacterial septicemia diseases, has also been observed in some cases, perhaps with secondary or pre-existing bacterial infections) (Miyazaki et al., 2008). It is not surprising, therefore, that KHV has a high rate of mortality of 80- 100% in most cases (Bergmann et al., 2010a; Dixon et al., 2009; Hedrick et al., 2000).

KHV infects multiple cell types and tissues during the productive infection. The most significant histologic changes are seen of the gills, characterized by hyperplasia and severe necrosis of gill epithelium and fusion of lamellae. Lamellar epithelial cells display vacuoles and cell swelling with nuclear degeneration, karyorhexis, pale coloration of the nucleus, and intranuclear inclusion bodies. The nucleus of infected cells is characterized by margination of chromatin (Cheng et al., 2011; Miwa et al., 2007). The kidney is another major organ targeted during KHV infection. The main affected cells in the kidney are hematopoietic cells, with mild changes in epithelial tubular cells. Similarly in the liver only mild changes of hepatocytes are seen as a cloudy appearance, and very few cells show intranuclear inclusion bodies. The heart has lesions associated with acute KHV disease as well, with nuclear degeneration and pyknosis of myocardiocytes and the formation of intranuclear inclusion bodies. The spleen develops areas of hemorrhage in the pulp, and splenocytes and splenic macrophages are also affected which develop necrosis and intranuclear inclusion bodies (Miyazaki et al., 2008).

Transmission

KHV is extremely contagious and easily transmissible, as it has been shown that just a single derived plaque is enough to induce disease in experimentally infected fish (Pikarsky et al., 2004). Recently, a European group investigated the major portal of entry of KHV in naïve fish. Experimentally infected carp were immersed with a luciferase-tagged recombinant virus then analyzed by bioluminescence imaging at various times post infection; it was determined that the skin covering the fins and body was the major portal of entry for KHV in all age-groups of carp (Costes et al., 2009; Ronsmans et al., 2014). This information is contrary to the prior belief that the gills were the major portal of entry by some researchers, which was further disqualified by the continued research of Costes and colleagues who designed an interesting experiment where the front half of the fish was separated from the back half of the fish by a latex diaphragm, and each half was exposed to virus independently through a "U-tube" tank apparatus. They adamantly showed that even when the gills do not see the virus, the [caudal-half only] exposed fish tested positive for viral invasion into tissues.

This is an interesting facet of host-and-microbe interaction as the skin of fish is covered by an ever-secreting layer of mucus, which is a powerful first line of defense and protects from the harsh aquatic environment and pathogens therein. Fish mucus has multiple immunologic components equipped to battle environmental pathogens such as lymphocytes, phagocytes, immunoglobulins, lyzosyme, complement proteins, C-reactive protein, lectins, and other proteolytic enzymes (Subramanian et al., 2007), and if compromised leaves the fish susceptible to invasion by parasites, bacteria, fungi, and viruses. It is not surprising, therefore, to find that carp skin mucus inhibits binding of KHV to epidermal cells and that KHV entry via the skin was drastically increased in areas of the skin that had mucus removed, with or without any associated epidermal lesion (Raj et al., 2011). In the arms race between host and microbe, this does not come without kickback from the virus. A reduction of the expression of carp genes associated with mucosal integrity was observed after infection with KHV such as mucin 5B, beta defensin 1 and 2, and claudin 23 and 30, important genes for tight

junction integrity. A decrease in the number and robustness of goblet cells was also observed in KHV infected fish compared to non-infected fish. These decreased expressions lead to an induced disruption of the mucus layer, making the fish more susceptible to secondary bacterial infections, and can also begin to explain viral tissue penetration after initial infection of the skin (Adamek et al., 2013). The skin is not the only portal of transmission for KHV; one study showed that the virus was transmissible by feeding infected materials (such as virus-soaked kibble) to naïve fish. Through these methods, the pharyngeal periodontal mucosa was determined to be the major portal of entry for orally contracted virus (Fournier et al., 2012).

The ease of transmission of KHV has major implications for spread of disease throughout intensively reared fish. Transmission between wild fish and cultured fish has been demonstrated in experimental settings, which has dire implications for areas of pond-cultured fish and wild carp populations exist in close vicinity (Fabian et al., 2013). Wild carp have tested positive in the Great Lakes of United States and Canada, as well as in Europe and Japan (Cornwell et al., 2012; Minamoto et al., 2012; Uchii et al., 2009; Xu et al., 2012). KHV DNA has been detected from rivers and pond water, from the sediment of lakes and ponds, and even from planktonic rotifers in the aquatic ecosystem (Haramoto et al., 2007; Honjo et al., 2012; Minamoto et al., 2011). KHV was also detected in the droppings of infected fish, which illustrates the ease of spreading infectious virus particles to piscine cohorts (Dishon et al., 2005). New collection methods from Japan have improved on the recovery of virus particles from waterways using cation-coated filter method; these methods were comparable to the ultrafiltration method of virus surveillance. Using this method, KHV viral loads were quantified by Taqman assay during and after a KHV outbreak and were found in waterways as much as three years after an observed outbreak; it should be noted, however, that they also found viral levels which were comparable to other water systems with outbreaks just one month prior, suggesting that an outbreak may have occurred unnoticed more recently than within the previous three years for the investigated ecosystem. It is accepted that viral DNA could be detected from

waterways consistently within three months after an outbreak (Honjo et al., 2010; Minamoto et al., 2009).

A key environmental factor influencing KHV transmission and disease outbreak is water temperature (Gilad, 2003; Gilad et al., 2004; Yuasa et al., 2008). When grown *in vitro*, optimum growth on cell lines is 15-25°C, with no growth or minimal growth at 4, 10, 30, or 37°C. Experimentally infected koi at a water temperature of 23°C resulted in mortality of 95.2%. Disease still progressed rapidly at 28°C but with lower mortality of 89.4%, and mortality at 85% was even seen at 18°C. No mortality was seen of experimentally infected koi at 13°C, however when the fish held at 13°C were shifted up to 23°C over 10 days, a rapid onset of disease and mortality was observed (Gilad, 2003). This relationship between viral replication and water temperature begets a form of seasonality observed with KHV outbreaks. Depending on geographic locale, it is not uncommon to see KHV break in the spring, when water temperatures are increasing and are permissive for viral replication (Uchii et al., 2014).

Diagnosis

There are several methods of diagnosing KHV. An enzyme-linked immunosorbant assay (ELISA) has been developed at University of California Davis which detects circulating antibodies against KHV in the serum of potentially infected fish (St-Hilaire et al., 2009). The development of monoclonal antibodies against immunostimulatory protein ORF68 (Aoki et al., 2011) and capsid protein ORF72 (Tu et al., 2014) have contributed to the development of immunohistochemistry assays for detecting viral proteins in infected tissues. In situ hybridization has been described as a valuable tool in detecting early infections and acute disease in cases where circulating antibodies have not yet been generated. In experimentally infected carp, a signal by in situ hybridization was detected as early as 1 hour post infection from the gills and the gut, with strong signal from many tissues detected at 6-8 hpi (Monaghan et al., 2014a).

Presence and concentration of KHV DNA in koi tissues has been assessed in many different PCR assays. One of the first developed was a PCR assay to distinguish KHV from carp pox herpesvirus (CyHV-1) and channel catfish virus (IcHV-1) (Gilad et al., 2002). Since then, many assays have been developed including methods to amplify regions of the TK gene (Bercovier et al., 2005), DNA polymerase gene and major envelope protein gene (Ishioka et al., 2005). Nested and semi-nested PCR assays were developed to improve the sensitivity of the diagnostic assay (Bergmann et al., 2010b; El-Matbouli et al., 2007b), and are sensitive enough to detect genomic DNA from tissues 70 days after recovery from productive infection. Bergmann and colleagues compared sensitivity of molecular tools used for virus detection and found highest sensitivity with the modified real-time PCR, suggested as the "gold standard", nested PCR and one-tube semi-nested PCR (Bercovier et al., 2005; Bergmann et al., 2010b); these methods can detect very small amount of virus, from 5-10 virions. It was determined that other published or unpublished PCR methods recognized much higher copy numbers and may under diagnose KHV if persistent or latent infections are deemed false negatives.

KHV genome copy numbers can be quantified in a sample by a highly sensitive and specific real-time TaqMan PCR, which was able to detect 10 genome copies in 10^6 host cells (Gilad et al., 2004). The greatest virus concentrations were found in the gill, kidney, and spleen, with high levels found in the mucus, liver, gut, and brain. Survivors of the infection at 64 dpi still showed small quantities of virus particles in gill, kidney, and brain tissues. These methods have recently been compared and assessed for their efficacy in detecting early KHV infections by both lethal and non-lethal methods (Monaghan et al., 2014b); non-lethal methods include sampling of skin mucus or fin base swabs, peripheral blood leukocytes, or droppings. An additional RT-PCR assay was designed to differentiate the replication stage of KHV by primers on the exon junction of the terminase gene mRNA, indicating whether the virus is actively replicating or not, which can be a valuable tool in determining latent or persistent infections from active infections (Yuasa et al., 2012).

Loop-mediated amplification methods have also been employed in the development of assays for the detection of KHV DNA. These tools are coveted at times of an outbreak to get a rapid diagnosis of KHV, and if they are isothermal, they can be performed at pond- or tank-side, or in remote rural areas (Gunimaladevi et al., 2004; Soliman and El-Matbouli, 2005, 2009; Yoshino et al., 2009). There are also current developments to relay the amplified product through an inexpensive lateral flow device in order to encourage testing by non-scientific users such as hatchery managers, pond managers, or veterinarians who may not have extensive training in molecular techniques (Soliman and El-Matbouli, 2010; Vrancken et al., 2013).

Virus isolation from infected tissues has been successful on several cell lines that are both fibroblastic and epithelial in origin, such as koi-fin 1 (KF-1) cells, common carp brain cells (CCB), silver carp fin cells (Tol/FL) and goldfish fin cells (AU), and *C. carpio* gill cells, with less suscebtibility of fathead minnow (FHM) and *Epithelioma Papulosum Cyprini* (EPC) lines (Michel et al., 2010a). EPC cells, FHM, and channel catfish ovary (CCO) cells were found to not be affected by viral implementation even at 2moi (Davidovich et al., 2007). Interestingly, however, confirmed KHV from the outbreak in New York was successfully isolated on FHM cells that have previously been shown to be restrictive to KHV (Grimmett, 2006). Typical CPE of inoculated KHV is observed within 10-14 days post inoculation and is characterized by fusion and retraction of the substrate, vacuole formation, and cell detachment (Hedrick et al., 2000). Devastating losses have spurred the community to develop more tools in order to thoroughly and efficiently diagnose KHV. Recently the development of new cell lines show promise into further elucidating the biology of KHV (Dong et al., 2011; Lin et al., 2013; Zhou et al., 2013).

Virion structure

All herpesviruses are characterized by four morphologic qualities: the core, the tegument, the capsid, and the envelope. KHV has been analyzed by transmission electron microscopy both in infected cultured cells and in cells from naturally occurring infections (Choi et al., 2004; Grimmett, 2006; Hedrick et al., 2000). Virions

are described as either round or hexagonal with a core diameter of approximately 110 nm (Fig. 1.5). KHV virions seem to exist in two forms, intact virion with an envelope layer, and a damaged virion with clear capsid with an electron dense core. Whereas mature virions are observed predominately in the cytoplasm of the infected cell, immature virions are observed in both the nucleus and the cytoplasm (Miwa et al., 2007; Yi et al., 2014). Empty capsids have also been seen in the cytoplasm of an infected cell. The tegument, a proteinacious matrix that is a component of all herpesviruses, appears as thread like structures on the core surface by electron microscopy (Pikarsky et al., 2004). The core with a capsid gains an envelope upon budding from the nucleus into the perinuclear space that then quickly fuses with the outer nuclear membrane. Naked capsids are released into the cytoplasm (Miyazaki et al., 2008), and the final envelope or tegument formation of KHV occurs in the cytoplasm via the host cell trans-golgi membrane, which is consistent with other herpesviruses (Miwa et al., 2007). The enveloped virion with surface viral glycoproteins is 170-200 nm in diameter (Michel et al., 2010a).

Classification

Classification of herpesvirus is based on the virion morphology, genome structure, and location of replication within the cells. With an ever-increasing effort towards sequencing and comparing herpesvirus genomes, three major clades of herpesviruses have emerged as: herpesviruses of mammals, birds, and reptiles; herpesviruses of fish and amphibians; and herpesviruses of invertebrates. In 2007, Aoki et al. proposed that these clades be reclassified as three families: *Herpesviridae*, *Alloherpesviridae*, and *Malacoherpesviridae*, respectively, and that all three families would be part of an order, the *Herpesvirales* (Aoki et al., 2007a; Davison et al., 2009). KHV is phylogenetically similar to other members of the *Alloherpesviridae* family such as cyprinid herpesvirus 1 and 2, ictalurid (channel catfish) herpesvirus 1, and ranid (frog) herpesvirus 1 and 2 (Waltzek et al., 2009). It is now accepted as a member of the *Herpesvirales* order and in the family *Alloherpesviridae* (Waltzek, 2005). *Alloherpesviridae* can be further characterized into four genera: *Batrachovirus,*

Cyprinivirus, Ictalurivirus, and *Salmonivirus*. Based on similarity, KHV belongs to the genus *Cyprinivirus*, alongside other herpesviruses of cyprinids, CyHV-1 and CyHV-2, as well as anguillid herpesvirus 1, a herpesvirus of freshwater eels (Davison et al., 2012).

KHV has at least three main genotypes: one which originated from Japan (strain J) and two others that are more similar to each other, from Israel (strain I) and United States (strain U); therefore, these strains have been classified as Asian (-J) or European (-I and -U) strains of KHV (Avarre et al., 2011; Kurita et al., 2009). These differ from each other at 217 loci; -J differs from -U and -I at 181 loci; -I differs from -J and -U at 32 loci, and -U differs from -I and -J at 4 loci. These differences make on average one nucleotide change every 1.5 kbp. These differences in strain help to identify origin and transmission dynamics of the virus, and also decree implications upon vaccine considerations. Several new genetic strains have been described. Upon phylogenetic analysis of the thymidine kinase (TK) gene of KHV between 14 clinical strains from Indonesia with 12 other KHV strains, 12/14 Indonesia strains did not group with either the Asian or European strain, but clustered together. The Asian lineage has an AT insertion 10 nt upstream from the termination site, causing a frameshift in the TK gene extending it by 24 nucleotides when compared to the European lineage. The original Asian lineage, A1, differs from the proposed Indonesian lineage, A2, by a single nucleotide polymorphism 35 nt downstream from the termination codon (Sunarto et al., 2011). There are reports describing variation in envelope glycoproteins of a Korean strain of KHV that do not group with –I, -U, or –J strain, but each of three glycoprotein genes investigated cluster separately (Han et al., 2013), which may be indicative of a novel genotype, -K. Further work is being done to identify three additional novel strains of cyprinid herpesvirus that may diverge from KHV. These strains displayed significant divergence (95-98%) from known KHV strains in the TK gene, the target gene for a common diagnostic PCR assay, and were not associated with any known outbreak of clinical disease; therefore the authors describe these as non-pathogenic strains of KHV (Engelsma et al., 2013).

Genome

The genome of KHV is linear double-stranded DNA of 295 kb, with a unique region of 251 kb flanked by repeat regions on the terminal ends of 22 kb each (Fig. 1.6), making it the largest herpesvirus genome sequenced to date (Aoki et al., 2007b; Davison et al., 2012; Michel et al., 2010a). The overall GC content is 59.2% G+C. The genome has 156 open reading frames (ORFs); 148 ORFs in the unique region and 8 ORFs in each terminal repeat. Counting the terminal repeat once, the ORF density is 0.57 per kbp compared to the largest human herpesvirus, cytomegalovirus, of 165 genes in the entire genome of 236 kb or 0.70 per kb (Aoki et al., 2007b). Some of these 156 ORFs have homology to other herpesvirus genes; a few have homologues of poxviruses, iridoviruses, and other large DNA viruses (Michel et al., 2010b). Predictions for putative genes have been made for approximately 70 of the ORFs. This includes a number of genes that are conserved between IcHV-1 such as those involved in capsid morphogenesis, structural components of the capsid shell, nucleotide metabolism, DNA replication, and DNA packaging. Other conserved genes are glycoproteins and other proteins with unknown functions. 13 ORFs have conserved sequences with ranid herpesvirus sequences. The fish herpesvirus clade is significantly more divergent than the mammalian herpesvirus clade, with 43 genes inherited from a common ancestor and displays frequent rearrangement, that even though the genes are conserved the order of the genes is not conserved (Aoki et al., 2007b). Forty proteins are involved in the structure of mature virions (Michel et al., 2010b). Of these 40, liquid chromatography and tandem mass spectrometry were used to describe 3 capsid proteins, 13 envelope proteins, 2 tegument proteins, and 22 unclassified proteins associated with the mature virion.

ORF112 of KHV encodes for a novel Zalpha domain (Tome et al., 2013). Zalpha proteins are found in vertebrate animals that detect foreign antigen and activate the interferon response. Poxviruses also encode similar proteins, such as E3L, which function as potent inhibitors of interferon. The product of KHV ORF112 binds CpG repeats in a left-handed conformation and has similar structural folds to E3L gene in

its crystal structure. This novel protein of KHV forms a dimer through a unique domain-swapping mechanism, which is quite different from other Zalpha-domain proteins; hence it is considered a new member of the Z-domain DNA binding family of proteins.

The envelope glycoprotein genes have very little homology to those of other herpesviruses and have generated an interest in further studies of entry into host cells and tissue tropism. ORF81 is thought to be one of the most immunogenic major membrane proteins of KHV (Michel et al., 2010b; Rosenkranz et al., 2008). It is a homologue to ORF59, the major envelope protein of IcHV-1. The pORF81 consists of a 256 amino acids with a molecular mass estimated to be 26 kDa and appears to be non-glycosylated. This protein is predicted to have four transmembrane helices, and the C-terminal domain of the type III membrane protein is very hydrophilic, which is why it has such a high antigenic index and its characterization has greatly opened up the doors of further investigating KHV infection (Rosenkranz et al., 2008).

The strong immunostimulatory effect of pORF81 has prompted the investigation of and its interaction with other KHV and host proteins and its potential development for therapeutic use (Gotesman et al., 2013). Antibody-based purification techniques coupled with mass spectrometry were used to identify proteins targets during acute KHV disease in carp. A total of 78 host proteins were identified that were involved in KHV infection or propagation, which includes members from cytoskeletal proteins, host defense proteins, protein modification enzymes or inhibitors, globins, transferrins, as well as unclassified proteins. Five immunogenic KHV protein targets were identified as glycoprotein pORF150 AMP binding and zinc-finger ring type, major capsid protein ORF92, ORF22, and ORF23 which has an interleukin 10 (IL-10) domain; these may serve as important targets for future therapeutic developments. Additionally, structural proteins and other membrane proteins are being investigated for their use as immunostimulatory antigenic properties towards vaccine development (Fuchs et al., 2014).

IL-10 is an immunomodulatory cytokine that actively suppresses a number of cytokines and chemokines, including tumor necrosis factor alpha, gamma interferon, IL-1β, IL-2, IL-3, IL-6, and MHC II. Many viruses, such as poxviruses, adenoviruses, and herpesviruses evade the host immune system by making viral IL-10 (vIL-10) which have been described for KHV as well as AngHV-1 (van Beurden et al., 2011). The KHV vIL-10, encoded by ORF134, spans 624 bp which contains an 83 bp intron which is translated to a 179 aa product (Aoki et al., 2007a). Transcripts of ORF134 were found to be in high levels in fish tissues during times of initial acute infection and reactivation; however, they were found at lower levels during low-temperature induced persistence (Sunarto et al., 2012). Deletion mutants of ORF134 did not significantly affect viral replication or virulence in vitro or in vivo, suggesting vIL-10 does not contribute significantly to the progression of clinical disease for carp, although the authors note that the protein may be important in other aspects of KHV biology or in circumstances other than those investigated in their study (Ouyang et al., 2013).

To elucidate which genes are involved in disease progression, recombinants of KHV were developed and investigated by infectivity studies on cell culture (Fuchs et al., 2011). Recombinants of KHV were made possessing deletions of viral ribonucleotide reductase, thymidine kinase, dUTPase, or a combination of thymidine kinase and dUTPase genes and their corresponding rescuants. All mutants were capable of replicating in cultured cells, however only mutants of ribonucleotide reductase showed decrease in viral plaques and titers. Although these genes are nonessential for *in vitro* replication of virus, the functions of these genes are still poorly characterized during acute in *vitro* KHV infections. It is also interesting that disrupting the TK gene (encoded by ORF55) by inserting a BAC cassette into ORF55 lead to an attenuated KHV infection (Costes et al., 2008).

Host response and interaction

Once infected with KHV, the carp host responds quickly to mount a defense against the viral intruder. Acute phase proteins have been identified as C-reactive

protein genes (*crp1* and *crp2*), as well as genes from the classical, alternative, and lectin pathways of the complement system (*cirs, c3, bf/c2,* and *masp2*) which were identified as increased in the carp after experimental infection with KHV (Pionnier et al., 2014). When host-proteins were assessed by fish tissue homogenate interacting with KHV glycoprotein (ORF56) on a monoclonal antibody membrane spin column, 78 host proteins were identified which fall in the categories of cytoskeletal proteins, host defense proteins, protein modification enzymes or inhibitors, globins, transferrins, and unclassified (Gotesman et al., 2013). The host interferon (IFN) response has been investigated in regards to infection with KHV. A type I interferon response was observed in carp cells *in vitro* when infected with KHV. Expression kinetics of cell response genes were quantified by RT-qPCR and increases were observed for IFN-a1, IFN-a2, and IFNa1S in cultured head kidney leukocytes but not in CCB cell lines. This indicates an IFN type I response in the head kidney cells, but a potential anti-IFN response may be happening in the CCB cell lines (Adamek et al., 2012). A host interferon type I response is also supported by significant upregulation of the IFN type I pathway TLR9 and TBK1, and IFN regulation factors IRF3 and IRF7. These results are corroborated by *in vivo* studies in which gene expression analysis supports an IFN type I response in the carp; the observed increase in IFN gene expression was generally greater in the skin compared to the head kidney, and the IFN type I response increased with additional viral load (Adamek et al., 2014). A wide microarray analysis of carp gene expression dynamics was performed on two different cell lines: Line K (a more susceptible line) showed changes in 581 genes (330 up-regulated and 251 down-regulated), while line R (a more resistant line) showed changes in only 107 genes (77 up-regulated and 30 down-regulated) (Rakus et al., 2011). These differences in gene expression may highlight immune responses and contribute to vaccine development and therapeutic intervention for KHV disease. Interestingly, an allele in the *C. carpio* MH IIB (*Cyca-DAB1*) showed to be linked to KHV-resistance in carp, which may infer a decrease in the ability of MHC to identify KHV and initiate an immune response (Rakus et al., 2009).

Vaccine Development

Controlling the virus by vaccination has been a recent goal for scientists in order to protect both aquacultured carp and ornamental koi, and much of the vaccine-related research has centered on KHV-resistant strains of carp. Upon developing an ELISA for diagnosing KHV in exposed fish, it was observed that fish with circulating antibodies of KHV were not protected against reactivated infection upon the increase of water temperature and again broke with clinical signs (St-Hilaire et al., 2009). This suggests that the antibodies are not protective and do not confer immunity to exposed fish against reactivation. However, in 2003, a group out of Israel found that sera from some apparently disease-resistant common carp demonstrated high levels of virus-specific antibodies. The attenuated virus that stimulated antibody response in that group of carp has been isolated and has been administered to fish and lead to high levels of protective antibody (Ronen et al., 2003).

These findings lead to the commercialization of an effective KHV vaccine that has been in use in North America. Safety and effectivity studies on this vaccine have proven to be safe for larger fish, with no mortality of fish weighing over 87 grams, but a ~10% mortality was observed when fish weighing less than 87 grams were exposed. When vaccinated fish were exposed to wild type virus challenge, 16.7% of vaccinated fish died, whereas 96.7% of unvaccinated fish died, which indicates a favorable protective efficacy of the vaccine against KHV (Weber III et al., 2014). When vaccinated fish were challenged 13 months post vaccination, a reduced mortality of 36% was observed compared to unvaccinated fish, which showed 100% mortality. Vaccinated fish that died in the study had a significantly prolonged mean survival time than unvaccinated fish at 17 days compared to 11 days (O'Connor et al., 2014). There are downfalls, however, to vaccinating fish every year with a modified-live virus; foremost, the practicality of vaccinating all fish every year as there is negligible horizontal transmission of protection, and secondly, there is no differentiation between vaccinated fish compared to infected fish complications would arise in diagnosing any disease outbreak should it occur among vaccinated fish. In a different direction

towards vaccine development for KHV, a group of researchers from Indonesia started vaccine developments using ORF124, an envelope glycoprotein. Prediction analysis of potential immunostimulatory epitopes of the glycoprotein ORF124 revealed ten epitopes recognizable by host B cells and eleven to fourteen epitopes recognizable by host T cells, which suggests that ORF124 could be an excellent candidate for a subunit vaccine (Murwantoko et al., 2012).

Latency of KHV

As previously discussed, latency is the persistence of the viral genome without the production of viral particles, and excludes the transcription of early or late genes. There are multiple members of the *Alloherpesviridae* for which latency has been identified (Hanson et al., 2011), including cyprinid herpesvirus 1, salmonid herpesvirus 2 (Gou et al., 1991), and ictalurid herpesvirus 1 (Gray et al., 1999). Latency of koi herpesvirus has been an important topic since its first emergence in the late 1990s (Hedrick et al., 2000); latency has major implications for transmission, detection and diagnosis, therapies, prevention, and management of herpesviruses. However very little has been discovered or even investigated to elucidate the mechanisms of KHV latency.

Latency and reactivation have been described for KHV as reappearance of clinical disease in fish that have been experimentally exposed to KHV that initially broke with disease, and then recovered, only to break with disease once again when water temperatures were increased (St-Hilaire et al., 2005). Virus transmission from the reactivated fish to naïve fish was also observed when they were cohabitated, and this reactivation lead to 100% mortality of naïve fish as well as 57% mortality of the reactivated fish. This confirms the ability of KHV to persist in fish tissues and then reactivate, leading to clinical disease as well as the ability of reactivated virus transmission.

Persistence and reactivation has also been described *in vitro*. Viral replication was investigated in cell culture during permissive and non-permissive temperatures. Cultured cells were maintained for 30 days at 30°C, and vacuolization and changes in

cell morphology were observed with multiple plaques seen, demonstrating CPE. Following an increase in temperature above permissive temperatures, the cells recovered to normal morphology and viral plaques disappeared. During this time, transcription of a handful of viral genes was turned off when cells were shifted to a non-permissive temperature. Expression of these viral genes returned after cells were returned to permissive temperatures (Dishon et al., 2007). Another study investigated KHV viral transcription in experimentally infected fish during three different phases of infection: productive, induced persistence, and reactivation. During the period of induced persistence, where fish were kept at 11°C for 24 days, a decrease but not cessation of viral transcription was observed. Once water temperatures were again increased to permissive temperatures, viral transcription was again observed for genes that had previously decreased, and mortality was observed in the reactivated fish. Although some viral transcription has been identified during times of induced persistence when experimentally infected fish were kept at non-permissive temperatures for 28 days (Sunarto et al., 2014), this may not reflect viral transcription during a truly latent infection since viral particles still have been observed 64 days post infection in gill, kidney, and brain tissues of surviving fish (Gilad et al., 2004).

KHV latency has also been confirmed molecularly (Eide et al., 2011a, 2011b). Ornamental koi with no clinical signs of disease were investigated for presence of viral genome by PCR and confirmed by Southern blotting. Genomic DNA from KHV was detectable in peripheral white blood cells of healthy koi without detectable transcription of viral DNA polymerase gene or major capsid gene. The fish were then subjected to physiologic stressors by a rapod increase in water temperature from 12°C to 23°C in at the rate of 1°C per day, and copy number of KHV virions was measured by RT-PCR, which showed peak virus reactivation at 8-12 days post temperature increase in gill and fecal swabs. This confirms that latent KHV can be reactivated and detected under stressful conditions *in vivo*, and that there is consistent genome detection from peripheral white blood cells during latency. White blood cells as the primary site of latency is reminiscent of betaherpesviruses and gammaherpesviruses which also become latent in WBC.

Latency has also been observed in wild carp populations in transient water sources in rural Oregon. Wild carp were captured from flood ponds in the Willamette Valley, Oregon, and blood was collected in 2010 and 2011 from fish with no clinical signs. Status of KHV in these carp was previously unknown. DNA from white blood cells was extracted and genomic DNA was detected by nested PCR KHV in 17/24 (70%) carp investigated (Xu et al., 2012). When analyzed for strain identification, the variant of the wild carp was unique and shared characteristics of both Asian and European strains of KHV. This is an important observation as it may provide insight as to the origin of KHV in these free-ranging wild fish. Because of the variation in genotype, KHV may have already existed in wild common carp before emergence and spread of KHV disease. Similarly, ornamental koi from a local fish supplier were surveyed for presence of KHV genome which revealed 4/8 (50%) ornamental koi were positive. None of these fish showed clinical signs of disease, suggesting that latent KHV infection was present in these fish (Xu et al., 2012).

The molecular program of KHV latency, its establishment, maintenance, or reactivation, has not been investigated. Establishment, maintenance, and reactivation are all under the molecular control of viral gene expression, and in some cases viral protein expression, and their interactions with the host cell. There are many unknowns for the latency program of KHV, but gaining an understanding of latency could facilitate control or elimination of KHV disease in koi and common carp. It can also unveil conserved molecular patterns of latency that can be applied to other herpesviruses leading to novel intervention techniques for devastating diseases in the veterinary and medical field.

Conclusions

KHV is a newly emerged viral disease of common carp and koi carp that causes significant mortality and disease that has devastated the aquaculture industry as well as the ornamental koi industry, killing hundreds of tons of fish and costing tens of millions of dollars in damage since its emergence. Although the size of KHV genome is not typical of previously known herpesviruses, based on its morphology and

location of replication, KHV has been classified as a member of the *Herpesvirales*. Researchers are just starting to understand how this pathogen interacts with host, its molecular biology, and potential mechanisms to control and mitigate infection. This understanding is crucial for anti-viral drug development as well as vaccine development. Researchers are also just beginning to investigate the latent pattern of infection, although there is currently no understanding of the molecular control of establishment, maintenance, or reactivation of latency. Because this herpesvirus is such a distinguished and divergent member of its order, it may be a cornerstone species in understanding the evolution of herpesviruses throughout the taxa. These unique qualities also make KHV a pointed candidate for modeling the molecular biology and therapeutic intervention of disease of other herpesviruses.

Scope of Dissertation

The most remarkable property of a herpesvirus, latency, is by its nature a barricade preventing the host from curing a herpesvirus infection and is the driving force for establishing a lifelong persistence in the host. If we can unlock these barriers, we can intervene by preventing latency from being established, therefore allowing the immune system to clear the infection. Another method of manipulating latency would be to prolong latency indefinitely, preventing reactivation and associated diseases. In order to make headway in the medical community towards eliminating herpesvirus infections indefinitely, there is a need for an appropriate animal model. The ideal natural-host animal model would be long-lived and inexpensive; it would provide repeatable non-lethal access to latently infected cells; it would be translatable to diseases of concern for human medicine as well as the veterinary community. The KHV and koi model fits these criteria.

The studies that comprise this dissertation describe investigations into the molecular program of latency for KHV in its natural host model the koi. In the following chapters, the identification of the specific cell type in which KHV becomes latent in koi; the viral transcripts expressed during latency, and the presence of a viral protein being expressed during latency are described. The findings presented here

have not been described before for a member of the *Alloherpesviridae*. KHV latency in koi may provide a groundbreaking model that can usher in a new season of the elimination of herpesvirus latency.

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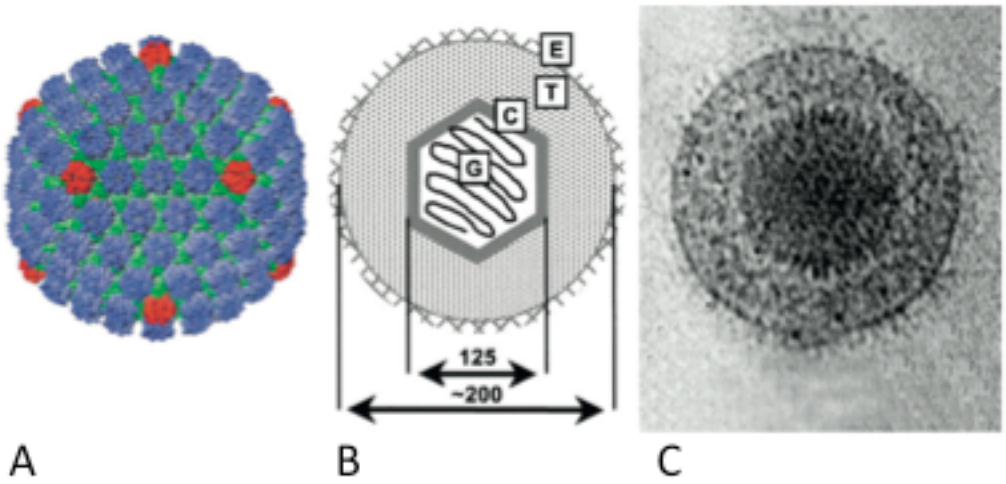

Figure 1.1. Herpesvirus morphology. **A.** Nucleocapsid of HHV-1 showing capsomeres. **B.** Diagram of HSV-1 virion with sizes in nm; (E) envelope, (T) tegument, (C) capsid, and (G) genome. **C.** Transmission electron micrograph of enveloped HHV-1 virion. Used from Rixon et al, 1993.

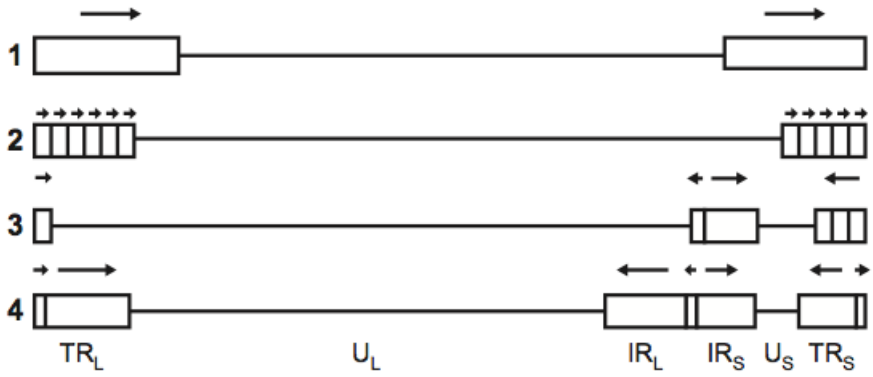

Figure 1.2. Examples of genome structure of different herpesviruses. Repeat sequences are in boxes. U_L is unique long region; U_S is unique short region; IR_L is internal repeat long; IR_S is internal repeat short; TR_L is terminal repeat long; TR_S is terminal repeat short. From Fenner's Veterinary Virology Fourth Ed. Elsevier, 2011. Page 182.

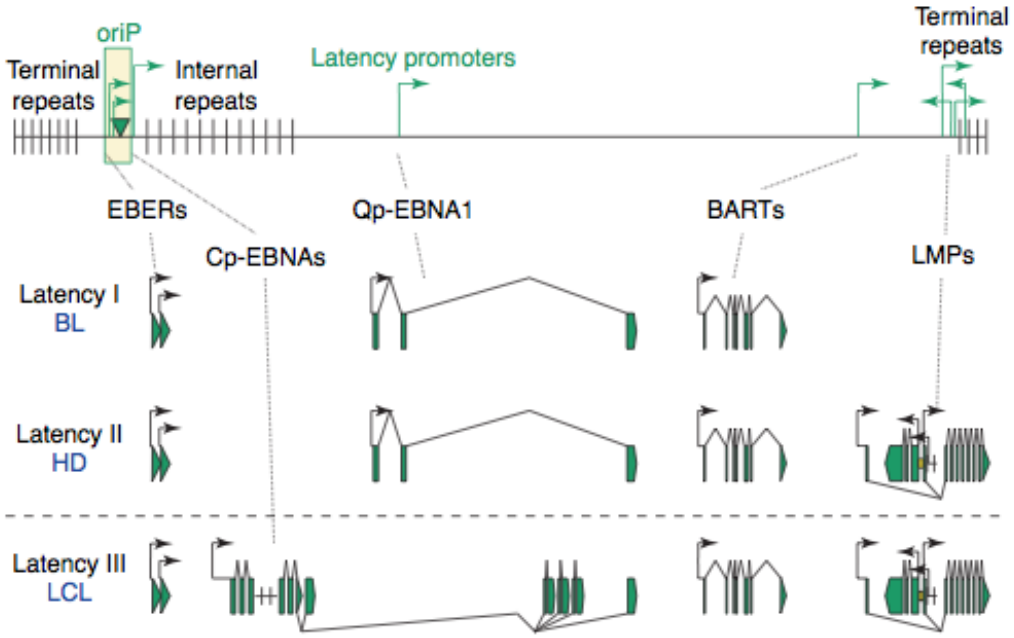

Figure 1.3. Epstein Barr virus latency classes. Linear genome with terminal repeats depicted on top. EBER (EBV-encoded small RNA), Cp (C promoter), Qp (Q promoter), BART (BamHI A rightward transcript), LMP (latent membrane protein), EBNA (Epstein Barr virus nuclear antigen), HD (Hodgkins Disease), LCL (lymphoblastoid cell line), BL (Burkitt's lymphoma), oriP (origin of plasmid replication). From Niller et al, 2004.

Figure 1.4. Clinical signs of koi herpesvirus disease. **A.** Gill ulceration and necrosis (Gotesman 2014); **B.** Enophthalmia (image from koipondguide.com) **C.** Dermal ulceration and hemorrhage, erythema (image from koiphen.com) **D.** Petechiation, nephritis, and death (image from koicrisis.com).

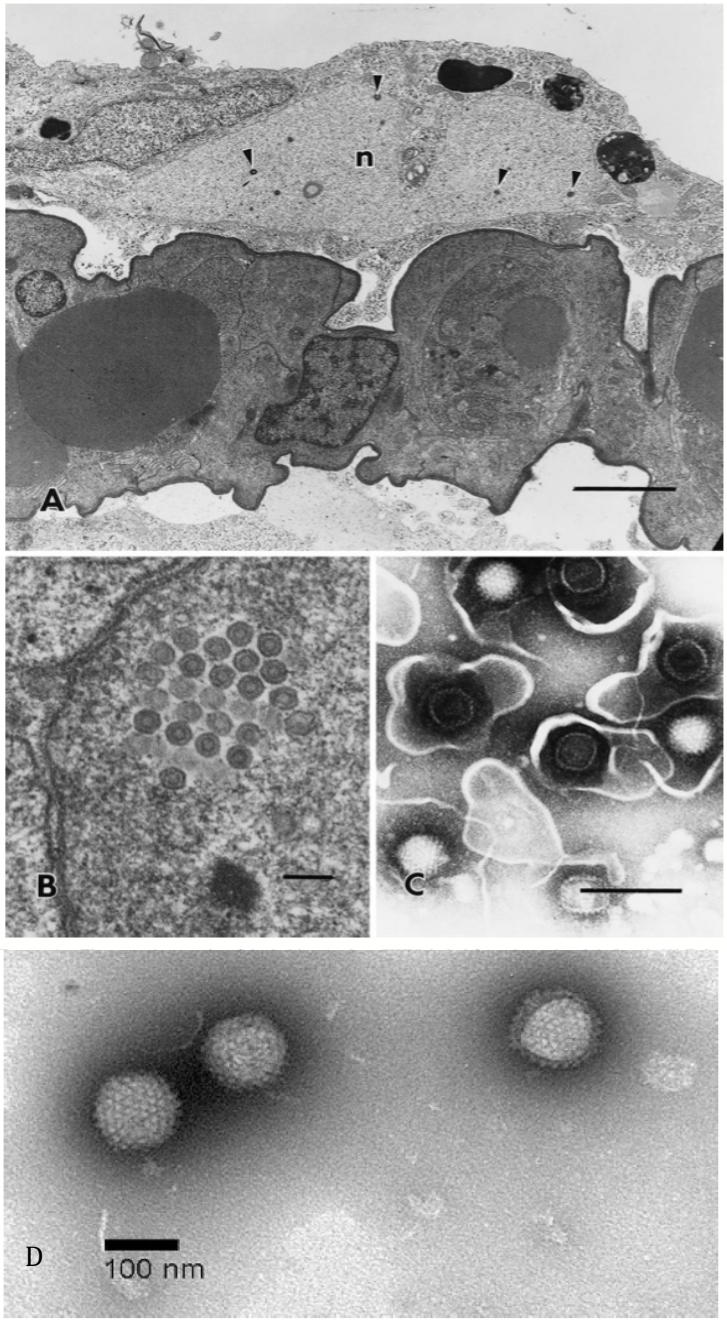

Figure 1.5. Transmission electron microscopy of KHV. **A**. Branchial epithelial cell with virions underneath arrowheads, n = nucleus, bar = 5 um. **B.** Nucleocapsids of KHV, bar is 200 nm. **C.** Negative staining of purified virions from KF-1 cells, bar =200 nm. (A, B, and C from Hedrick et al, 2000). **D.** Naked capsids cultured from infected koi tissues on FHM cells (From Grimmet et al, 2006).

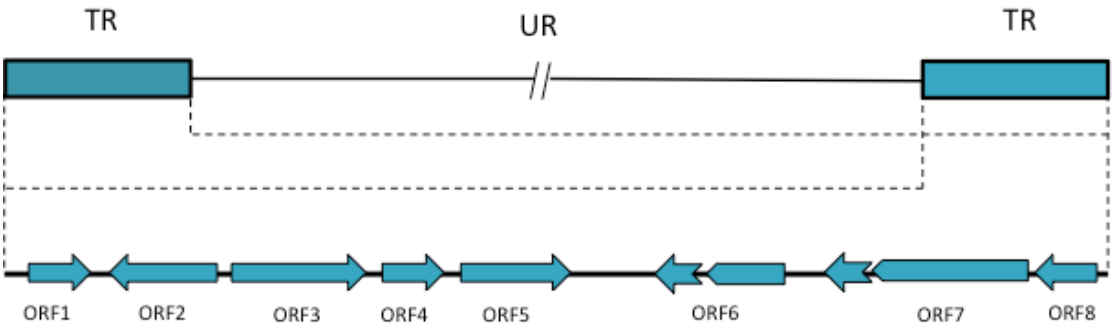

Figure 1.6. KHV genome structure. Total genome length for KHV-U is 295 kb. UR is unique region of 251 kb, contains ORF9 – ORF156. TR is terminal repeat each at 22 kb, contains ORF1 – ORF8 depicted by blown-up diagram, broken arrows depict introns for ORF6 and ORF7.

Chapter 2. Identification of B Cells as a Major Site for Cyprinid Herpesvirus 3 (CyHV-3) Latency

Aimee N. Reed[1,2], Satoko Izume[1], Brian Dolan[1], Scott LaPatra[3], Michael Kent[1,2], Jing Dong[1,] and Ling Jin[1,2]

[1]Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331

[2]Department of Microbiology, College of Science, Oregon State University, Corvallis, OR 97331

[3]Research Division, Clear Springs Foods, Inc, Buhl, ID 83316

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Abstract

Cyprinid herpesvirus 3 (CyHV-3), commonly known as koi herpesvirus (KHV), is a member of the *Alloherpesviridae* and is a recently discovered emerging herpesvirus that is highly pathogenic for koi and common carp. Our previous study demonstrated that CyHV-3 becomes latent in peripheral white blood cells (WBC). In this study, CyHV-3 latency was further investigated in IgM^+ WBC. The presence of the CyHV-3 genome in IgM^+ WBC was about 20-fold greater than in IgM^- WBC. To determine if CyHV-3 expressed genes during latency, transcription from all 8 ORFs in the terminal repeat was investigated in IgM^+ WBC from koi with latent CyHV-3 infection. Only a spliced ORF6 was found to be abundantly expressed in IgM^+ WBC from CyHV-3 latently infected koi. The spliced ORF6 transcript was also detected *in vitro* during productive infection as early as 1 day post-infection. The ORF6 transcript from *in vitro* infection begins at -127 bp upstream of the ATG and ends +188bp downstream of the stop codon, +20 bp downstream of the polyadenylation signal. The hypothetical protein of ORF6 contains a consensus sequence with homology to a conserved domain of EBNA-3B and ICP4 from Epstein Barr Virus and herpes simplexvirus 1, respectively, both members of the *Herpesviridae*. This is the first report of latent CyHV-3 in B cells and identification of gene transcription during latency for a member of the *Alloherpesviridae*.

Importance

This is the first demonstration that a member of the *Alloherpesviridae,* koi herpesvirus (CyHV-3), establishes a latent infection in the B cells of its host, *Cyprinus carpio*. In addition, this is the first report of identification of gene transcription during latency for a member of *Herpesvirales* outside the *Herpesviridae*. This is also the first report that the hypothetical protein of a latent transcript of CyHV-3 contains a consensus sequence with homology to a conserved domain of EBNA-3B from Epstein Barr Virus and ICP4 from Herpes simplex virus 1, which are genes important for latency. These strongly suggest that latency is evolutionally conserved across vertebrates.

Introduction

Cyprinid herpesvirus 3 (CyHV-3), commonly known as koi herpesvirus (KHV), is a recently emerging pathogen of koi and common carp (*Cyprinus carpio*) that causes significant disease and a high mortality in infected fish (Gilad et al. 2002). The most prominent clinical signs from the active viral infection are seen in the gills, characterized by hyperplasia and severe necrosis of the gill epithelium. Other clinical signs include sunken eyes and pale patches on skin, ulcerative skin lesions, lethargy, anorexia, increased respiration, and uncoordinated swimming and movement; the disease is further characterized by interstitial nephritis, splenitis, and enteritis (Gilad et al. 2004). Mortality rates have been reported as high as 80-100% in naïve fry (Iida and Sano 2005; Grimmett et al. 2006; Bondad-Reantaso et al. 2007). Due to the severity of the disease, CyHV-3 has devastated the carp aquaculture population around the world and affected the koi trade, costing hundreds of millions of dollars in damage and loss.

CyHV-3 has been classified as a member of *Alloherpesviridae*, which consists of herpesviruses of fish and amphibians, and is under the order *Herpesvirales* (Waltzek et al. 2005; Davison et al. 2013). Latency, a hallmark of herpesviruses, is the persistence of the viral genome in host cells for the life of the host in the absence of productive infection and viral replication. Reactivation from latency, largely triggered by physiologic host stressors, leads to virion production which produces clinical disease as well as transmission of the virus to naïve hosts. CyHV-3 latency has also been demonstrated in koi that have recovered from an initial viral infection (St-Hilaire et al. 2009; Eide et al. 2011).

Latency has classically been divided into establishment, maintenance, and reactivation phases. Although the mechanism of latency is not fully understood, it has been studied in many different herpesviruses: from animals, such as bovine herpesvirus type 1, equine herpesvirus type 1, and porcine herpesvirus type 1 (Jones et al. 1990; Priola and Stevens 1991; Jones 2003; Cohrs and Gilden 2011); and from humans, such as human herpesvirus type 1 and type 2 (HSV-1 and -2); Varicella

Zoster virus, human cytomegalovirus (CMV), and Epstein-Barr virus (EBV) (Jin and Scherba 1999; Jones 2003; Grinde 2013). In all the herpesviruses studied thus far, latency is characterized by a mostly dormant viral genome and limited gene expression. For alphaherpeviruses, such as HSV-1 and BHV-1 which become latent in the trigeminal ganglion, the only viral gene expressed during latency is the latency associated transcript (LAT) or latency related transcripts (LR) (Stevens et al. 1988; Jones, Delhon et al. 1990; Priola and Stevens 1991; Jin et al. 2000). The LAT of *Simplexvirus,* such as HSV-1 (Zwaagstra et al. 1991) and HSV-2 (Krause et al. 1991), is expressed from the unique long inverted repeats, while the LR of the *Varicellovirus*, such as VZV (Sadzot-Delvaux et al. 1997), BHV-1 (Jones, Delhon et al. 1990) and PrV (Jin and Scherba 1999), is expressed from unique short inverted repeats. For betaherpesvirus latency, such as in human CMV (F. D. Goodrum et al. 2002; Cheung et al. 2006), a few more genes are reportedly expressed, such as the CMV latency transcripts, US 28, vIL10, LUNA, and UL138 loci (Kondo and Mocarski 1995; Kondo et al. 1996). Although, CMV has a relative short inverted repeats, the latency associated UL138 loci (F. Goodrum et al. 2007) is close to the unique long inverted repeats. During gammaherpesvirus latency, in the case of EBV, up to nine virally encoded proteins are expressed in latently infected B cells which include the EBV nuclear antigens (EBNA-1, -2, -3A, -3B, -3C, and -LP) and the latent membrane proteins (LMP-1, -2A, and -2B) (Niedobitek et al. 2000; Speck and Ganem 2010). Most of the EBNA are expressed from the repeat region near the end of the genome. In the case of Kaposi sarcoma herpesvirus (KSHV), the major latency antigen (LANA) (Olsen et al. 2000) is expressed from the direct repeat region 7 (DR7). Genes that are identified during latency can be divided into three functional groups: 1) transcription regulation, such as EBNA, which regulates viral gene expression during latency (Speck and Ganem 2010), 2) anti- apoptosis, such as LAT, which can block apoptosis during virus replication (Perng et al. 2000; Knickelbein et al. 2008), and 3) immune modulation, such as vIL10, which can limit immune detection and mitigate clearance of latently infected cells (Jenkins et al. 2008; Reeves and Sinclair

2010). These genes have important roles in the latency establishment, maintenance, and reactivation.

Although CyHV-3 is classified as a member of *Alloherpesviridae*, the biology of CyHV-3 latency and reactivation is unknown (St-Hilaire et al. 2005; Eide, Miller-Morgan et al. 2011). Understanding how CyHV-3 establishes latency in koi would provide information on the evolution of this seemingly conserved yet complex process. In this study, CyHV-3 latency was further investigated in IgM^+ WBC and IgM^- WBC. To further prove that B cells are the preferred latency site, we investigated whether CyHV-3 transcribes any genes during latency. Since latency associated genes from many herpesviruses are located in the inverted repeats, it is likely that CyHV-3 also expresses latency associated genes from the inverted repeat region. Therefore, we chose to screen the inverted repeat region for genes associated with latency.

Materials and Methods

Koi and sampling. Ten adult koi fish were acquired from a local distributor in Oregon with a history of CyHV-3 exposure. After a 30-day quarantine and acclimation period, fish found to have no clinical signs of CyHV-3 were used in our study. Koi with CyHV-3 latent infection were screened for CyHV-3 by nested PCR as reported previously (Xu et al. 2013). All koi were kept and maintained at 12^{o}C in 4 ft diameter tanks at Oregon State University Salmon Disease Research Lab (OSU-SDL) in accordance with the Animal Care and Use Committee regulations. All blood samples were collected via venipuncture of the caudal vein after anesthetizing the koi with 90 ppm MS-222 Tricaine buffered with an equal amount of sodium bicarbonate. Whole blood was immediately transferred into tubes containing 3.2% sodium citrate to prevent both coagulation and erythrocyte lysis.

CCB and KF-1 cell lines. Both common carp brain cell line (CCB) and koi fin cell line (KF-1) (gift from Dr. Ronald Hedrick, University of California, Davis) were

cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Sigma-Aldrich, Inc., St. Louis, MO) and incubated at 22°C. The United States strain of CyHV-3 (KHV-U) was a gift from Dr. Ronald Hedrick.

Antibodies. Anti-common carp IgM monoclonal antibody was purchased from Aquatic Diagnostics Ltd (Stirling, Scotland, UK). Anti-mouse IgG microbeads were purchased from Miltinyi Biotec (Bergisch Gladbach, Germany). Anti-Pax5 polyclonal antibody specific to the paired domain of trout Pax5 was a gift from Dr. Patty Zwollo (College of William and Mary) (Zwollo et al. 1998). Secondary antibodies used were: DyLight 649 donkey anti-mouse IgG antibody (Thermo Fisher Scientific, Rockford, IL), AlexaFluor 488 goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR), and Texas Red goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR). Nuclear staining was performed with Vectashield Mounting Media with DAPI (Vector Labs, Burlingame, CA).

IgM^+ WBC Isolation. White blood cells (WBC) were collected after layering whole blood on a Ficoll-Paque PLUS gradient according to manufacturer's instructions (GE Healthcare, UK) and washed twice with HBSS. Total WBC were stained first with anti-carp IgM monoclonal antibody (Aquatic Diagnostics Ltd) at 1:100 dilution on ice for 60 min and rinsed twice with HBSS. WBC were then stained with anti-mouse IgG microbeads (Miltinyi Biotec) at a 1:4 dilution at 4°C for 30 min and washed once. Stained WBC were passed through an LS column on a magnet according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The non-selected cells that flowed through the magnetized column were collected and labeled as "IgM^- WBC"; the column was then removed from the magnet and selected cells were washed off the column, collected, and labeled as "IgM^+ WBC".

Flow cytometry and confocal microscopy. Populations of pre-sorted WBC, IgM^+ WBC, and IgM^- WBC were analyzed by fluorescence activated cell sorting (FACS) and confocal microscopy. Each population of cells was fixed with 4%

paraformaldehyde and permeabilized with 0.1% saponin buffer in PBS with 1% BSA. Each population was then stained with primary anti-carp IgM monoclonal antibody and anti-Pax5 polyclonal antibody (from rabbit) at 1:100 dilutions at 4°C for 30 min and rinsed twice with saponin buffer. Cells were then stained with secondary DyLight 649 donkey anti-mouse IgG antibody and AlexaFluor 488 goat anti-rabbit IgG antibody at a 1:500 dilution. A subset of each cell population was stained with only secondary antibodies to serve as a negative control. Stained cells were then analyzed by FACS with the BD Accuri C6 flow cytometer and 20,000 events were recorded for each cell population. Data was analyzed with BD Sampler Analysis software. For visualization by confocal microscopy, cells were stained with secondary Texas Red goat anti-mouse IgG antibody and AlexaFluor 488 goat anti-rabbit IgG antibody at a 1:500 dilution. DAPI was applied to cells before imaging for nucleus visualization. Cells were then examined in Zeiss LSM510 META with Axiovert 200 motorized microscope with with LSM software v3.2. Confocal images were analyzed with ImageJ software v1.46r.

Total DNA and RNA extraction from WBC or CCB cells. Total DNA was extracted from an equal number of cells from IgM^+ and IgM^- WBC using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions. Total RNA was extracted from IgM^+ and IgM^- WBC populations using TRIzol (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Total RNA from *in vitro* infection was extracted from CCB cells infected with 1 moi CyHV-3 and harvested at 1, 3, 5, 8, 13, and 21 days post-infection with TRIzol.

Primers. Selection of primers for CyHV-3 sequence amplification was based on DNA sequences of CyHV-3 (NC_009127.1) available at NCBI. The primers used for screening for the presence of CyHV-3 DNA in koi WBC were selected as previously described (Xu, Bently et al. 2013). Real-time PCR primers specific for CyHV-3 were selected as previously described (Gilad, Yun et al. 2004). The primers and TaqMan probe for *Cyprinus carpio* glucokinase gene were selected as reported

previously (Gilad, Yun et al. 2004). The primers used for screening ORF1-8 gene expression during latency are described in Fig. 2.1 and Table 2.1. The 3' RACE primer and 5' RACE primers are also shown in Fig. 2.1 and Table 2.1.

DNA Probe. ORF6 RT-PCR products amplified with primers ORF6s-F291 and -R291 were cloned into TOPO TA vector (Life Technologies, Carlsbad, CA). The plasmid containing the spliced ORF6 DNA at 310 bp (vs. 396 bp of unspliced DNA) was confirmed by DNA sequencing. The ORF6 plasmid DNA was used as the template to generate the DIG-labeled DNA probe as shown in Fig. 2.1D. The DIG-labeled probe was made by PCR with primers ORF6s-216F and ORF6s-216R using DIG DNA labeling kit according to manufacturer's instructions (Roche Diagnostics, Indianapolis, IN).

PCR Amplification. PCR was performed in a 25 µl reaction consisting of 0.5 unit of Amplitaq Gold 360 and 12.5 ul of 2X buffer (Life Technologies, Carlsbad, CA), 5 µl Q-solution enhancer (Qiagen, Germantown, MD), 400 µM each primer, and 0.5 µg of DNA or 2 ul of RT product. The mixture was subjected to 94 C for 2min, and 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec, followed by a 5 min elongation at 72°C after the final cycle. The nested PCR was ran by using the same conditions as above, the nested set of primers and 2 µl of the PCR product as template. Products were visualized on a 1.2% agarose gel with electrophoresis at 80V and stained with ethidium bromide.

Real-time qPCR. CyHV-3 DNA was quantified in each sample by real-time PCR using primers CyHV-3 86F and CyHV-3 163R and TaqMan probe CyHV-3 109P (Gilad, Yun et al. 2004). Amplification was performed on the BioRad CFX96 thermocycler (BioRad Laboratories, Hercules, CA) using a 25 µl reaction consisting of 12.5 µl Platinum qPCR Supermix-UDG with ROX (Invitrogen, Carlsbad, CA), 0.5 µl of each primer (20nM) and probe (10 mM), and 5 µl (approximately 1 µg total) of DNA template; the reaction was subjected to 50°C for 2 min, 95°C for 2 min, followed by 60 cycles of 95°C for 15 sec and 60°C for 60 sec; a slower ramp time was adjusted to 2°C/sec to facilitate higher sensitivity. Data analysis was performed

using the associated CFX Management Software suite (BioRad Laboratories, Hercules, CA).

RT- PCR. Extracted RNA was treated with DNase (Life Technologies, Carlsbad, CA) according to manufacturer's instructions before using in RT-PCR. cDNA was synthesized with either random primers or gene specific primers using SuperScript III cDNA synthesis kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. As an internal control, an equal amount of RNA was treated in parallel without addition of reverse transcriptase. The cDNA was subsequently amplified in PCR using primers specific for ORF1 to ORF8 listed in Table 2.1. RT-PCR products were visualized on a 1.2% agarose gel with electrophoresis at 80V and stained with ethidium bromide. The 18S ribosomal RNA amplification was performed as internal control to ensure comparable levels of input RNA were used in RT-PCR, according to manufacturer's instructions (QuantumRNA Classic II Universal 18S Internal Standard kit, Ambion, Life Technologies, Carlsbad, CA).

Southern Blot. RT-PCR products generated from RNA of IgM^+ or IgM^- WBC were electrophoresed through a 1.5% agarose gel, transferred to a nylon membrane (Jin, Schnitzlein et al. 2000), and then UV cross-linked to the membrane. The membrane was prehybrizided with prehybridization buffer (Roche Diagnostics, Indianapolis, IN) at 68°C, and then hybridized with the dig-labeled DNA probe specific for ORF6 (Fig. 2.1D) at 68°C overnight. After incubation with the probe, membranes were washed with 0.1% sodium dodecyl sulfate and 10% 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) before incubation with an anti-digoxigenin antibody conjugated with peroxidase. The membrane was then developed by incubation with a chemiluminescent peroxidase substrate (Roche Diagnostics, Indianapolis, IN). The blots were exposed to film (Kodak) at room temperature for 30 min to 2 h. The molecular masses of the resulting bands were estimated by DNA Molecular Weight Marker VII, DIG-labeled (Roche Applied Science, Indianapolis, IN).

3' RACE. The 3' end of the ORF6 transcript was analyzed by the 3' RACE System (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Briefly,

the cDNA synthesis was performed in a 20 µl reaction consisting of 1 µg of DNase-treated RNA isolated from CyHV-3-infected CCB cells was combined with 2 µl 10× PCR buffer, 500 nM adapter primer (AP), 500 µM each dATP, dCTP, dGTP, dTTP, 10 mM DTT, 2.5 mM $MgCl_2$, and incubated at 42°C for 5 min before adding 1 µl SuperScript RT II, then continued to incubate at 42°C for 50 min; the reaction was then terminated at 70°C for 15 min. The resulting poly (dT)-cDNA was treated with RNaseH and incubated at 37°C for 20 min. 2 µl of this cDNA was used to amplify 3'RACE products by PCR as described above, using 200 nM abridged universal amplification primer (AUAP) and 400 µM gene-specific primer ORF6-3RACE112 in the first reaction, and 2 µl of the PCR product was amplified again with the AUAP and semi-nested gene-specific primer ORF6-3RACE94.

5' RACE. The 5' end of the ORF6 transcript was mapped by the 5' RACE System (Life Technologies). First-strand cDNA synthesis was carried out as described above with the gene-specific primer ORF6-5RACE599, and cDNA was treated with RNaseH as described above. cDNA was purified by the protocol adapted to Roche High Pure PCR Product Purification Kit (Roche, Indianapolis, IN); the purified cDNA was then tailed with dCTP by Terminal Deoxynucleotidyl Transferase (TdT) according to manufacturer's instructions. The 5' end of the ORF6 transcript was amplified first with primers ORF6-5RACE455 and AAP (abridged anchor primer) and then with AUAP and semi-nested primer ORF6-5RACE293 (Fig. 2.1D) as described above for 3'RACE products.

PCR DNA Sequencing. PCR products were cleaned with the Charge Switch PCR Clean-up Kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions and submitted for Sanger sequencing at the Center for Genome Research and Bioinformatics (CGRB) at Oregon State University, Corvallis, OR. RT-PCR products were cloned in TOPO TA vector (Invitrogen, Carlsbad, CA), and plasmids were submitted for Sanger sequencing at CGRB. Sequences were aligned to the CyHV-3 genome by using Geneious Software.

Results

Isolation of koi B cells. In a previous study, we tentatively identified the peripheral WBC as the latency site for CyHV-3 (Eide, Miller-Morgan et al. 2011). To determine which leukocyte population harbor latent CyHV-3, the peripheral WBC of koi were further sorted into IgM^+ and IgM^- using magnetic beads to isolate cells labeled with an anti-carp IgM monoclonal antibody. Pooled WBC isolated from whole blood collected from 5 koi (2 ml from each fish) were stained first with antibody and separated via magnetic column (Miltinyi Biotec). On average, about 25 % of total WBC were selected by the magnetic beads. To confirm the selection was specific for IgM^+ B cells, presorted WBC, IgM selected, and non-selected cells were co-stained by antibodies against IgM and transcription factor Pax5, an additional marker specific for B cells (Zwollo et al. 2008), and analyzed by FACS. As shown in Fig. 2.2A, before cells were sorted, the IgM^+ portion of the total WBC population was about 26.8%, the $Pax5^+$ cells were 25.0%, and the portion positive for both IgM and Pax5 was about 19.1% of total WBC (Fig. 2.2A). The FACS analysis confirms that the magnetic column is capable of isolating the majority of IgM^+ WBC. Of the cells that were selected via magnetic column, the portion that was IgM^+ was 96.5%; the portion that was $Pax5^+$ was 94.1%; and the portion that was positive for both IgM and Pax5 was 92.9% (Fig. 2.2B). Of the non-selected cells only 14.0% were IgM+ while 16.4% of cells were Pax5+ (Fig. 2.2C). The vast majority of magnetically selected cells were identified as double-positive for IgM and Pax5 by FACS, which confirms they were an enriched population of B lymphocytes. The co-expression of these two B cell markers in these WBC was also examined by confocal microscopy. In agreement with the FACS analysis, the majority of magnetically selected WBC had IgM staining on the cell surface and Pax5 staining localizing to the nucleus (Fig. 2.3A). In the IgM^+ portion of WBC, a small portion of cells had IgM^+ staining without Pax5 staining. Very few non-selected cells had IgM surface staining and Pax5 staining (Fig. 2.3B), and only a small portion of unsorted cells had IgM and Pax5 staining (Fig. 2.3C).

CyHV-3 genome assessment in IgM^+ B cell and IgM^- WBC. To determine what type of leukocyte is preferentially targeted in CyHV-3 latent infection, real-time PCR was used to compare CyHV-3 genome copy numbers between IgM^+ B cells and IgM^- WBC isolated from latently infected koi. Genome copy numbers from real time PCR were extrapolated from a standard curve established by using 10-fold serial dilutions of CyHV-3 DNA from 10^9 to 10^5 genome copies (Fig. 2.4A). Real-time PCR was performed with an equal amount of DNA from IgM^+ B cells and IgM^- WBC and demonstrated that the presence of CyHV-3 genomes in IgM^+ B cells was about 20 fold more abundant than in IgM^- WBC (Fig. 2.4B, open bar). Comparable levels of *Cyprinus carpio* glucokinase gene were amplified from both IgM^+ and IgM^- populations of WBC when evaluated by real-time PCR, confirming equal amounts of DNA were examined in each sample (Fig. 2.4C, hashed bar).

Viral gene expression in latently infected B cells. To investigate whether CyHV-3 has any gene expression during latency, all 8 open reading frames (ORFs) from the terminal repeat were investigated by RT-PCR (Fig. 2.1 and Table 2.1). Total RNA was extracted from IgM^+ B cells from latently infected koi and used in cDNA synthesis with random primers, which were then used in RT-PCR with primers specific for ORF1-8 (Fig. 2.1B, Table 2.1). Amplification from ORF1-5 and ORF7-8 was not observed (data not shown). However, using primers ORF6s-F291 and ORF6s-R291 flanking the intron of ORF6 (Fig. 2.1D), a spliced transcript was detected in IgM^+ B cells from latently infected koi. To confirm the amplimer is the spliced ORF6 transcript, the RT-PCR product was hybridized with a DNA probe specific for the spliced ORF6 by Southern blot (Fig. 2.1E and Fig. 2.5). In the RT reaction with reverse transcriptase, a spliced product of 310 bp was hybridized by the ORF6 probe (Fig. 2.5, lane 1). In the RT reaction without reverse transcriptase, an unspliced product of 396 bp was also hybridized by the probe, which may derive from the genomic CyHV-3 DNA amplification (Fig. 2.5, lanes 2 and 4). No spliced DNA product was hybridized by the ORF6 probe in the RT-PCR from IgM^- cells (Fig. 2.5, lane 3). Both spliced and unspliced ORF6 transcripts were amplified from total RNA from CyHV-3 infected CCB cells (Fig. 2.5, lane 7), however only unspliced

product was amplified from RNA from CyHV-3 infected CCB cells without the addition of reverse transcriptase, which may also come from the genomic DNA or the unspliced transcript (Fig. 2.5, lane 6). Since the spliced product is only about 86 bp shorter than the unspliced product, they are too close to be clearly separated on the gel (Fig. 2.5, lane 7). The unspliced DNA amplification control is KHV-U DNA (Fig. 2.5, lane 5). No product was amplified from the control reaction containing no RNA template (Fig. 2.5 lane 8).

Expression of ORF6 transcripts during productive infection of cultured cells. To determine whether ORF6 is expressed during productive infection, ORF6 transcription was investigated in CyHV-3 infection *in vitro*. Total RNA was extracted from CyHV-3 infected CCB cells or mock infected CCB cells on day 1, 3, 5, 8, 13, and 21 post-infection (p.i.) and reverse transcribed to cDNA using the gene-specific primers ORF6s-R291. Spliced and unspliced ORF6 cDNA was detected via PCR with primers ORF6s-F291 and ORF6s-R291 designed to amplify a 310 bp spliced or a 396 bp unspliced sequence spanning the intron region of the ORF6 (Fig. 2.1D). To confirm specificity of the ORF6 detection, the RT-PCR product was probed by Dig-DNA probe specific for ORF6 (Fig. 2.1E). A 310 bp product, the spliced ORF6, could be detected at 1 day p.i. (Figs. 2.6A and 2.6B, dpi 1). Thereafter, the spliced transcript was detectable in CyHV-3 infected CCB cells to 21 day p.i. (Figs. 2.6A and 2.6B). A 396 bp product, the unspliced ORF6, could be detected at 5 day p.i. and thereafter to 21 day p.i. Both spliced and unspliced transcription increased over the progression of viral infection, with strongest expression at days 13 p.i. (Fig. 2.6A). Comparable 18S RNA amplifications were observed in RNA samples from either CyHV-3 infected cell or mock infected cells examined from different time points (Figs. 2.6C and 2.6F). Amplification did not occur when reverse transcriptase was omitted from the RT reaction (Fig. 2.6A) or when amplification using RNA from mock-infected cells (Fig. 2.6D). No products were hybridized when reverse transcriptase was omitted from the RT reaction (Fig. 2.6B) or when amplification using RNA from mock-infected cells (Fig. 2.6E). Thus, the spliced ORF6 transcript is expressed immediately following infection.

Sequence analysis of the ORF6 RT-PCR products. To determine whether the intron boundary is as predicted by the sequence annotation, the spliced RT-PCR products from CyHV-3 infected CCB cells were sequenced directly by Sanger Sequencing. The RT-PCR product from latently infected IgM^+ B cells (Fig. 2.6) was cloned into TOPO-TA vector and then sequenced by Sanger sequencing. As shown in Fig. 2.7, the 86 bp intron boundary from both active and latent infection was identical to the predicted boundary (Aoki et al. 2007). The amplimers generated from the total RNA of infected CCB and latent IgM^+ B cells were found to have over 99% homology with the corresponding region of the processed ORF6 (Fig. 2.7).

ORF6 predicted aa conserved domains. To investigate the putative function of this predicted 725 aa ORF6, a search of conserved protein domains was performed (Marchler-Bauer et al. 2011). The aa sequence was submitted to the Conserved Domain database (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and queried against the database PRK v6.9, which encompasses 10,885 PSSMs (position-specific scoring matrix) and compounds conserved domain footprints from phage (PHA) proteins. As shown in Fig. 2.8, predicted ORF6 residues 347-472 have homology to the EBNA-3B consensus domain (Epstein-Barr nuclear antigen 3B; PHA03378; multi-domain hit; bit score 41.59; e-value 8.73e-04; pssmid: 223065) between residues 683 and 808. This consensus domain is derived from 3 gammaherpesvirus proteins (Ambinder et al. 1991; Sjoblom et al. 1995; Jiang et al. 2000). The same region of the predicted ORF6 aa sequence (residues 342-468) also showed homology to multi-domains of the herpesvirus transcriptional regulator protein ICP4 consensus domain (PHA03307; multi-domain hit; bit score: 38.23; E-value: 9.14e-03; pssmid: 223039) between residue 103 and 225. This consensus domain is derived from 36 alphaherpesvirus proteins (Lee and Schaffer 1998).

Characterizing the viral ORF6 transcript. To define the 5′ end of ORF6 RNA expressed *in vitro*, 5′ RACE was used. Total RNA isolated from CyHV-3-infected CCB cells at 13 day p.i. was analyzed. Primer 5RACE599, complementary to the DNA sequence 599 bp downstream of the ATG of ORF6, was used for the first-

strand cDNA synthesis (Fig. 2.1D). Primers 5RACE455 and 5RACE293 upstream of primer 5RACE599 were used in PCR with AAP and AUAP, respectively (Fig. 2.1D). No visible PCR product was observed when the primer pair AAP and 5RACE455 was used (Fig. 2.9A, lane 1). However, an approximate 400 bp product could be readily observed when the primary PCR product was re-amplified with AUAP and the semi-nested primer 5RACE293 (Fig. 2.9A, lane 2). Sequencing of the 417 bp product revealed that the ORF6 transcript starts -127 bp upstream of the ATG of ORF6 (Fig. 2.10A).

To define the 3′ end of ORF6 RNA expressed *in vitro*, 3′ RACE was used. Similar to 5' RACE, total RNA isolated from CyHV-3 infected CCB cells at 13 day p.i. was used. An oligo-dT adapter primer was used to synthesize the cDNA. The resulting cDNA was amplified by PCR with abridged universal amplification primer (AUAP) and gene-specific primer 3RACE112. A product close to the predicted size was produced, but a non-specific product was also amplified (Fig. 2.9, lane 1). The primary PCR product was re-amplified with AUAP and a nested gene-specific primer 3RACE94 which amplified a 273 bp product from the 3' end of the transcript (Fig. 2.9B, lane 2). Sequencing of this 273 bp product revealed a 189 nt untranslated region (Brutlag et al.) downstream of the stop codon, and a polyadenylation signal was identified (AATAAA) 20 bp upstream of the 3' end of the transcript (Fig. 2.10B).

Discussion

In this study, CyHV-3 latency was investigated in IgM^+ B cells that were sorted by monoclonal antibodies specific to common carp IgM using a magnetic column (Figs. 2.2-2.4). By real-time PCR, the presence of CyHV-3 in IgM^+ B cells was found to be about 20 fold higher than in IgM^- WBC (Fig. 2.4). This suggests that at least one site of CyHV-3 latency site is in the IgM^+ B cell. There are a variety of B cell subsets in teleost fish. In catfish (*Ictalurid sp*.), three B cell subsets have been identified: IgM^+/IgD^-, IgM^+/IgD^+ and IgM^-/IgD^+ (Edholm et al. 2010), while in

rainbow trout, only two subsets have been described thus far: $IgM^+/IgD^+/IgT^-$ and $IgM^-/IgD^-/IgT^+$ (Zhang et al. 2010). In general, the circulating B cells have been shown to be more abundant in teleost fish compared to humans. In most of the analyzed teleost species, B cells represent an average of ~30-60% of all peripheral blood leukocytes (PBL) (Sunyer 2012). In contrast, the percentage of circulating B cells for humans is only ~2-8% of PBL (de Jong and Zon 2005). In koi, lymphocytes are categorized as small (<8 um), medium (8–10 um), or large (>10 um) cells based on their diameter (Tripathi et al. 2004). The distribution of B cells within the peripheral WBC of common carp has not yet been well characterized. In our study, about 25% of IgM^+ B cells were recovered from the total peripheral WBC, which suggests the IgM^+ B cells may represent only a subset of circulating B cells of koi and common carp. The majority of CyHV-3 genome present during latent infection was detected in the IgM^+ B cells, and very little was detected in the IgM^- WBC population, which is comprised of other B cell subsets, T cells, and other leukocytes (Fig. 2.4).

To confirm the identity of the IgM^+ WBC selected by the MACS MicroBeads as B cells, an antibody specific for a B cell transcription factor was used in our study. Transcription factor Pax5 is a master regulator of B cell development and has been identified in both mammalian and non-mammalian species (Adams et al. 1992) (Zwollo 2011). Pax5 is mostly expressed in the vertebrate B cell lineage (Cobaleda et al. 2007) including teleost species such as rainbow trout, puffer fish, and zebrafish (Pfeffer et al. 1998; Ohtani et al. 2006; Zwollo, Haines et al. 2008). Within the B cell lineage, Pax5 transcripts and their encoded products have been detected in pro-B, pre-B, and mature B cell lines, whereas either very low or undetectable levels were found in the various plasma cell lines studied (Cobaleda, Schebesta et al. 2007). The functional domains of the Pax5 are highly conserved between vertebrate species (Barr et al. 2011). As shown in Figs. 2.2 and 2.3, the polyclonal anti-paired domain antibody ED-1 specific to trout Pax5 can cross-react with Pax5 of koi B cells. Visible Pax5 staining was also observed in some IgM^- cells (Figs. 2.3B and 2.3C), which suggests IgM^- subsets of B cells may exist in koi. The expression of Pax5 was localized to the nucleus as expected for a transcription regulator protein. Our study

demonstrated that Pax5 is also expressed in common carp and can be used to identify B cells of common carp.

In this study, the transcript from ORF6 is shown to be expressed in B cells from latently infected koi. In latently infected fish, only the spliced CyHV-3 ORF6 transcript is detectable (Fig. 2.6). However, as shown in Fig. 2.7, both unspliced and spliced ORF6 transcripts are detectable during productive infection, wherein the unspliced transcripts are more abundant than the spliced ORF6 transcript as the productive infection progresses (Fig. 2.6A, dpi 13 and 21). This is the first study to show that a spliced ORF6 transcript is abundantly expressed during CyHV-3 latency. It is possible that both spliced and unspliced ORF6 transcripts code a protein and play a role in both productive and latent infections whereas the spliced transcript alone is sufficient for latent infection. Future studies will have to address what the role of the translated products of spliced and un-spliced ORF6 have during the infection cycle.

A conserved domain (aa 342-472) identified in the predicted 725 aa of CyHV-3 ORF6 has homology to consensus sequences of EBNA-3B conserved domain (residue 683 to 808) and the N-terminal regulator domain of ICP4 (residue 103 to 225) (Fig. 2.8). EBNA is a family of *Epstein-Barr virus* (EBV)-latent antigens which are only expressed in EBV latently infected B cells. It is interesting to discover that CyHV-3 expresses a gene during latency with a conserved domain with homology to EBNA-3B. During latency, EBV expresses three related nuclear proteins: EBNA-3A, -3B, and -3C, which are hypothesized to function as transcriptional transactivators. Both EBNA-3A and -3C genes are essential for EBV immortalization, but EBNA-3B is dispensable *in vitro* (Tomkinson and Kieff 1992; Tomkinson and Kieff 1992). Since EBNA-3B is not necessary for EBV-induced B-cell immortalization *in vitro*, it is speculated that EBNA-3B is important for successful EBV infection *in vivo*, perhaps by regulating expression of cell genes important for acute or latent EBV infection (Jiang, Cho et al. 2000). It is possible that ORF6 plays a similar role as EBNA-3B during latency of CyHV-3. Additionally, the conserved domain showed homology to N-terminal regulator domain of ICP4, which is also a transcriptional

transactivator and has homologs that can be found in many alphaherpesviruses (Grundy et al. 1989; Fuchs et al. 2000; Afonso et al. 2001; Kenyon et al. 2001; Van Opdenbosch et al. 2012). ICP4 contains discrete functional domains which determine DNA binding, dimerization, nuclear localization, and transcriptional activation (Bates and DeLuca 1998). It is interesting to find that ORF6 contains a conserved domain with homology to the ICP4 transcription activation regulatory domain near the amino terminus (aa 143-210) which are necessary for ICP4 function (Bates and DeLuca 1998). This suggests that the conserved domain in ORF6 may be essential for transcription activation, and ORF6 may function as a transcription activator perhaps by regulating expression of cell genes important for acute or latent CyHV-3 infection.

Based on the transcription regulatory site search by Softberry, many transcription regulatory elements are found to be present in the putative ORF6 promoter as illustrated in Fig. 2.10A and Table 2.2. An E-box binding site is present -403 to -394 nt upstream of the ATG, which normally regulates responses to cell growth and differentiation, apoptosis, and immune response (Carter et al. 1997; Giangrande et al. 2003; Giangrande et al. 2004). There are three activator protein 1 (AP-1) binding sites upstream of the ATG of ORF6, which regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stressors, and bacterial and viral infections (Sanyal et al. 2002; Ameyar et al. 2003). Two serum response elements (SRE), which are binding sites of serum response factor (SRF), were also present in the promoter region. SRF normally regulates the activity of many immediate-early genes, for example c-fos, and thereby participates in cell cycle regulation, apoptosis, cell growth, and cell differentiation (Norman et al. 1988). This suggests that ORF6 expression can be regulated under many conditions, such as cell growth, cell differentiation, apoptosis, immune responses, and bacterial and viral infections. A number of promoter regulatory elements found in HSV-1 LAT promoter were also present in the putative ORF6 promoter (Kenny et al. 1997). These include a cyclic AMP response element binding site (CREB), a CCAAT box, three Sp1 DNA-binding consensus sequences, and two upstream stimulatory factor (USF) binding sites (Kenny, Millhouse et al. 1997). In addition, a glucocorticoid receptor (GR)

binding site is also present in the ORF6 promoter region. It has been shown GR is associated with cortisol-induced reactivation of BHV-1from latency (Frizzo da Silva et al. 2013). Similar to EBV LMP1 promoter regulatory region, a PU box and GCN4 binding site are present in ORF6 putative promoter region. This suggests the ORF6 promoter may be regulated similarly as LAT, LR, and LMP1 during latency. Up to four CCAAT-enhancer-binding protein (C/EBP), or NF-IL6, binding sites are predicted in the ORF6 promoter region. NF-IL6 has roles in regulation of IL-6 and genes involved in acute-phase reaction, inflammation, and hematopoiesis (Akira et al. 1990; Hirano et al. 1990). It will be interesting to know what role NF-IL6 plays in CyHV-3 ORF6 gene expression during latency. Additional transcription binding sites for insulin response (IRE), interferon response (Smith et al.), and oxidative stress (GA-1) were also present in the ORF6 putative promoter region. These suggest the ORF6 gene can be regulated by many host transcription factors involved in cell growth and immune response, and viral transcription factors during infection. It will be interesting to find how each of the regulatory elements function during acute and latent CyHV-3 infection.

In summary, this is the first report of identification of CyHV-3 latency in B cells and gene transcription during latency for a herpesvirus in the *Alloherpesviridae*. The hypothetical protein sequence of ORF6 contains a conserved domain, which has homology to a conserved domain of EBNA-3B and ICP4. These data suggest that ORF6 may have a conserved function similar to EBNA-3B and ICP4, and may play a critical role in understanding the mechanism and evolution of herpesvirus latency.

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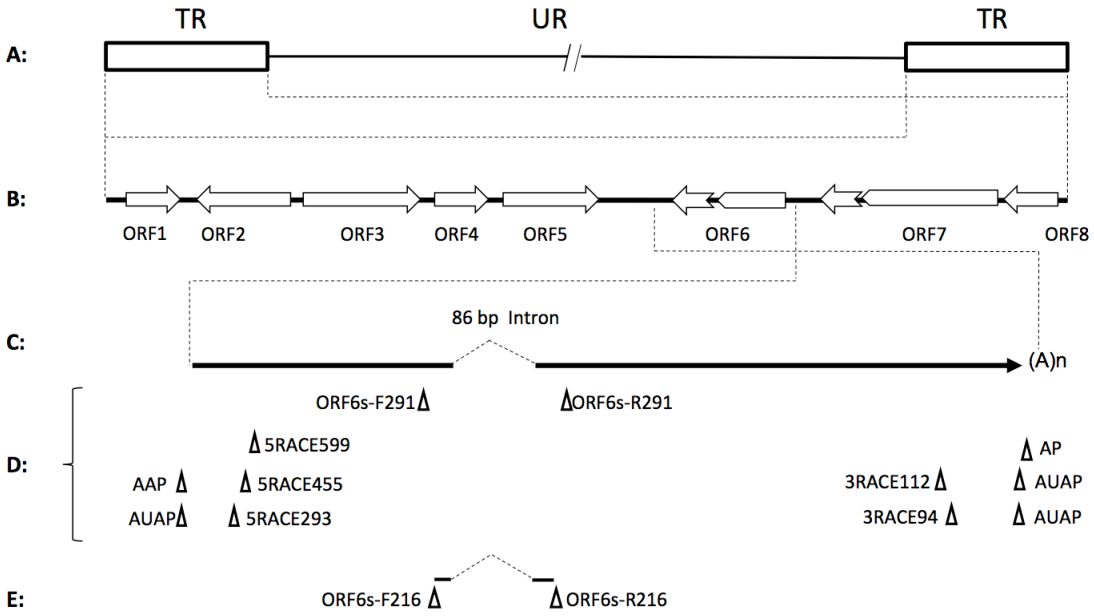

Figure 2.1. Schematic of CyHV-3 genome and location of primers and DNA probes. A: CyHV-3 genomic structure. TR indicates the viral terminal repeats (containing ORF1-8); UR indicates unique regions containing ORF9-156. B: The expanded TR with ORF1-8; broken arrows indicate intron for ORF6 and ORF7. C: The expanded 5'-3' ORF6. The dashed line indicates the 86 bp intron. D: The relative location of the primers used in 5' RACE, 3'RACE, and primers specific for the ORF6 flanking the intron. E: The relative location of the DNA probe specific for the spliced and unspliced ORF6. The expected sizes of the PCR products for the spliced ORF6 are indicated above the bar.

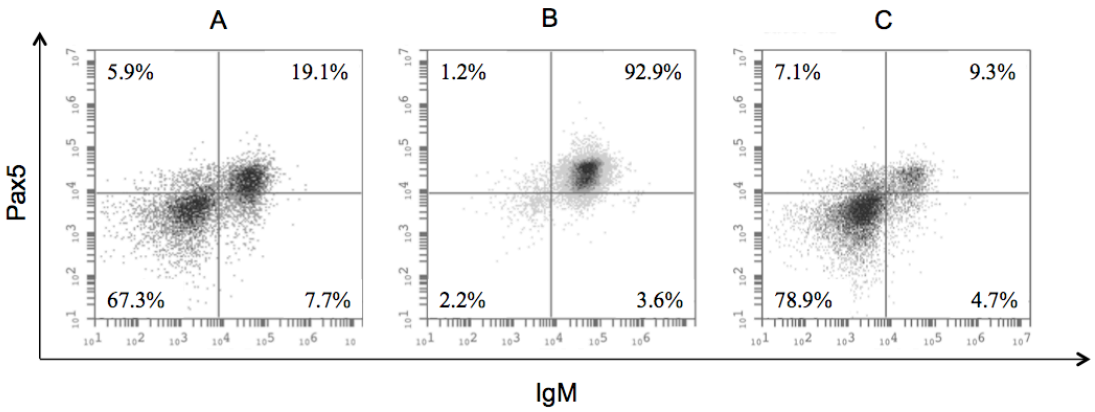

Figure 2.2. Fluorescence-activated cell sorting (FACS) of koi peripheral WBC. After sorting on a magnetic column, cells were stained with mouse anti-carp IgM antibody and polyclonal rabbit anti-Pax5 antibody, followed by secondary goat anti-rabbit IgG antibody which fluoresces at 488 nm (fluorescence intensity measured on Y axis) and secondary donkey anti-mouse IgG antibody which fluoresces at 649 nm (fluorescence intensity measured on X axis). 20,000 events were recorded for each cell population. Panel A: Peripheral WBC before magnetic sorting; panel B: IgM^+ cells selected by magnetic column; panel C: IgM^- non-selected cells that passed through magnetic column.

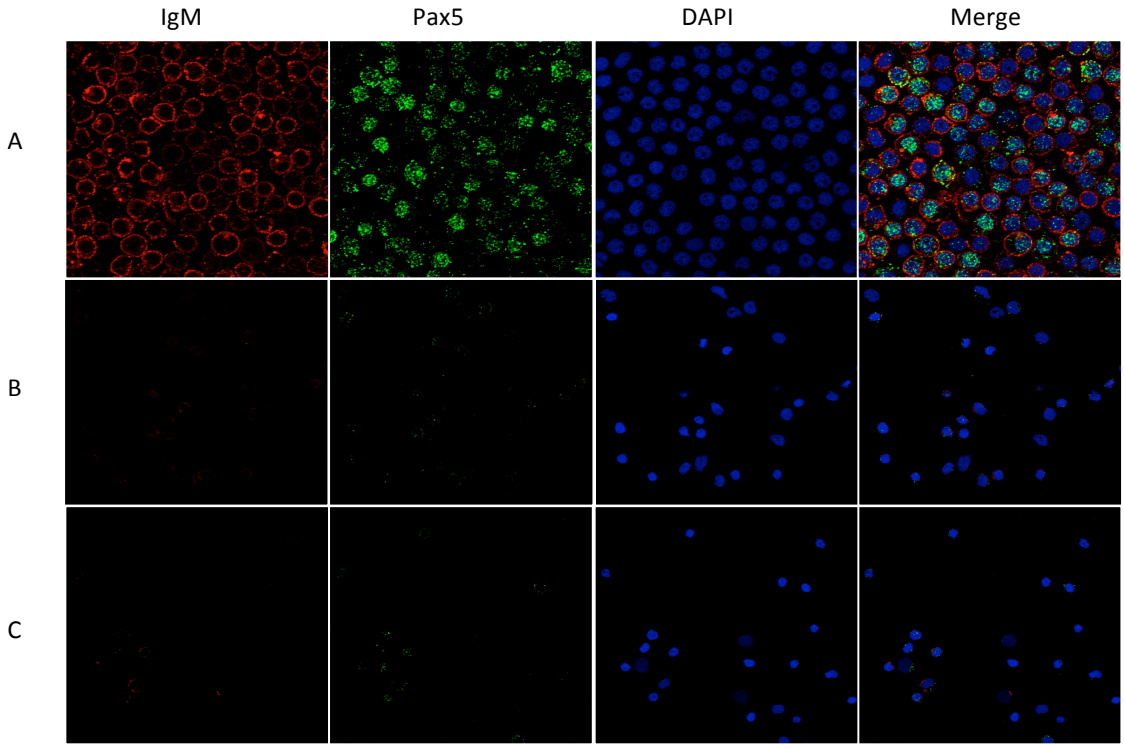

Figure 2.3. Confocal micrographs of koi peripheral WBC. IgM^+ cells identified by anti-carp IgM monoclonal antibody and secondary goat anti-mouse IgG coupled with Texas Red (red); Pax5+ cells identified by anti-Pax5 polyclonal antibody and secondary goat anti-rabbit IgG antibody coupled with AlexaFluor 488 (green); nucleus identified with DAPI (blue); merge shows all three staining. A: IgM^+ WBC selected by magnetic column; B: IgM^- non-selected cells that passed through magnetic column; C: Peripheral WBC before sorting on magnetic column.

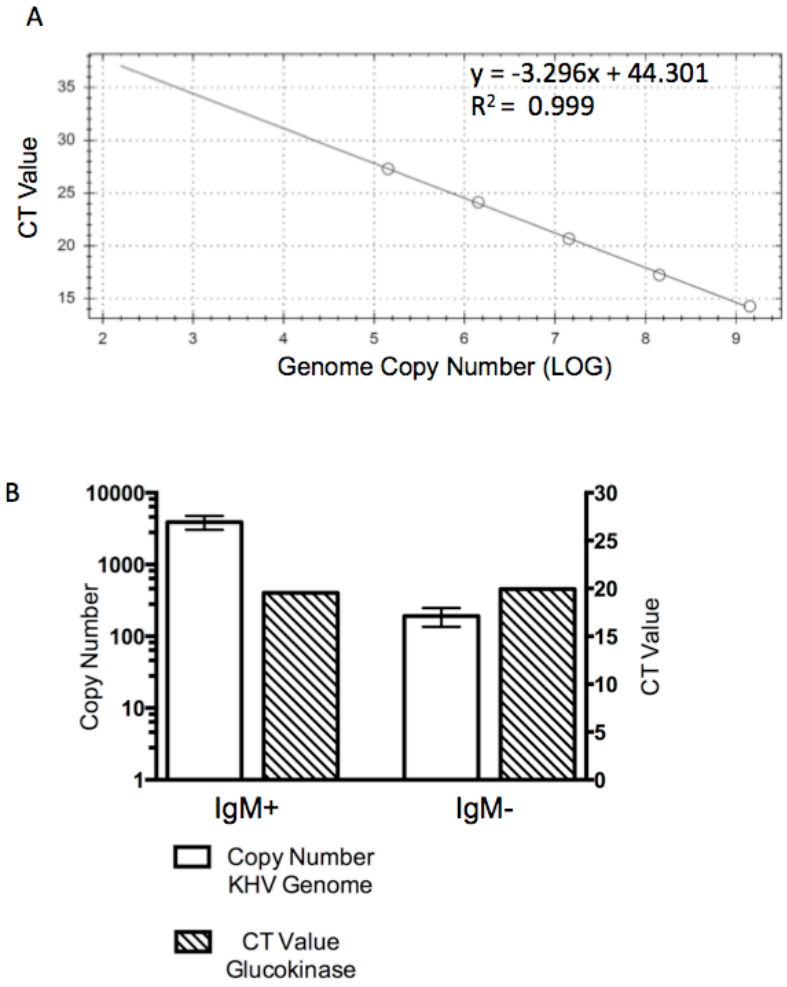

Figure 2.4. Real-time PCR of koi WBC DNA. A: Standard curve using threshold cycles to calculate the analytic sensitivity of CyHV-3 genome equivalents with the real-time TaqMan PCR. B: Open bars are copy number of CyHV-3 genomes detected from 1×10^6 cells of selected (IgM^+, B cells) and non-selected (IgM^-) WBC from CyHV-3 latently infected koi. Mean genome copy numbers: $IgM^+ = 3917.0 \pm 496.0$; $IgM^- = 191.9 \pm 32.4$; n=3. T test, two-tailed p = 0.0017. Hashed bars are cycle threshold (C_T) values for housekeeping gene *Cyprinus carpio* glucokinase from 1×10^6 cells from selected (IgM+, B cells) and non-selected (IgM-) WBC from CyHV-3 latently infected koi. Mean C_T of housekeeping gene *Cyprinus carpio* glucokinase: $IgM^+ = 19.55 \pm 0.055$; IgM^- cells = 19.93 ± 2.78; n=2. T test, two-tailed p = 0.9026.

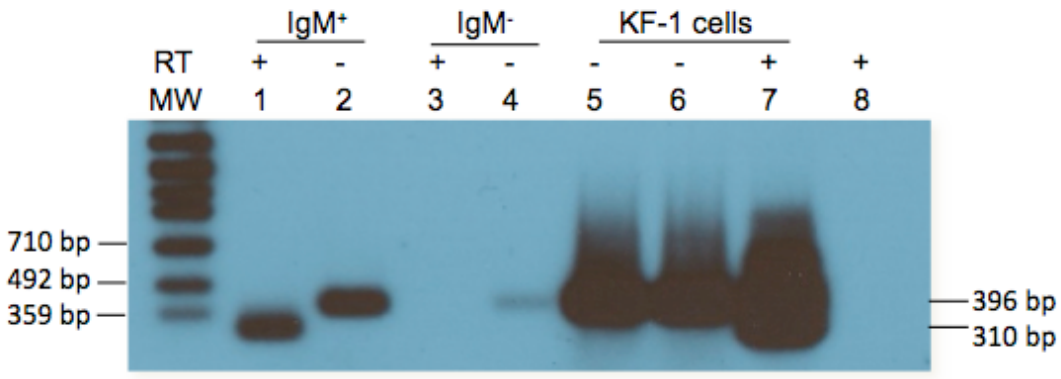

Figure 2.5. Autoradiogram of CyHV-3 ORF6 RT-PCR amplicons. cDNA was synthesized with random primer from total RNA isolated from CyHV-3 latently infected koi: sorted IgM^+ WBC (B cells, lanes 1-2), IgM^- WBC (lanes 3-4) and KF-1 infected (lanes 6-7); lane 5 is the positive control (P), CyHV-3-U DNA. N: negative control without nucleic acid (Lane 8). The "+" or "-" indicates the presence or absence of reverse transcriptase, respectively, in the cDNA synthesis reaction. PCR primers are ORF6s-F291 and ORF6s-R291. Membrane was probed with DIG-DNA probe described in Fig. 2.1E, which is specific for ORF6 and internal to RT-PCR primers. MW: DNA Molecular Weight Marker VII, DIG-labeled (Roche Applied Science).

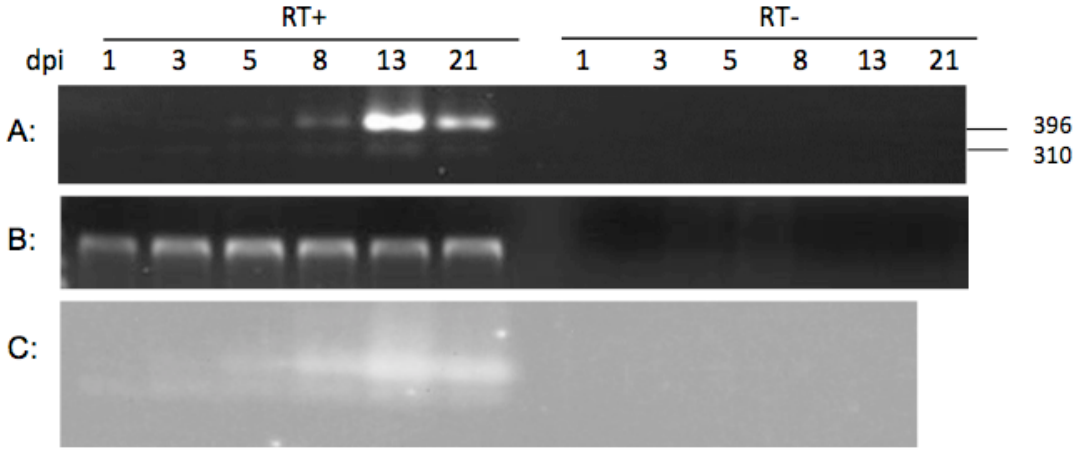

Figure 2.6. Temporal expression of ORF6 during CyHV-3 infection in CCB cells. A&D: RT-PCR using total RNA harvested from infected CCB cells (A) or mock infected cells (D) at 1, 3, 5, 8, 13, and 21 days post infection (dpi), with (RT+) and without (RT-) the presence of reverse transcriptase; The cDNA was synthesized with ORF6 specific primer, ORF6s-R291. B&E: Southern blot of RT-PCR in A (B) and in D (E), probed with Dig-ORF6 DNA probe described in Fig. 2.1E. 310 bp: the spliced product, 396 bp: the unspliced product. C&F: RT-PCR amplification of 18s ribosomal RNA showing 324 bp product from each time point as in A and D. P: CyHV-3-DNA positive control.

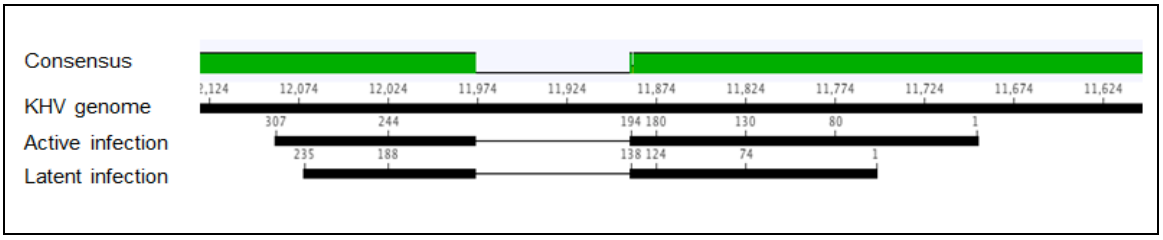

Figure 2.7. Alignment of CyHV-3 lytic and latent ORF6 transcripts with CyHV-3 genome. CyHV-3 Genome with predicted 86 bp intron (11888-11974), NCBI accession no NC_009127.1. A: CyHV-3 genomic DNA (NC_009127.1) . B: Sequence of RT-PCR product (291 bp) from lytic *in vitro* infection of CCB cells using primers ORF6s-F291 and ORF6s-R291. C: Sequence of RT-PCR product (216 bp) from latently infected IgM+ B cells using additional nested primers ORF6s-F216 and ORF6s-R216.

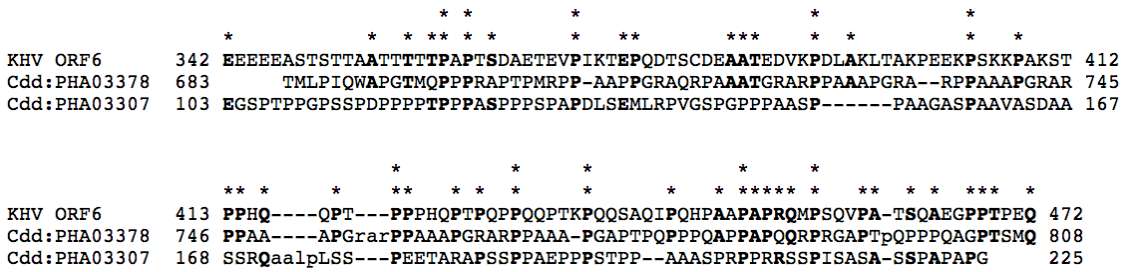

Figure 2.8. Alignment of ORF6 conserved domain with consensus sequences from the Conserved Domain database (cdd; www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). PHA03378 is Epstein-Barr nuclear antigen 3B consensus sequence derived from 3 gammaherpesvirus proteins. PHA03307 is transcriptional regulator ICP4 consensus sequence derived from 36 alphaherpesvirus proteins. Residues in bold are conserved; one star above the ORF6 residue indicates consensus with one referenced domain, and two stars indicate consensus with both referenced domains. Lower case letters indicate unaligned residues; dashes indicate variation in sequence length. The numbers before and after the aa sequence indicate the span of the sequence data that was imported from the complete consensus record (Marchler-Bauer, Lu et al. 2011).

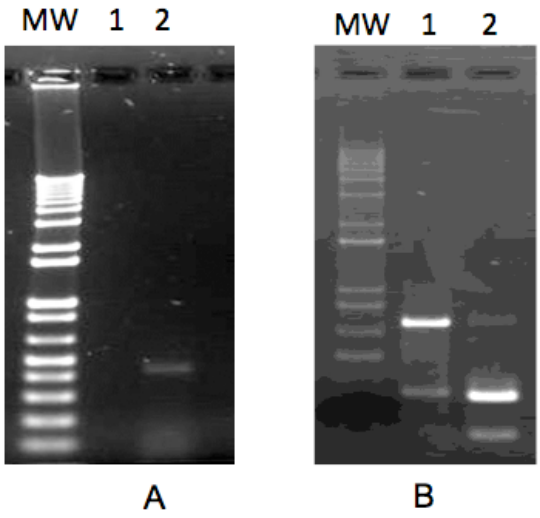

Figure 2.9. ORF6 RACE products generated by using RNA from CyHV-3 infected CCB cells at 13 d p.i. A: The 5′ RACE product amplified with primers ORF6-5RACE455 and AAP (lane 1) and semi-nested primers ORF6-5RACE293 and AUAP (lane 2). B: The 3′ RACE ~290 bp product amplified with primers ORF6-3RACE112 and AUAP (lane 1), and ~270 bp product with semi-nested primers ORF6-3RACE94 and AUAP (lane 2). MW is molecular weight marker (1Kb Plus, Invitrogen).

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5'RACE PRODUCT        ------------------------------------------------------------

KHV ORF6         14316 AATACTTAGACATAGACTATTATGACCAGGGTGTACTATACAGCGTACATGGCCAGGGTG 14257
                                          AP-1
5'RACE PRODUCT        ------------------------------------------------------------

KHV ORF6         14256 TAGCGTACAGTCAGGGGCAATACATGGCCAGGGCATACATGGCGAAGGAGTGAAGTGACT 14197
                                   SRE            SRE
5'RACE PRODUCT        ------------------------------------------------------------

KHV ORF6         14196 AGATTAGATTACGAGGAAAGGGTGACTCGATTACGAGGAAAAAGGTGTGACTGTTGCCAC 14137
                        ATCT                              PU BOX   E-BOX
5'RACE PRODUCT        ------------------------------------------------------------

KHV ORF6         14136 TGATTGCGACACTGACCGTTAATGACGCATTTAACGGCGATTACGAGGAAAGGATGACTA 14077
                         C/EBP              USF II                          CCAAT AP-1
5'RACE PRODUCT        ------------------------------------------------------------

KHV ORF6         14076 ATAAAGGGTGTGACTGTTGCCACTGATTGCGACACTGACCGTTAATGACGCATTTAACGC 14017
                       GCN4                        C/EBP              USF II
5'RACE PRODUCT        ------------------------------------------------------------

KHV ORF6         14016 CTATTGATGTGGTAGCATTGTGGCAATAAGGGAGGGTCTGACGTCAAAGAGGAGGGTTTT 13957
                                    C/EBP      SP1       CREB   SP1  C/EBP
5'RACE PRODUCT        ------------------------------------------------------------

KHV ORF6         13956 GAAGGCGAAGAGGGAGGATCAGTTTCGGGTTGTGGTGGGCGGCGCTGCCCTTGATAAGGG 13897
                                      GR    ICS        SP1
5'RACE PRODUCT       1 ---------------CTCTCAATTTCTGACACTTTTGCGCGAGGGTGAAACTCTGTCCGT    45
                                       ||||||||||||||||||||||||||||||||||||||||||||
KHV ORF6         13896 CGTCGAGCGTCGTACCTCTCAATTTCTGACACTTTTGCGCGAGGGTGAAACTCTGTCCGT 13837

5'RACE PRODUCT      46 TTGTACAACGAGAGTCATTGACATACTCTGTCTTTTTTTACTTTCTCGCCTTGTCTCTTC   105
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
KHV ORF6         13836 TTGTACAACGAGAGTCATTGACATACTCTGTCTTTTTTTACTTTCTCGCCTTGTCTCTTC 13777
                             AP-1                TBP    GA-1    IRE
5'RACE PRODUCT     106 TCTCAACCTCAACACTTGAGCCATGCGTTTCTACGTGCACTCGTACGAGGGTGATGCCTT   165
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
KHV ORF6         13776 TCTCAACCTCAACACTTGAGCCATGCGTTTCTACGTGCACTCGTACGAGGGTGATGCCTT 13717

5'RACE PRODUCT     166 CTGGATCAAACCTCGCAAGAATGGTGACTGGGAATGGAAGAAGTTCAGCGCTGGTGCCAG   225
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
KHV ORF6         13716 CTGGATCAAACCTCGCAAGAATGGTGACTGGGAATGGAAGAAGTTCAGCGCTGGTGCCAG 13657

5'RACE PRODUCT     226 ACACAGGAGCACGGTAAAGAGTTTTGGTCCTAAACTTGCAAACCCTAGAAAGGCTTTCAA   285
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
KHV ORF6         13656 ACACAGGAGCACGGTAAAGAGTTTTGGTCCTAAACTTGCAAACCCTAGAAAGGCTTTCAA 13597

5'RACE PRODUCT     286 CGATTCGAAGAATGACATTGAGTGTTATCATGTGACGTGTGGAAACAGTGGTTATTTGAT   344
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
KHV ORF6         13596 CGATTCGAAGAATGACATTGAGTGTTATCATGTGACGTGTGGAAACAGTGGTTATTTGAT 13537

5'RACE PRODUCT     345 TAGAAAGAGACTGACTGA------------------------------------------   362
                       ||||||||||||||||||
KHV ORF6         13536 TAGAAAGAGACTGACTGATGGGTGGTGGTGTGCTAAGAACGCTGAGGGTACTCCGTACCA 13477
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Figure 2.10. Sequence alignment of the ORF6 transcript ends with CyHV-3 Genome at ORF6 (NCBI ref seq NC_009127.1); the numbers before and after the sequence indicate the span of the genome that was imported from the complete consensus record. A. 5' RACE product mapped to CyHV-3 genome; numbers before and after the sequence indicate length of the PCR product; start codon ATG is in bold; underlined bases are predicted binding sites in putative promoter region and can be cross-referenced in Table 2.2. B. 3' RACE product mapped to CyHV-3 genome; numbers before and after the sequence indicate length of the PCR product; stop codon TAA is in bold; underlined bases AATAAA are predicted polyadenylation signal. Alignments performed using Geneious Software.

Table 2.1 Primer pairs used to detect KHV gene transcription and mapping. F/R is forward or reverse primer.

Assay	Primer Name	KHV Target	5'-Primer sequence- 3'
RT-PCR	ORF1-F340	ORF1	TGAGCACAGGACTGCTGATT
	ORF1-R430		AGGGACCAGGGTCTTCATCT
	ORF2-F377	ORF2	ATCATGGTCTGGCTGAGGAC
	ORF2-R377		GAGATATCCCCTGCCACTGA
	ORF3-F433	ORF3	AGCTGCTGAGAAAGCTGAGG
	ORF3-R433		CCAGGTGCAGAGTTGTCAGA
	ORF4-F399	ORF4	TGGTTCCTATGGCTGGGTAG
	ORF4-R399		CCGTCTCTGGGATGAGTGTT
	ORF5-F365	ORF5	TCAGCAGGACCAGGACTCTT
	ORF5-R365		ACAGCTCGTCGTACACGTTG
	ORF6s-F291	ORF6	GACCCAGGGGACAGCTCTAT
	ORF6s-R291		AGTGGTACAAGTGGCGCTTC
	ORF7s-F316	ORF7	CATCACTCAGCTGTGCCACT
	ORF7s-R316		CGCAAGAGAGCAGTGATGAA
	ORF8-F360	ORF8	TAAGACGTCGTGCTGTCAGG
	ORF8-R360		CAGGCTGAAGTGTGAGGTGA
Southern Blot	ORF6s-F216	ORF6	CAGCAGACTGAGACGCTGAA
	ORF6s-R216		TGCACCATGGACAGACAGAT
RACE	ORF6-3RACE112	ORF6	AAGAAGCACAGGAGCGAGAG
	ORF6-3RACE94		AGGGAGAGGAGCGAGAAGAG
	ORF6-5RACE599		GTGAACTGCACCCAGTCAAA
	ORF6-5RACE455		GTGCCCAGCTCAAACTTCTC
	ORF6-5RACE293		GCAACAGCGTGTCTCTGGTA

Table 2.2 Predicted binding sites in putative promoter of ORF6 as labeled on Fig. 2.10. Mapped location is number of nucleotides upstream from ATG start site. Sequence is 5' to 3'. Length is in nucleotides.

Fig. Label	Sequence	Location from ATG			Name	Length
AP1	GATGACTAAT	-330	to	-321	Activator protein 1	10
AP1	AGAGTCA	-72	to	-66	Activator protein 1	7
AP1	ATGACCAGG	-541	to	-533	Activator protein 1	9
ATCT	TAGATTAGAT	-443	to	-434	ATCT motif	10
C/EBP	TGTGGCAAT	-244	to	-236	CCAAT-box enhancer binding protein	9
C/EBP	GAGGAGGG	-214	to	-207	CCAAT-box enhancer binding protein	8
C/EBP	ATTGCGACA	-380	to	-372	CCAAT-box enhancer binding protein	9
C/EBP	ATTGCGACA	-297	to	-289	CCAAT-box enhancer binding protein	9
CCAAT	GGATGACTAATAAAG	-331	to	-317	CCAAT box	15
CREB	TGACGTCA	-224	to	-217	cAMP response element	8
E BOX	AAAAGGTGTG	-403	to	-394	E-box	10
GA-1	TTACTTTCT	-45	to	-37	GA-1	9
GCN4	ATGACTAAT	-329	to	-321	GNC4 recognition site	9
GR	CAGTTTCGGGTTGTGG	-183	to	-168	Glucocorticoid receptor	16
ICS	AGTTTCGGGTTG	-182	to	-171	Interferon consensus sequence	12
IRE	TACTTTCTCGCCT	-44	to	-32	Insulin response element	13
PU BOX	ACGAGGAAAA	-410	to	-401	PU box	10
SP1	TGGGCGGCGCTG	-167	to	-156	Specificity-protein 1	12
SP1	AAGGGAGGGT	-235	to	-226	Specificity-protein 1	10
SP1	AAGAGGAGGG	-216	to	-207	Specificity-protein 1	10
SRE	GCAATACATGGC	-486	to	-475	Serum response element	12
SRE	GGCATACATGGC	-471	to	-460	Serum response element	12
TBP	CTGTCTTTTTTTACTTT	-55	to	-39	Tata-box binding protein	17
USF II	TGACGCA	-360	to	-354	Upstream stimulatory factor	7
USF II	TGACGCA	-277	to	-271	Upstream stimulatory factor	7

Chapter 3. Transcriptome analysis of latent KHV from koi cells obtained by a nanoflare probe specific for KHV ORF6 RNA

Aimee N. Reed[1,2], Timothy Putman[2], Christopher Sullivan[3], Ling Jin[1,2]

[1]Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331

[2]Department of Microbiology, College of Science, Oregon State University, Corvallis, OR 97331

[3]Center for Genome Research and Biocomputing, Oregon State University, Corvallis, OR 97331

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Abstract

Cyprinid herpesvirus 3, also known as koi herpesvirus (KHV), is an important and deadly pathogen of koi and common carp, *Cyprinus carpio*. Acute infection can cause up to 100% mortality in exposed fish, and fish that survive become latently infected, harboring the viral genome in B lymphocytes that can reactivate under stressful conditions. During latency, KHV ORF6 transcripts can be detected in peripheral B lymphocytes from latently infected koi. In this study, a nanoflare probe was generated specific to ORF6 RNA and used to separate live KHV latently infected cells from total peripheral white blood cells. Using the nanoflare ORF6 probe, about 1% of peripheral WBC from latently infected koi were identified and collected by the ORF6-specific nanoflare probe. When this enriched population of KHV^{+} latently infected cells was examined by RNA-seq, the ORF6 transcript was found to be the only viral transcript that consistently mapped to the KHV reference genome. This study demonstrated that a nanoflare RNA probe could be used to enrich latently infected cells, which can subsequently be used to characterize gene expression during KHV latency.

Importance

This is the first demonstration of isolating an enriched live population of herpesvirus latently infected cells based on the latency-associated transcript. Live peripheral WBC were non-lethally collected and separated into KHV+ and KHV- populations based on the KHV latency-associated transcript ORF6. This study investigated the genome-wide viral gene expression of a latent herpesvirus and detected expression from one gene, ORF6.

Introduction

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV) is a recently emerged pathogen of koi and common carp, *Cyprinus carpio*, that is spread worldwide and belongs to the family *Alloherpesviridae,* which includes herpesviruses of fish and amphibians (Davison et al., 2009; Waltzek, 2005). The severity of clinical signs, such as gill necrosis, nephritis, and dermal ulcers, leads to a mortality rate of up to 80-100%, and is the cause of millions of dollars in loss and damage to the aquaculture industry as well as the ornamental koi trade (Bondad-Reantaso et al., 2005; Gilad et al., 2002; Hedrick et al., 2000). Fish that do not succumb to a fatal infection from KHV and recover become lifelong carriers of the herpesvirus, which exists in the host cells with a persistent pattern of infection that is latency (St-Hilaire et al., 2005). During herpesvirus latency there is no production of virus particles and the viral genome alone persists inside the host cell with limited gene transcription. Upon physiologic stress, however, the latent viral genome can enter productive replication and begin making infectious particles. Reactivation of latent KHV can lead to transmission to naïve hosts resulting in up to 100% mortality in naïve hosts as well as over 50% mortality in reactivated hosts (Eide et al., 2011; St-Hilaire et al., 2005).

Latency of herpesviruses is under a strict regulatory molecular program and has been classically divided into three phases: establishment, maintenance, and reactivation (Jones, 2003). Genes that are expressed by the virus during latency are involved in managing the latent infection within the host cell, and their functions typically fall into three categories: 1) transcription regulation, such as EBNA of EBV which regulates viral gene expression during latency (Speck and Ganem, 2010), 2) anti-apoptosis, such as LAT of HSV-1 which can block apoptosis of infected cells during virus replication (Jones, 2003; Perng et al., 1994), and 3) immune modulation, such as vIL10 of HCMV which can limit immune detection of the virus and mitigate clearance of latently infected cells (Jenkins et al., 2004; Sinclair and Reeves, 2013). The investigation into gene expression during latency is critical to understanding the molecular control of latency. Recently, efforts to interfere with the herpesvirus

infections have focused on latency associated transcripts to direct therapeutic intervention of disease caused by acute and reactivated herpesvirus infections (Moerdyk-Schauwecker et al., 2009).

To understand how latency is regulated, it is critical to know what genes are expressed during the latent infection. During herpesvirus latency, very few cells harbor the latent herpesvirus genome. In humans, the latent HSV-1 genome resides in latently infected neurons with an infection frequency that ranges from 300 to 10,000 copies per 100,000 cells from the trigeminal ganglia (Cohrs et al., 2000) which can only be collected post-mortem. It is challenging to profile viral gene expression in tissues where the latency occurs in such a small portion of cells. The lytic transcriptome of HSV-1 and HSV-2, as well as anguillid herpesvirus 1, another member of the *Alloherpesviridae*, have been profiled with relative ease due to the abundance of productive viral transcription during lytic infections (Aguilar et al., 2006; van Beurden et al., 2012). The latent herpesvirus transcriptome, however, has not yet been investigated for many herpesviruses.

Our previous studies discovered that ORF6 mRNA is expressed in the IgM+ B cells from koi latently infected with KHV (Reed et al., 2014). In this study, a nanoflare probe is designed to specifically target ORF6 mRNA and used to separate KHV+ cells from peripheral WBC of latently infected koi. The viral gene expression profile from this enriched population of $ORF6^{+}$ cells was further examined by RNA-seq.

Materials and Methods

Koi and sampling. Ten adult koi fish were acquired in July 2011 from a local distributor in Corvallis, Oregon with a history of KHV exposure. Koi were screened for KHV by nested PCR as reported previously (Xu et al., 2012). Nine adult koi were generously donated by a distributor in Eugene, Oregon in March 2014 with no history of KHV exposure and were negative for KHV antibodies when tested by ELISA upon receipt to the OSU-SDL. Koi from each distributor were kept separately and maintained at 12°C in 4-foot diameter tanks at Oregon State University Salmon

Disease Research Lab (OSU-SDL) in accordance with the Animal Care and Use Committee regulations. No fish in this study have had any clinical signs since their arrival at the OSU-SDL. Koi were anesthetized with MS-222 at 90 ppm buffered with an equal amount of sodium bicarbonate and phlebotomized by venipuncture of the caudal tail vein then recovered. 2 ml whole blood was collected into tubes with 3.2% sodium citrate anticoagulant.

WBC isolation. Peripheral WBC were isolated on a gradient of Ficoll-Paque PLUS (GE Healthcare). Briefly, 2 ml of anticoagulated whole blood was diluted with an equal amount of HBSS and gently layered onto 3 ml Ficoll-Paque in a 15 ml conical tubes. Tubes were centrifuged at 400 × g at 20°C for 40 minutes. WBC formed a visible layer on the Ficoll-Paque which were then removed and washed twice with RMPI with 5% fetal bovine serum.

RNA nanoflare probe design. The internal ORF6 RNA probe ORF6-Cy5 was designed to target a 27 nt region of the KHV ORF6 transcript (5'-CGCACACTCTGACTGCTGAGGAGAAGT-3') (Fig. 3.1A) and used to isolate KHV+ latently infected koi WBC. The RNA-specific flare (SmartFlare by EMD Millipore) (Seferos et al., 2007) is bound to a gold nanoparticle and is initially quenched upon endocytosis; when it competitively binds to the transcript of interest the Cy5 flare is released and subsequently fluoresces (Fig. 3.1). An Uptake-Cy5 probe, which is always fluorescent (Fig. 3.1B), was used to establish the effectiveness of the cells' ability to endocytose the probe. An additional control probe, Scramble-Cy5 probe which is always quenched (Fig. 3.1C), was used to identify background fluorescence from the probe as well as autofluorescence from cells. Additionally, WBC were treated in parallel with no added probe as a negative control. The three lyophilized probes (Uptake-Cy5 probe, Scramble-Cy5 probe, and ORF6-Cy5 probe) were reconstituted according to manufacturer's instructions by slowly adding 50 µl sterile water in a dropwise fashion. Each probe was diluted at 1:20 with PBS then added separately to cells in a 48-well plate at 1 µl per 3×10^5 cells in RMPI media with 5% FBS. Plates were incubated at room temperature in the dark for 20 hours.

Flow cytometry and cell sorting with nanoflare probe ORF6-Cy5. Live cells incubated with nanoflare probes were sorted directly on the BD InFlux cell sorter at Oregon Health and Sciences University (OHSU) Core Flow Lab in Portland, OR. For sorting, WBC were collected from 3 fish with no known KHV exposure (Koi 1-3) and from 3 fish with known KHV exposure (Koi 4-6) and each sample was sorted independently. Cells were excited by a red laser (640 nm) and analyzed by Cy5 fluorescence. To assess probe uptake efficiency, cells with no probe were used to set a gate for the assessment of the cells incubated with Uptake-Cy5 which is always fluorescing. To assess flare signaling and estimate background fluorescence, cells incubated with Scramble-Cy5, which is always quenched, were used to set a gate for sorting cells incubated with ORF6-Cy5, which were then sorted below (Gate A) and above (Gate B) the gate set by the cells incubated with Scramble-Cy5. The ORF6-Cy5 cells of extremely high fluorescence intensity were sorted separately for each sample (Gate C).

Confocal Microscopy. KHV latently infected WBC were incubated with nanoflare probes as described above and nuclear staining was performed with Vectashield Mounting Media with DAPI (Vector Labs, Burlingame, CA). Live cells were then examined on a Zeiss LSM510 META with Axiovert 200 motorized microscope using LSM software v3.2. Confocal images were analyzed with ImageJ software v1.46r.

Real-time Quantitative PCR. Total DNA was extracted from 5,000 cells incubated with ORF6-Cy5 from each Gate A, B, and C with the E.Z.N.A. DNA extraction kit (Omega BioTek) according to manufacturer's instructions and eluted in 40 µl TE buffer. KHV DNA was quantified in each sample by real-time PCR using primers KHV 86F and KHV 163R and TaqMan probe KHV 109P (Gilad et al., 2004). Amplification was performed on the BioRad CFX96 thermocycler (BioRad Laboratories, Hercules, CA) using a 25 µl reaction consisting of 12.5 µl Platinum qPCR Supermix-UDG with ROX (Invitrogen, Carlsbad, CA), 0.5 µl of each primer (20 nM) and probe (10 mM), and 10 µl (approximately 500 ng total) of DNA template; the reaction was subjected to 50°C for 2 min, 95°C for 2 min, followed by

60 cycles of 95°C for 15 sec and 60°C for 60 sec; a slower ramp time was adjusted to 2°C/sec to facilitate higher sensitivity. Data analysis was performed using the associated CFX Management Software suite (BioRad Laboratories, Hercules, CA). A standard curve that was previously established for this assay was used to calculate genome copy numbers for obtained C_T values (Y= -3.296X + 44.301) (Reed et al., 2014). To determine genome copy numbers for the entire 5,000 sample (in 40 µl), values obtained from the 10 µl sample were multiplied by four.

RNA extraction and Illumina library preparation and RNA-seq. Total RNA was isolated with PureLink RNA mini kit from 2×10^5 cells sorted by ORF6-Cy5 probe from Gate C from Koi 1-6 (n = 6) according to manufacturer's instructions (Ambion Life Technologies). RNA was then treated with DNase (Invitrogen) as described previously (Reed et al., 2014) and purified by ethanol precipitation with sodium acetate and glycerol according to manufacturer's instruction (Ambion Life Technologies). Ribosomal RNA was depleted using the Ribo-Zero Magnetic Gold kit (Epicentre) according to manufacturer's instructions. A cDNA library was prepared by using the Smarter Stranded RNA-seq kit (Clontech Laboratories) with random hexamer primers according to manufacturer's instructions, which recommend an optimum final fragment length to be around 300 bases. For RNA-seq, paired-end reads of 100 bases were obtained by the Illumina HiSeq 2000 at the Center for Genome Research and Biocomputing (CGRB, Oregon Sate University, Corvallis Oregon) (Wang et al., 2009).

Qualitative and quantitative analysis of RNA and cDNA library. RNA was analyzed on the NanoDrop spectrophotometer (Thermo Scientific) as well as by the Qubit fluorometer (Life Technologies). qPCR was used with primers for Illumina adapters to quantify the cDNA as well as to assess average size of cDNA fragments in each sample. Each cDNA sample was also analyzed by the Agilent 2100 Bioanalyzer to further obtain electropherograms on a High Sensitivity DNA chip.

Bioinformatics analysis of Illumina reads. A spliced and truncated reference genome was prepared from the CyHV-3 genome (NCBI GenBank Accession

NC_009127.1 GI: 131840030) using Geneious software. The second terminal repeat was eliminated to minimize a bimodal assembly of genes in the terminal repeat regions. Additionally, introns were removed at predicted splicing sites according to the annotated genome from all ORFs with predicted introns (6, 7, 10, 90, 131, 134, 139, and 155) with the exception of ORF33 because the intron sites span multiple other coding regions. Returned reads were parsed by sample Koi 1-6 according to their indexed barcode. Illumina adapters were removed with the cutadapt script using stringency of minimum cut-length 45 bases; reads shorter than 45 bases were removed from analysis. Because of the overall suboptimal quality, the reads were not trimmed based on quality or phred scores. Reads were aligned to the reference KHV genome using Bowtie2 software (Langmead and Salzberg, 2012), and resulting .sam files were converted to .bam files using samtools. Alignment files were visualized by Geneious software v 7.1.5 (Fig. 3.5).

Results

Enriched population of live KHV+ latent WBC sorted by latency associated transcript ORF6. To separate KHV+ latently infected koi WBC, cells were incubated with ORF6-Cy5 and visualized first by confocal microscopy and then sorted by flow cytometry. When viewed by confocal microscopy, a few cells incubated with ORF6-Cy5 displayed fluorescence in the cytoplasm (Fig. 3.2B) whereas no Cy5 signal was detected from cells incubated with the Uptake-Cy5 (Fig. 3.2A). WBC incubated with the Uptake-Cy5 showed a significant increase in mean fluorescence intensity when compared to cells that were not incubated with any probe, demonstrating the ability of koi peripheral WBC to effectively endocytose the gold nanoparticle and be sorted by the Cy5 fluorescent signal (Fig. 3.3A). Cells that were incubated with Scramble-Cy5 demonstrate background signal from the Cy5 fluorophore and autofluorescence (Fig. 3.3B, Gate A). When cells that were incubated with ORF6-Cy5 were sorted and compared to the Scramble-Cy5 cells, the majority of cells showed significant increase in fluorescence (Fig. 3.3B, Gate B). Additionally, a small and separate population of intensely fluorescing cells with significantly increased MFI

was observed in the ORF6-Cy5 cells, which accounted for less than 1% of analyzed cells (Fig. 3.3B, Gate C).

To confirm specificity of ORF6-Cy5 and to its ability to identify KHV+ latently infected cells, DNA from ~5,000 cells from each gate (Gates A, B, and C) was extracted and KHV genome copy numbers were quantified by real-time qPCR (Table 3.1). The amount of detected KHV genomes was most abundant from Gate C in which 1240 genome copies were detected in 5,000 cells; this is about twelve-fold more abundant than the number of genome copies detected in the same number of cells from Gate B (Fig. 3.4). There was no detection of KHV genomes in 5,000 cells from Gate A. This shows that the majority of latent KHV genomes resides in cells from Gate C and confirms the ability of the internal RNA probe ORF6-Cy5 to separate KHV^+ WBC in the latently infected koi.

Qualitative and quantitative analysis of RNA, cDNA, and raw reads. To investigate the transcriptome in KHV+ latently infected WBC, RNA was extracted from 200,000 cells sorted from ORF6-Cy5 Gate C from each fish (n = 6). Qualitative and quantitative analysis of the ribosomal-depleted RNA on the NanoDrop spectrophotometer revealed poor quality (260/280 readings from 1.54-1.84) and very little quantity (7.05 – 15.75 ng/µl, in a total volume of 20 µl). Analysis on the Qubit fluorometer failed to detect sufficient RNA to analyze (minimum required concentration is 10 ng/µl). Despite the poor quality of the total RNA extracted from those sorted cells, cDNA libraries were made from those RNA as templates. An average fragment size in the cDNA library ranged between 365-422 bases; Agilent Bioanalyzer detected cDNA concentrations of 165.2 – 648.0 pg/µl. Also, more than 12 peaks for each sample were observed from corresponding electropherograms (Supplemental Table S3.1). Samples were subsequently sequenced on the Illumina HiSeq 2000. Post-run analysis of reads for fish Koi 1 – Koi 6 (Table 3.2) yielded a range of 3792 to 6463 Mbases from 51,935,260 to 79,018,010 individual paired-end reads of 100 bases each. The mean quality score of reads ranged from 23.36 to 31.87,

with the percent of bases with a quality score over 30 ranged from 61.17% to 80.07% for each sample.

Latency-associated transcript ORF6 identified by RNA-seq. Bioinformatics analysis of reads from each sample Koi 1- Koi 6 (Table 3.2) identified very few reads that mapped to the truncated spliced reference KHV genome, from 47,624 to 230,667, the majority of which mapped to repeated regions within the KHV genome or had higher identity to the *Cyprinus carpio* genome. After visual inspection of each read that mapped, reads that were determined to be artifactually mapped to the KHV genome were disregarded. The resulting reads mapped consistently to ORF6 for every sample. The number of reads from each sample that mapped to ORF6 are 28 reads, 121 reads, 68 reads, 46 reads, 236 reads, and 162 reads for Koi 1-Koi 6, respectively. Koi 1 had two reads that mapped to ORF81 and two reads that mapped to ORF114; Koi 2 had 4 reads that mapped to the DNA polymerase gene ORF79; Koi 3 had 8 reads that mapped to the major capsid protein ORF92; and Koi 5 had 2 reads that map to ORF4. Reads that aligned to genes other than ORF6 always mapped to the same 100 bp region of the genome and contigs were never assembled; these reads were also queried through the blastn suite (ncbi.nlm.nih.gov) and all returned matches were specific for KHV. The Illumina data suggests that ORF6 is the only gene present in KHV+ latently infected WBC.

Discussion

In this study, KHV latently infected koi peripheral WBC were successfully separated by the ORF6-Cy5 probe specific to KHV ORF6 mRNA. Latency-associated genes were investigated in the ORF6-enriched cells by RNA-seq.

The SmartFlare probe allows sorting of cells based on the presence of a specific RNA transcript (Seferos et al., 2007). This novel technology facilitates the collection of live cells with a similar gene expression profile based on a common transcript. As applied in this study, it separated an enriched population of live WBC that are latently infected with KHV based on presence of the latency-associated transcript ORF6. According to the manufacturer, the Scramble-Cy5 should be used to

detect background of the endocytosed probes. However, our analysis revealed that a true background was seen in cells incubated with ORF6-Cy5 that was different from Scramble-Cy5, and that true RNA-specific Cy5 signal was identified from a very small population of cells with an intensely fluorescent signal within the ORF6-Cy5 treated cells. The cells from Gate C have an average fluorescence that is about six fold greater than the majority of the ORF6-Cy5 cells (Gate B) and over one hundred fold more intense then the Scramble-Cy5 cells (Gate A). This agrees with the qPCR data, which shows very little KHV in the ORF6-Cy5 cells in Gate B while most KHV is in cells from Gate C. It is possible that the probe is interacting in part with some ubiquitous transcript in the koi WBC; this is, however, unlikely since the probe was chosen with specificity for KHV. These data support the ability of koi WBC to take up nanoflare probes by endocytosis, as well as the nanoflare probe's ability to enrich a cell population based on the presence of a specific viral transcript, ORF6.

When the KHV genomes were quantified in the cells sorted by ORF6-Cy5, 1240 copies were quantified from DNA extracted from 5,000 WBC (Table 3.1, Fig. 3.4). In theory, all the cells sorted from Gate C should contain ORF6 DNA, however this is not what was determined by the qPCR assay. It is possible that some KHV^- cells were collected in the C gate; tightening up the Gate C boundaries could alleviate any dilution factor caused by non-infected cells in the DNA extraction. Another possibility is that the percentage of total DNA recovery is relative low, which results in fewer copy numbers per cells than was expected. If the blood volume of an adult 200 g fish is 20 ml (10% of its body weight), and the average lymphocyte population in koi is 17.3×10^3 cells per µl (Tripathi et al., 2008), the total number of peripheral blood lymphocytes would be 3.52×10^8. Therefore, with an estimated infection frequency of 0.25% as estimated by qPCR results, the number of latent KHV genomes in one 200 g fish would be estimated at 880,000 copies in peripheral WBC of one fish. This low infection frequency is consistent with infection frequencies of other latent herpesviruses, such as KHSV which has as few as 2 latently infected cells per 1×10^7 lymph node cells (Campbell et al., 2005) or HSV-1 that has 300 to 10,000 latent genome copies per 100,000 cells from the trigeminal ganglia (Cohrs et al., 2000).

There were several regions of the KHV genome that enabled reads to artifactually map to the reference genome. These regions contained several repeating nucleotides with which single reads aligned, providing an abundance of coverage but not contigs. An example of this would be the 74 base-long repeat of "GTGTGT" that spans bases 37880 – 37993 of the KHV genome that has mapped regions of 116, 924X coverage although no reads align with 100% identity to the KHV genome. Another example would be the string of "GAGAGA" nucleotides that span 162 bases, from KHV genome positions 177,568-177,729 that has 12,632X coverage. These single-read alignments were considered an artifact due to the repeating nature of the KHV genome and were not considered true viral transcription in this analysis and were disregarded. This type of alignment artifact accounts for the majority of the reads mapping to the KHV genome from the Bowtie2 alignment (Table 3.2). Possibly more stringent alignment parameters could be employed to reduce this kind of artifact but it most likely would not be completely eliminated due to the repeating nature of the KHV genome. Another source of non-specific mapping artifact is the homology between the *C. carpio* genome and the KHV genome. Some reads that had 99% homology to the *C. carpio* genome also had 90-95% homology to the KHV genome due to the co-evolution of the herpesvirus with its host and therefore erroneously mapped to the KHV genome. To facilitate distinguished mapping of reads with homology to *C. carpio* and KHV, stricter mapping parameters could alleviate some of this non-specific alignment.

Reads from all six fish in this study were mapped to the KHV genome. Although KHV exposure from Koi 4-6 was previously known, Koi 1-3 were from a facility with no previous history of KHV exposure and tested negative by ELISA. It is interesting that the latency associated transcript ORF6 was identified in these fish, but with generally fewer reads and less coverage for ORF6 than the Koi 4-6. It is possible that these fish are KHV latently infected but their infection was not detected due to the low sensitivity of the diagnostic test. Our previous study detected KHV in 17 of 24 wild common carp with no clinical signs of KHV from two different natural ponds in the Malheur National Wildlife Refuge in Oregon (Xu et al., 2012), demonstrating a

widespread prevalence of latent KHV. It is possible these are latently infected fish at lower copy number of KHV.

The RNA preparation from this study includes reads from host transcription from koi WBC with latent KHV infections. Previous studies have identified 78 host proteins that interact with antibodies against KHV glycoprotein (ORF56) from fish with productive KHV infection, and 12 host proteins that interact with the same monoclonal antibodies (glycoprotein ORF56) from fish with no clinical signs of KHV (Gotesman et al., 2013). Several immunomodulatory genes have been identified in the response to productive infection including ubiquitin-like proteins, STAT1, interferon-inducible proteins, C-type lectins, and IFN genes (Adamek et al., 2012; Rakus et al., 2012). It would be interesting to evaluate host transcripts that are involved in latent KHV infections from a population of enriched latently infected WBC and how their dynamics may interact with the latency program of KHV.

Recently, herpesvirus miRNA expression has been found to be involved in the control and maintenance of latency (Gupta et al., 2006; Jurak et al., 2011; Murphy et al., 2008; Umbach et al., 2008). Additionally, there has been a recent increase in efforts to detect transcription from non-coding areas of the genome, including antisense regions and miRNA expression during latency, which have been shown to play significant roles in the control of latency and reactivation (Marquitz and Raab-Traub, 2012; Xu and Ganem, 2010; Zhang et al., 2007). The RNA preparation methods for this current study excluded very short fragments from the cDNA library and discarded any RNA fragments less than 150 bases, which would eliminate any microRNA transcription from the analysis. It would be interesting to investigate the miRNA expression of KHV during latency from a population of enriched latently infected host cells.

In conclusion, this study describes successful identification and isolation of an enriched population of KHV^{+} latently infected WBC from koi using a nanoflare probe specific to the KHV latency associated transcript ORF6. The latent infection frequency was estimated to be one genome per 400 peripheral WBC, although this

could be an inaccurate estimation if the DNA recovery from the 5,000 cells was lower than expected. RNA-seq analysis of viral transcription from an enriched population of latently infected WBC revealed ORF6 to be the major gene expressed in KHV during latency. The ability to obtain a population of exclusively latently infected cells will facilitate further investigations into the molecular program of KHV latency.

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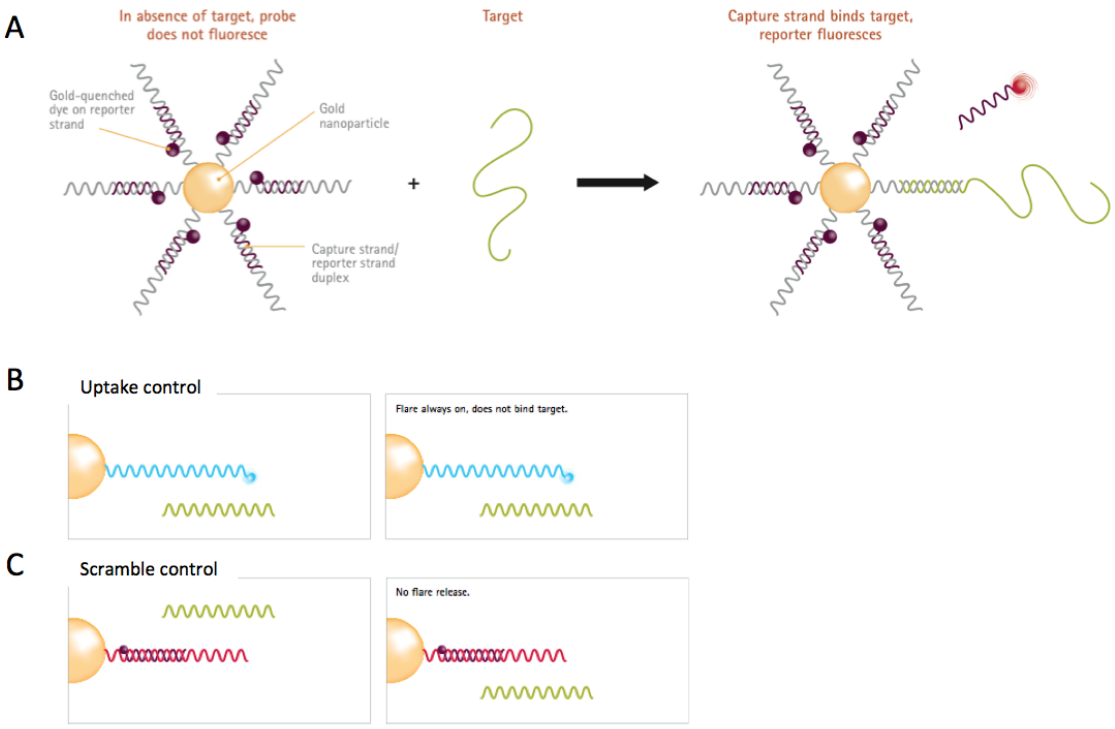

Figure 3.1. SmartFlare RNA probe. **A.** RNA-specific SmartFlare gold nanoparticle; gold-quenched dye on reporter strand bound to capture strand in the absence of target transcript. When the target transcript is present, it competitively binds to the capture strand, releasing the reporter strand which in turn fluoresces in the cytoplasm of the cell. **B.** Uptake control that has a Cy5 signal that always fluoresces upon endocytosis. **C.** Scramble control that has a Cy5 signal that is always quenched upon endocytosis for background fluorescence estimation. Copyrighted material EMD Millipore Corporation © 2012.

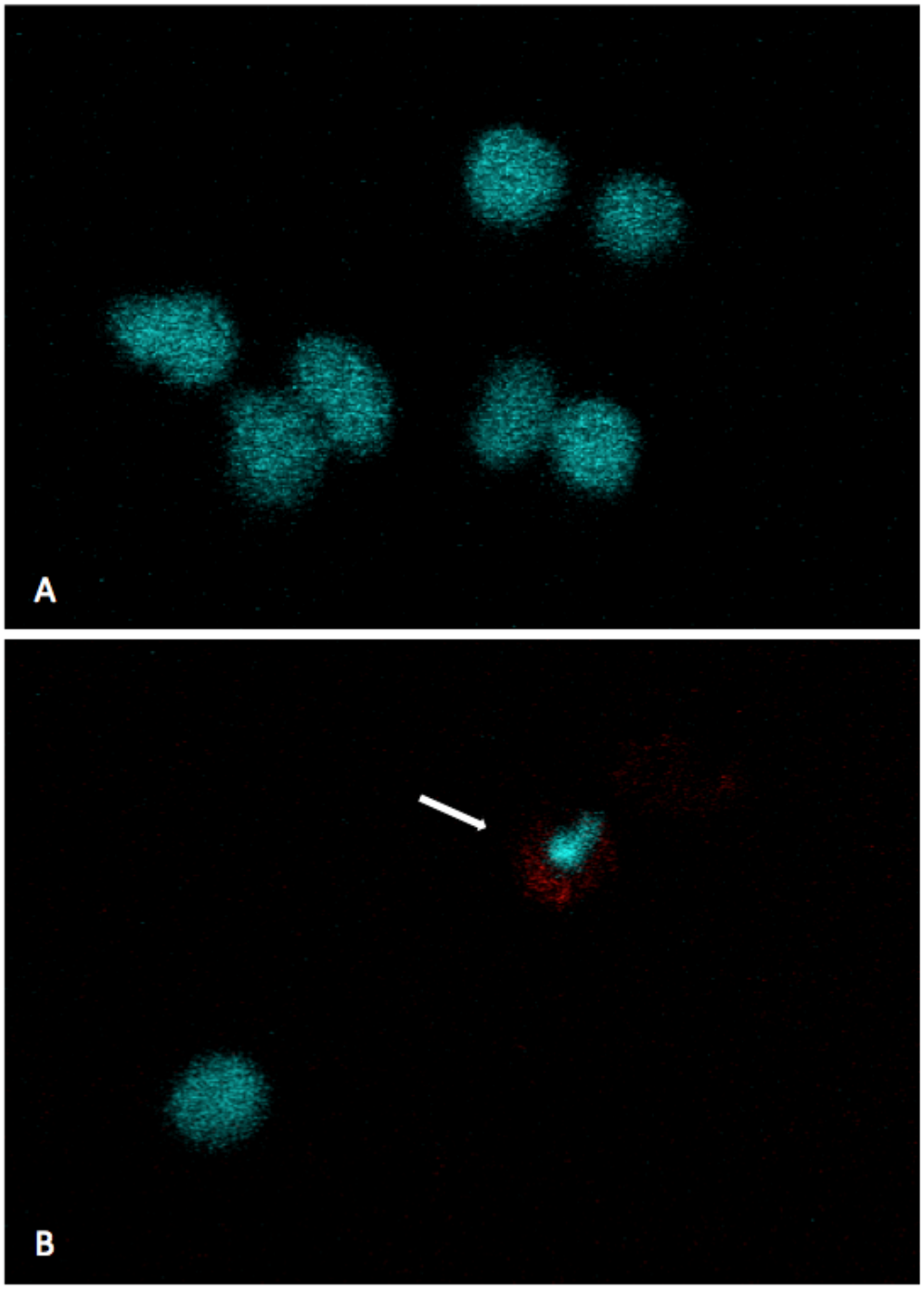

Figure 3.2. Confocal micrographs of live WBC incubated with nanoflare probes. **A.** Uptake-Cy5 probe or **B.** ORF6-Cy5 flare probe; arrow shows positive Cy5 signal. Cy5 appears red, DAPI appears cyan. Magnification 40X.

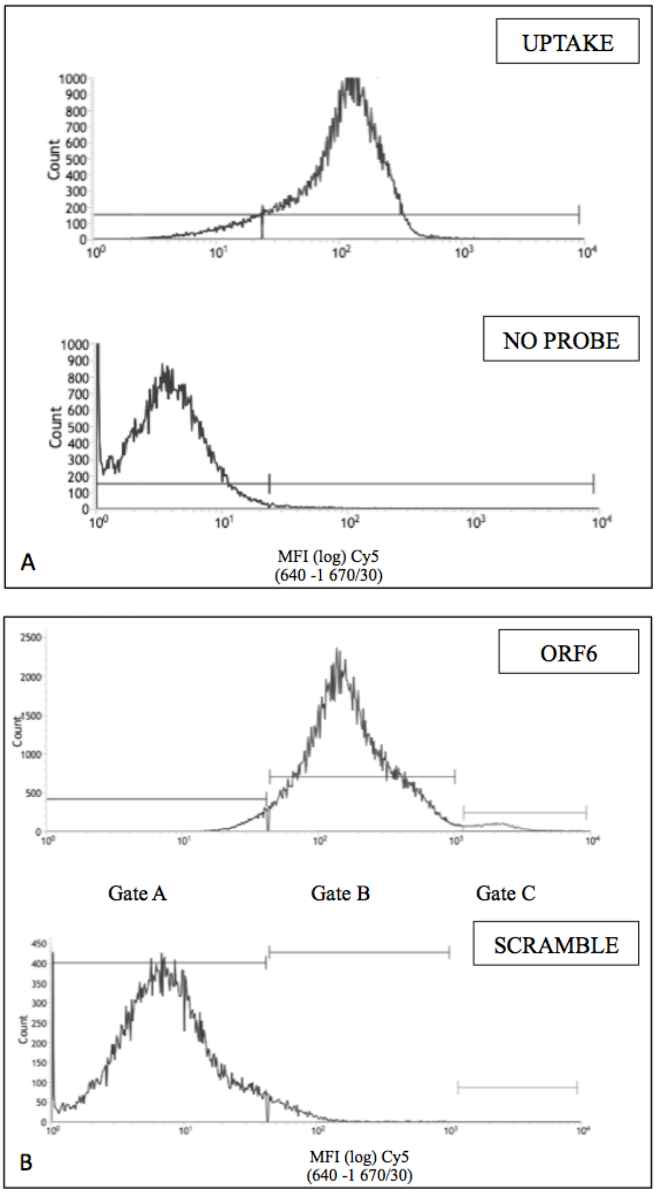

Figure 3.3. Flow cytometry analysis for WBC incubated with nanoflare probes. **A.** Top depicts cells incubated with Uptake-Cy5 probe; bottom depicts cells incubated with with no probe. **B.** Top shows cells incubated with ORF6-Cy5 probe, bottom shows cells incubated with Scramble-Cy5 probe. Gate A set to Scramble-Cy5 cells. Gate B set to majority of ORF6-Cy5 flare cells. Gate C set to top 1% of ORF6-Cy5 fluorescence intensity. Mean fluorescence intensity is on y-axis by a red laser at 640 nm (670/30).

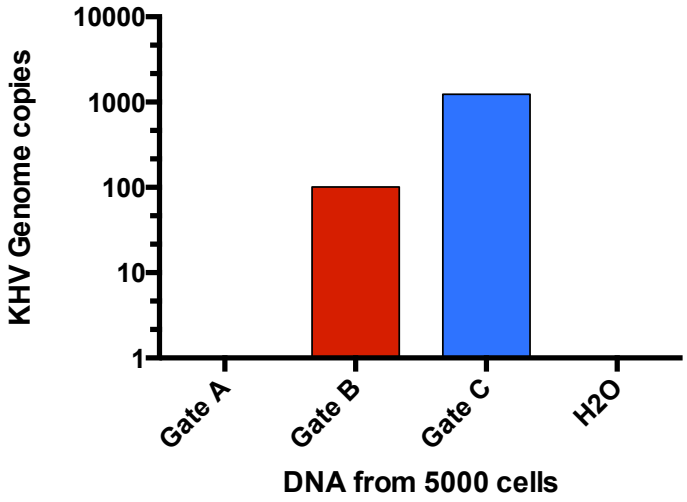

Figure 3.4. Quantitative Taqman PCR of KHV genome copies in cells sorted by flow cytometry by ORF6-Cy5 probe. DNA extracted from 5,000 cells from each Gate A, Gate B, and Gate C. Genomes were calculated using previously established standard Y= -3.296X + 44.301. Gate A = 0 genome copies; Gate B = 100.88 copies; Gate C = 1239.52 copies. H2O is negative control with no DNA template.

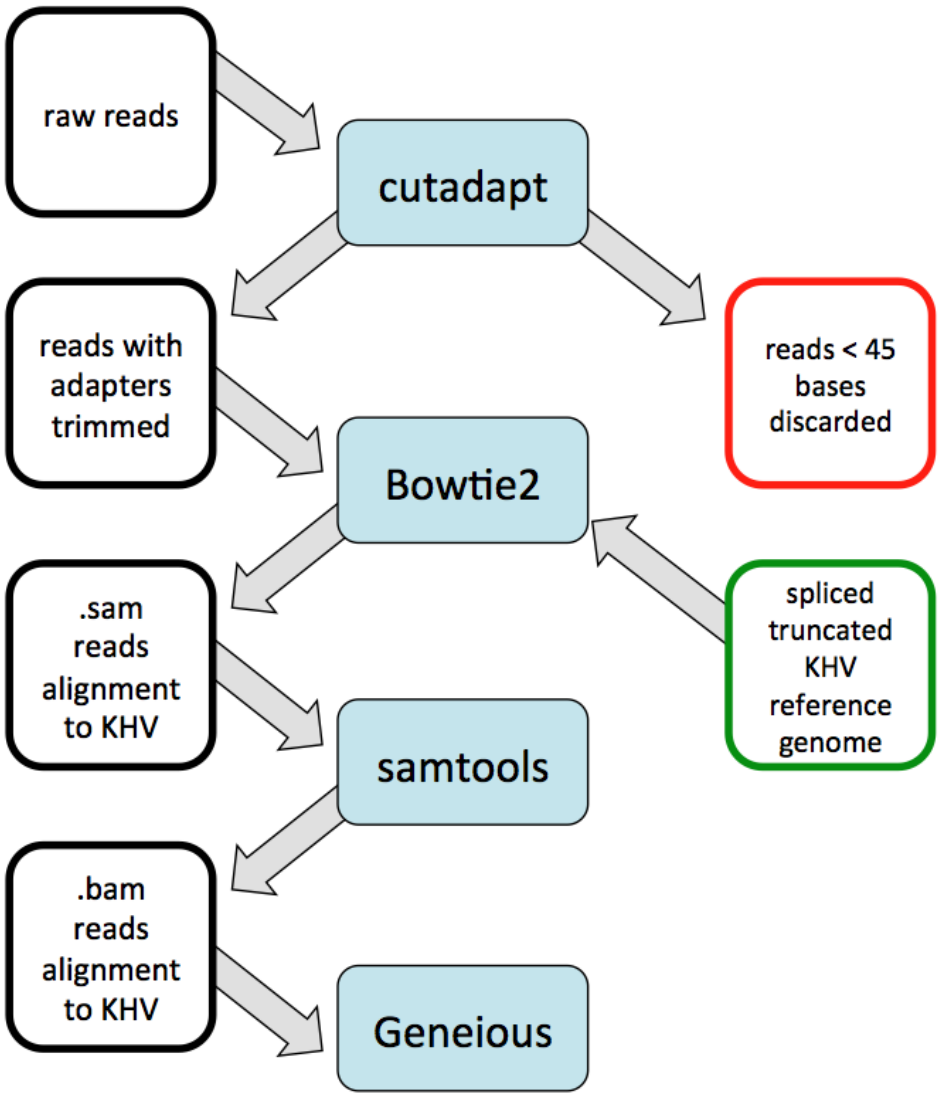

Figure 3.5. Bioinformatics analysis of Illumina reads flowchart. Raw reads from Illumina HiSeq 2000; cutadapt was used to remove TruSeq adapters and discard reads that were shorter than 45 bases post trim. Adapter-trimmed reads were inputted to Bowtie2 with the spliced truncated KHV reference genome and output was a .sam alignment file. samtools was used to convert .sam to .bam file. Identifications of aligned reads were inspected visually using Geneious software v 7.1.5.

Table 3.1. Quantitative Taqman PCR results for WBC in each gate sorted by ORF6-Cy5 probe. The previously established standard used is Y= -3.296X + 44.301.

DNA source	Ct value	Copy number in 10 uL sample	Copy number per 5000 WBC
KHV DNA (pos)	16.31	3.11E+08	n/a
Gate A	0	0	0
Gate B	39.68	25.22	100.88
Gate C	36.09	309.88	1239.52
Negative	0	0	n/a

Table 3.2. Post-run data from 100 base reads from Illumina HiSeq 2000.

	Koi 1	Koi 2	Koi 3	Koi 4	Koi 5	Koi 6
Yield (Mbases)	3,792	5,042	6,463	5,274	6,029	4,216
Number of reads	51,935,260	68,889,138	79,018,010	66,387,952	74,901,846	58,854,060
Mean quality score	23.36	25.76	31.49	30.82	31.87	25.65
% >30 quality bases	61.17	62.67	78.93	77.03	80.07	62.06
Reads aligned from Bowtie2	47,624	69,209	149,929	121,341	230,667	88,374
Reads aligned to KHV ORF6	28	121	68	46	236	162
Reads aligned to other KHV genes	4	4	8	0	2	0

Supplemental Table S3.1. RNA and cDNA pre-run quality analysis.

Parameter	Koi 1	Koi 2	Koi 3	Koi 4	Koi 5	Koi 6
Total number of cells	200,000	200,000	200,000	200,000	200,000	200,000
Mass of ribosomal depleted RNA (ng)	15.73	13.8	7.26	7.71	9.76	7.05
260/280 (NanoDrop)	1.62	1.66	1.84	1.67	1.54	1.71
Qubit concentration ng/ul **	<10	<10	<10	<10	<10	<10
Aligent Bioanalyzer average size (bp)	365	422	403	391	415	372
Concentration by qPCR (nM)	1.19	1.88	1.72	3.69	2.44	1.59
Aligent Bioanalyzer Molarity (pmol/l)	800.2	1350.7	2550.5	2987.8	2527.7	1203.7
Aligent Bioanalyzer concentration (pg/ul)	165.2	308.53	565.12	648	579.26	249.56
Number of peaks found on electropherogram	19	25	29	28	27	12

Chapter 4. Detection of the ORF6 protein during latent KHV infection

Aimee N. Reed[1,2], Claire Ostertag-Hill[1], Tim-Miller Morgan[1], Ling Jin[1,2]

[1]Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University Corvallis, OR 97331

[2]Department of Microbiology, College of Science, Oregon State University, Corvallis, OR 97331

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Abstract

Cyprinid herpesvirus 3, or koi herpesvirus (KHV), is a deadly virus that affects koi and carp worldwide. It causes severe gill necrosis and nephritis, dermal ulceration and hemorrhage, and mass mortality of up to 100% of affected fish. Fish that survive KHV infection are latently infected lifelong carriers. Little is known about the molecular mechanisms and control of latency for KHV. Our previous studies identified KHV ORF6 mRNA to be present during latent infection. In this study, the expression of viral protein from ORF6 was investigated by a polyclonal antibody specific to a synthetic peptide derived from predicted ORF6 protein (anti-ORF6). By immunofluorescence assay (IFA), positive staining to the anti-ORF6 was observed in both KHV-infected CCB cells *in vitro* and IgM^+ B cells *ex vivo* from koi latently infected with KHV. No IFA staining was observed in uninfected CCB cells nor IgM^- B cells from the same koi latently infected with KHV. The ORF6 protein expressed during productive infection was detected around 140 kDa, which is bigger than the ~80 kDa predicted protein. ORF6 protein at a similar size as the predicted protein was produced in an expression vector pet6XHN in *E coli*. Based on an analysis using software GPS-SUMO, 5 potential sumoylation sites were identified in the ORF6 protein sequence. Our study demonstrated that ORF6 protein is expressed during KHV latency in koi and may be sumoylated in infected cells.

Importance

This is the first demonstration that a member of the *Alloherpesviridae,* cyprinid herpesvirus 3 (CyHV-3), expresses a viral protein during latency, ORF6 protein. The protein appears to be around 140 kDa in molecular mass and may be sumoylated or post-translationally modified. ORF6 protein has homology to a conserved domain of EBNA-3B from Epstein Barr virus and ICP4 from herpes simplexvirus 1, genes important for latency. Our results suggest that ORF6 protein may have a potential role in regulating KHV latency.

Introduction

Cyprinid herpesvirus type 3, also known as koi herpesvirus (KHV), is a member of *Alloherpesviridae* (Waltzek et al. 2009), and it causes significant morbidity and mortality in its host, *Cyprinus carpio*. Acute KHV infection leads to severe gill and kidney necrosis, dermal ulceration, neurologic disease, and death with a reported 85-100% mortality rate (Hedrick et al. 1999). In the past 18 years since its emergence, KHV has devastated the carp aquaculture trade, as well as the ornamental koi industry and wild fish populations around the world, leading to millions of dollars in economic losses. Fish that recover from the initial infection become latently infected (Eide et al. 2011; Eide et al. 2011) then can reactivate and cause disease (Eide et al. 2011) as well as transmit the infection to naïve fish (St-Hilaire et al. 2005). Additionally, KHV has been shown to persist in natural wild populations of carp in North America (Cornwell et al. 2012; Xu et al. 2013), Europe (Baumer et al. 2013; Fabian et al. 2013), and Asia (Uchii et al. 2009).

Herpesviruses are ancient viruses that infect almost every kind of animal from invertebrates to humans. These enduring viruses can attribute their success to unique patterns of infection, of which their hallmark is latency: the persistence of viral genome within host cells with limited viral transcription activity and no production of virus particles. Although the mechanisms of latency are mysterious and complex, genes expressed during latency have been found to play important roles during latency establishment, maintenance, and reactivation. In general, latency associated genes can be divided into three groups: 1) transcription regulatory genes, such as LMP-1 of EBV, which regulates viral gene expression during latency (Docke et al. 2003; Speck and Ganem 2010); 2) anti-apoptosis genes, such as LAT of HSV-1, which can block apoptosis of infected cells during virus replication (Perng et al. 2000; Knickelbein et al. 2008); and 3) immunomodulatory genes (Forero et al. 2014; Iempridee et al. 2014; Matar et al. 2014), examples of which include vIL10 of EBV and M2 of MHV68, which can dampen immune detection of the virus and mitigate clearance of latently infected cells (Jenkins et al. 2008; Reeves and Sinclair 2010;

Matar et al. 2014). Although latency has been described for KHV, the molecular mechanisms of how KHV becomes latent are not yet well understood. Our previous study identified peripheral B lymphocytes as the main cell type in which KHV becomes latent (Reed et al. 2014), making KHV a lymphotropic herpesvirus. In addition, ORF6 mRNA was detected in IgM^+ B cells from KHV latently infected koi. The predicted protein sequence of ORF6 has homology with conserved domains of the C-terminal of transcription transactivators of other herpesviruses, EBNA-3B of EBV and ICP4 of HSV-1, which suggests that an ORF6 protein may play a similarly important role in regulation of the latent herpesvirus infection. To determine whether ORF6 protein is made during latency, ORF6 protein expression was investigated *in vitro* in KHV infected cells and *in vivo* in B cells from KHV latently infected koi by an antibody specific to peptides selected from the predicted ORF6 protein.

Materials and Methods

Koi and sampling. Ten 5 year old koi were acquired from a local distributor in Oregon with a history of KHV exposure and are designated as KHV^+ koi. KHV^+ koi were screened by nested PCR as reported previously (Xu et al. 2013), and no KHV disease has been observed in these KHV^+ koi in the past three years. Nine adult koi, 2-5 years old, with no KHV genome detected, designated as KHV^- koi, were donated by a distributor in Eugene, Oregon. All koi were kept and maintained at 12°C in 4-foot diameter tanks at Oregon State University John L. Fryer Salmon Disease Research Lab in accordance with the Animal Care and Use Committee regulations. All blood samples were collected via venipuncture of the caudal vein after anesthetizing the koi with 90 ppm MS-222 buffered with an equal amount of sodium bicarbonate and then were recovered. Whole blood was immediately transferred into tubes containing 3.2% sodium citrate to prevent both coagulation and erythrocyte lysis.

CCB cell lines and virus infection. Common carp brain (CCB) cell line (gift of Dr. Scott LaPatra) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products),

penicillin (100 U/ml), and streptomycin (100 µg/ml) (Sigma-Aldrich, Inc.) and incubated at 22°C. The United States strain of KHV (KHV-U) was a gift of Dr. Ronald Hedrick. CCB cells in 25 cm flasks were infected with KHV-U at 0.1 moi and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 5% fetal bovine serum (Gemini Bio-Products), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Sigma-Aldrich, Inc.) and incubated at 22°C.

B cell isolation and RNA extraction. Peripheral WBC of koi were isolated on a Ficoll Paque gradient as previously described (Reed et al. 2014). Peripheral WBC were sorted into IgM^+ B cells and IgM^- WBC by magnetic beads as previously described (Reed et al. 2014). Total RNA was extracted using TriZol (Invitrogen) according to manufacturer's instructions.

RT- PCR. Extracted RNA was treated with DNase (Life Technologies, Carlsbad, CA) according to manufacturer's instructions before using in RT-PCR. ORF6 mRNA was amplified with primers flanking the ORF6 coding region (Fig. 4.1) using the SuperScript®III One-Step RT-PCR System (Life Technologies) according to manufacturer's instructions. Briefly, a 25 µl reaction consisting of 12.5 ul of 2X Reaction Mix, 1 µl SuperScript®III RT/ Platinum®Taq Mix, 400 µM each of forward primer ORF6-5 and reverse primer ORF6-3, and about 50 ng, 150 ng, or 300 ng of total RNA as template. The mixture was subjected to 55°C for 30 min, then 94°C for 2 minutes, then 40 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 68°C for 3 minutes, followed by a 5 minute extension at 68°C. RT-PCR products were electrophoresed through a 0.8% agarose gel and then visualized by UV illumination after staining with ethidium bromide (1 µg/ml). Commercially available 1 kb Plus ladder (Life Technologies) served as size markers.

Southern Blot. RT-PCR products on the 0.8% agarose gel were transferred to a nylon membrane and then UV cross-linked to the membrane. The membrane was prehybrizided with prehybridization buffer (Roche Diagnostics, Indianapolis, IN) at 68°C and then hybridized with the dig-labeled DNA probe specific for ORF6 at 68°C overnight as previously described (Reed et al. 2014). After incubation with the probe,

membranes were washed with 0.1% sodium dodecyl sulfate and 10% 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) before incubation with an anti-digoxigenin antibody conjugated with peroxidase. The membrane was then developed by incubation with a chemiluminescent peroxidase substrate (Roche Diagnostics, Indianapolis, IN). The blots were exposed to film (Kodak) at room temperature for 30 min to 2 h. The molecular masses of the resulting bands were estimated by DNA Molecular Weight Marker VII, DIG-labeled (Roche Applied Science, Indianapolis, IN).

Generation of a polyclonal peptide antibody against ORF6. ORF6 antigenic peptides were selected by using Antigen Profiler software (http://www.pierce-antibodies.com/custom-antibodies/peptide-design-antigen-profiler.cfm) based on the predicted whole ORF6 protein. Two peptides were identified as the antigenic peptides that can be used to raise antibody for WB and IHC applications. One of the peptide sites near the c-terminal of the protein, (C)-KKSREYVEPDSDSDS, was selected and synthesized by solid state FMOC method (Pierce Antibodies, Thermo Fischer Scientific), conjugated and injected into two rabbits (Pierce Antibodies, Thermo Fischer Scientific). Serum containing the antibody harvested at day 35 from one of the two rabbits was used in this study. The polyclonal antibody is designated as anti-ORF6.

Indirect immunofluorescence assay. KHV infected and uninfected CCB cells were harvested by trypsinizing the monolayer off the flask then washed once in PBS and once in FA blocking buffer (1X PBS containing 1.5 % BSA) before applying to a Teflon-coated slide. IgM^+ B cells and IgM^- WBC were also washed once in PBS and once in FA blocking buffer before applying to the slides. Cells were fixed onto slides with 95% acetone / 5% methanol for 30 minutes, air dried and stored at -20°C overnight before continuing with IFA. Prior to applying the primary antibody, slides were first blocked with blocking buffer for 30 minutes at 37°C, then incubated with primary antibody (anti-ORF6) at either 1:100 or 1:50 dilution and incubated in a humidified chamber at 37°C for 30 minutes. A secondary donkey anti-rabbit IgG

antibody conjugated with FITC (Jackson ImmunoResearch Lab) was applied to slides at a dilution of 1:150 and incubated in a humidified chamber at 37°C for 30 minutes. Slides were washed gently 3-4 times in PBS containing 0.01% Tween 20 at room temperature for 5 min before and after application of the secondary antibody. Slides were then stained in Evan's blue counter stain for 10 min, rinsed briefly with double-deionized water. Finally slides were rinsed in 10% glycerol in PBS solution before adding a cover slip, and cells were then examined under a fluorescent microscope.

Construction of ORF6-pet6XHN expression vector. RT-PCR product amplified by primers ORF6-5 and ORF6-3 was first cloned into TOPO TA vector (Invitrogen). The correct insert was confirmed by restriction digestion and Sanger sequencing. The coding region of ORF6 excluding the ATG but up to and including the stop codon was amplified by primers PetF and PetR with *Eco*RI restriction sites added to the ends of the product (Figs. 4.1C and 4.1D). The PCR product amplified with primers PetF and PetR (Fig. 4.1E), as well as the expression plasmid Pet6xHN-N (Clontech) were digested first with *Eco*RI; the vector was then dephosphorylated with shrimp alkaline phosphatase according to manufacturer's instructions, and deactivated by heating at 85°C for 15 minutes. Following digestion and dephosphorylation, both vector and PCR products were electrophoresed, gel excised, and ligated with T4 ligase (Invitrogen). The correct ligation, designated as ORF6- Pet6xHN-N, was confirmed by restriction digestion and Sanger sequencing.

PCR. PCR product was amplified in a 25 µl reaction consisting of 12.5 ul of 2X Platinum PCR SuperMix (Life Technologies, Carlsbad, CA), 400 µM each primer, and 0.5 µg of DNA template. The mixture was subjected to 94°C for 2 min, and 30 cycles of 94°C for 30 sec, 59°C for 30 sec, and 72°C for 2 min 30 sec, followed by a 10 min elongation at 72°C after the final cycle. The amplicons were run in 0.8% agarose gel and then visualized by an UltraBright blue LED transilluminator after staining with SYBR Safe DNA gel stain (Invitrogen) (1:10,000 dilution). Commercially available 1 kb Plus Ladder (Life Technologies) served as size markers.

ORF6 protein expression and isolation *in vitro*. The ORF6-Pet6xHN-N plasmid was transformed into BL21 *E. coli* cells and grown in the presence of ampicillin (50 mcg per ml) to OD 0.60 at 600 nm, then induced with isopropyl-β-$_D$-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at 37°C for 5 hours. The culture was harvested by centrifugation at 3000 rpm for 10 min at 4°C. The bacterial pellet was first lysed in xTractor buffer (Clontech) and incubated on ice for 15 minutes and then treated with 2 U DNase I before centrifuging at 10,000 × g for 20 minutes at 4°C. The clarified supernatant was added to a His60 Nickel Superflow Resin and Gravity Column (Clontech) and incubated for 1 h by gentle rocking at 4°C. Non-tagged proteins were washed off the column with equilibration buffer (20mM imidazole) then followed by wash buffer (40 mM imidazole). His-tagged protein was eluted in elution buffer (300 mM imidazole). The elute fractions were analyzed by Bradford assay at 595 nm first before being analyzed by western blot.

Western blot. KHV-infected or mock-infected CCB cells grown in 25 ml flasks were washed, trypsinized, and pelleted by centrifuging at 10,000 × g and stored at -80°C. The purified his-tagged protein, KHV infected cell preparation, and BL21 cells transformed with the ORF6- Pet6xHN plasmid were lysed in Laemelli loading buffer with 5% β–mercaptoethanol or Laemelli buffer plus 1 mM DTT (Bio-Rad) then heated at 80°C for 5 min, or lysed in 2X 8M urea buffer before loading onto 10% SDS-PAGE gel (Bio-Rad) and electrophoresed for 60 minutes at 150V. Following SDS-PAGE electrophoresis, proteins were transferred to a nitrocellulose membrane via TransBlot SD semi-dry transfer cell (BioRad) at 15V for 15 min. Membranes were blocked in TBST (Tris buffer with 0.1% Tween 20) with 5% milk for one hour and washed twice with TBST. Membranes were incubated with anti-ORF6 antibody at a 1:500 dilution, or anti-6xHN polyclonal antibody (Clontech cat #631213) at 1:2000, or anti-panSUMO polyclonal antibody (Abgent cat #AP1290a) at 1:500 in 0.5% milk in TBST at 4°C overnight. Membranes were then washed three times with TBST and incubated with secondary IRDye 800 CW goat-anti rabbit IgG antibody (LI-COR Biosciences) at 1:10,000 for one hour at room temperature, rinsed twice with TBST for five minutes, then rinsed with PBS twice for five minutes to eliminate

excess background caused by Tween-20. The blotted membranes were analyzed on the Odyssey infrared imaging system (LI-COR Biosciences).

Results

Detection of full-length ORF6 transcript in KHV latently infected B cells. Our previous 5' RACE and 3' RACE results demonstrated that the ORF6 transcripts begins at -127 bp upstream of the ATG of ORF6 and end at +189 nt untranslated region downstream of the stop codon; a polyadenylation signal was identified (AATAAA) 20 bp upstream of the 3' end of the transcript (Reed et al. 2014). To confirm the entire mRNA transcript for ORF6 is produced in latently infected B cells, total RNA from IgM^+ B cell from KHV latently infected koi were investigated by RT-PCR with primers located +100 bp upstream of the start codon and -12 bp downstream of the stop codon (Fig. 4.1D). As shown in Fig. 4.2, a ~2.3 kb product (expected 2313 bp) was detected in total RNA isolated from IgM^+ B cells from KHV^+ koi (Fig. 4.2A, lanes 1-3) and CCB cells infected with KHV *in vitro* (Fig. 4.2A, lane 7). The RT-PCR product is smaller than the product amplified from the genomic DNA (Fig. 4.2A, lane 8), suggesting the intron has been spliced from the mRNA. No amplicon was detected from total RNA extracted from IgM^+ B cells from KHV^- koi (Fig. 4.2A, lanes 4-6). To further confirm the RT-PCR product is ORF6, RT-PCR products were hybridized by the ORF6 specific probe as described previously (Reed et al. 2014). As shown in Fig. 4.2B, both RT-PCR and PCR amplified product were hybridized (Fig. 4.2B, lanes 1-3, and lane 7-8). In addition, RT-PCR amplicon was also sequenced by Sanger sequencing, which confirmed the RT-PCR amplicon is the cDNA of ORF6 mRNA (data not shown).

Detection of ORF6 protein during productive infection *in vitro*. To determine whether the ORF6 protein is produced during productive infection in *vitro*, CCB cells from day 0, 1, 5 and 12 post-infection (pi) were examined by an indirect immunofluorescence assay (IFA) using the ORF6 peptide specific antibody (anti-ORF6). As shown in Fig. 4.3, positive staining was observed in several CCB cells on day 5 pi (Fig. 4.3C), with many more positive cells detected on day 12 pi (Fig. 4.3D).

Weak positive staining was detectable on day 1 pi (Fig. 4.3B). The positive staining intensity increased after day 5 pi, which suggests that production of ORF6 protein increases over time. The positive staining localized mostly around the nuclei of the infected cells (Fig. 4.3E). No staining was observed in the KHV infected cells harvested on day 0 pi (Fig. 4.3A) or in uninfected CCB cells (data not shown). These results demonstrate the expression of ORF6 protein during productive KHV infection.

Detection of ORF6 protein during latent infection *in vivo*. To determine if ORF6 protein is expressed during latent KHV infection, B cells from KHV^+ koi were examined by IFA using the ORF6 specific antibody. IgM^+ B cells and IgM^- WBC cells isolated from KHV^+ koi were stained with anti-ORF6 antibody first and then a secondary donkey anti-rabbit IgG antibody conjugated with FITC. As shown in Fig. 4.4, fluorescence was observed in numerous IgM^+ B cells from KHV^+ koi, whereas no fluorescence was detected in IgM^- WBC from the same KHV^+ latently infected koi (Fig.4 KHV^+). No fluorescence was detected in IgM^+ B cells or IgM^- WBC from KHV^- koi (Fig. 4.4 KHV^-). These IFA results demonstrate ORF6 protein is expressed in B cells of koi during a latent infection.

Detection of ORF6 protein by western blot. To confirm the IFA results, cell lysates from KHV infected CCB cells collected at day 12 post-infection were analyzed by western blot using the anti-ORF6 peptide antibody. As shown in Fig. 4.5C, a product at about 140 kDa was detected in KHV infected CCB cells (lanes 3-5). No band was detected in uninfected cells (Fig 5C, lane 6). The apparent size of ORF6 protein is bigger than the predicted product of ORF6 at ~81.5 kDa. To determine whether ORF6 is modified post-translationally *in vivo*, a comparison of protein expression was investigated *in vitro*. The coding region of ORF6 between ATG and TAA was placed between *Eco*RI cloning sites in the his-tagged expression plasmid pet6XHN-N. The expression vector was then transformed in BL21 cells and ORF6 protein expression was induced by IPTG and purified by His60 Nickel Superflow Resin and Gravity Column (Clontech). As shown in Fig. 4.5A, a band at about 85 kDa, similar to the predicted size (also in Fig. 4.5C lane 1), was detected

from induced cell lysates (lane 1 and lane 2) or protein purified from the His-tag Ni-column (lane 4) when stained with anti-ORF6, whereas no band was detected in non-induced cell lysates (lane 3). In agreement with ORF6 antibody staining, same sized protein bands were detected by anti-his6XHN antibody, which suggests that the protein that was detected by the ORF6 peptide antibody is derived from the expression vector. Since the protein expressed in eukaryotic cells is about 60 kDa bigger than the protein expressed in the bacteria, it is plausible that ORF6 protein expressed in the infected cells has post-translational modifications.

Detection of sumoylation of KHV proteins by western blot. To detect sumoylation of KHV proteins, KHV infected CCB cells were investigated by western blot using anti-panSUMO antibody. Sumoylation of several proteins were detected in KHV infected CCB cell lysates (Fig. 4.5D lanes 2-4 at arrows) whereas no sumoylation was detected from uninfected CCB cells (Fig. 4.5D lane 5). This supports the sumoylation of proteins during productive *in vitro* infection for KHV.

Discussion

In this study, the viral ORF6 protein was demonstrated in both productive infection and latent infection of KHV by a polyclonal antibody raised by synthetic peptides from the predicted ORF6 protein. The ORF6 protein produced in eukaryotic cells is around 140 kDa, which is significantly bigger than the predicted protein ~81.5 kDa and suggests that ORF6 proteins may be post-translationally modified.

Our previous study demonstrated that KHV, like many other herpesviruses, becomes latent in the host that has recovered from a primary infection (Eide et al. 2011), and additionally, that KHV becomes latent mainly in the B cell and that ORF6 RNA can be detected in KHV latently infected B cells (Reed et al. 2014). Using a polyclonal antibody specific to the ORF6 peptide located near the c-terminal, positive staining of ORF6 protein can be observed in KHV infected CCB cells *in vitro*, with an increased number of cells detected from day 1 to day 12 p.i. (Fig. 4.3), which

suggests that ORF6 protein is produced during productive infection. Positive staining was also detected in IgM^+ B cells from latently infected KHV^+ koi, which strongly supports ORF6 protein production during the latent infection (Fig. 4.4). The intensity of staining in latently infected B cells is similar to staining observed during productive infection, which suggests that ORF6 protein is abundantly expressed during latency. The positive staining by anti-ORF6 antibody localized mostly in the nucleus of infected cells or IgM^+ B cells (Fig. 4.3E and Fig. 4.4, KHV+ IgM+). However, there was some staining observed on the cell membrane of uninfected cells or IgM^- B cells. Since any observable membrane staining is significantly weaker than the nucleus staining of KHV infected cells (Figs. 4.3 and 4.4), the polyclonal antibody may have some non-specific staining to a membrane protein.

Our studies suggest that a viral protein is produced during KHV latency. Proteins made during latency are commonly key regulatory proteins during a herpesvirus infection. During MHV68 latency, the latency associated M2 protein has been shown to play a critical role in both the establishment of latency as well as reactivation (Herskowitz et al. 2008). An M2-null strain of MHV68 replicates with wild-type efficiency in mice following intranasal inoculation but exhibits a dose-dependent defect in the establishment of latency at day 16 p.i. (Jacoby et al. 2002). During EBV latency, up to nine latent proteins can be detected during latency. The main viral protein important during latency is latent membrane protein 1 (LMP1), which can block apoptosis and provide growth signals in latently infected cells (Kelly et al. 2006; Le Clorennec et al. 2008; Ndour et al. 2012). EBNA2 is required for protection of the latently infected cells against specific apoptosis stimuli (Lee et al. 2004). In addition, LMP1 can regulate interferon regulatory factor 7 by inducing its sumoylation in latently infected cells (Bentz et al. 2012). Another lymphotropic herpesvirus, KSHV, expresses two proteins during latency: the latency-associated nuclear antigens LANA1 and LANA2. LANA functions as both a transcriptional activator and a repressor depending on the context of promoters and cell lines interrogated (Campbell and Izumiya 2012; Toth et al. 2013). LANA1 is necessary to maintain nuclear association of latent genomes during replication, as it tethers the

viral episome to the chromosome of the daughter cell during cell division, thereby ensuring its persistence in replicating cells (Barbera et al. 2004; Ballestas and Kaye 2011). LANA1 has also been implicated in the promotion of S-phase entry via β-catenin upregulation (Fujimuro et al. 2003). LANA2 is a latent protein found in B cells of Castleman's disease and primary effusion lymphoma (PEL) and acts a potent inhibitor of p53 during latency transformation (Rivas et al. 2001). It will be interesting to investigate the function of ORF6 protein and its potential role in KHV latency.

The ORF6 protein detected in KHV infected CCB cells appears to have a molecular mass of ~140 kDa, which is greater than its predicted molecular mass of 81.5 kDa. It is not uncommon for proteins to be detected at an apparently greater molecular mass than predicted. Because the size difference is less than twice the predicted molecular weight, it is not likely to be a dimer, which is also supported by the evidence that treatment cell lysates from the KHV infected CCB cells with powerful reducing agents such as 8M urea did not change the apparent molecular mass. In addition, ORF6 protein lacks any predicted coiled coils or a leucine-rich area that would indicate a leucine zipper domain, which is a common dimerization domain in transcription regulator proteins (Landschulz et al. 1988). Post-translation modifications often lead to the increase in the apparent size of a protein. The most probable explanation in this case is the presence of post-translational modifications, such the addition of the small ubiquitin-related modifier, SUMO, an ~11.5 kDa protein, or glycosylation or phosphorylation (Gravel et al. 2004; Anckar and Sistonen 2007; Garaude et al. 2008). Post-translational modification with SUMO proteins, sumoylation, is one of the key regulatory protein modifications in eukaryotic cells. Hundreds of proteins involved in processes such as chromatin organization, transcription, DNA repair, macromolecular assembly, protein homeostasis, trafficking, and signal transduction are subject to reversible sumoylation (Gill 2004; Gill 2005; Flotho and Melchior 2013). When the ORF6 amino acid sequence was queried with the sumoylation site prediction software GPS-SUMO (Zhao et al. 2014), 5 potential sumoylation sites were identified (Table 4.1). With an added mass of five

11.5 kDa proteins it would explain the apparent augmented molecular mass from predicted molecular mass observed for ORF6 protein in infected CCB cells. Additionally, when KHV infected CCB cells were investigated for sumoylation by anti-panSUMO antibodies, multiple bands were detected from infected cells (Fig. 4.5D lanes 2-4), but bands were not detected from uninfected CCB cells (Fig. 4.5D lane 5) indicating KHV-specific modifications are present in infected cells. The anti-panSUMO antibody was purchased commercially and has recognition of SUMO2 and SUMO3 related proteins, and was generated from rabbits immunized with recombinant protein encoding full length human SUMO3. Although it is not specific for *C. carpio* SUMO proteins, sumoylation is considered a highly conserved mechanism from yeast to humans. This supports the possibility of ORF6 post-translational modification by SUMO during KHV infection.

Recently, sumoylation has been found to play a significant role in latency of HSV-1 and KHSV (Marcos-Villar et al. 2011; Campbell et al. 2012; Cai et al. 2013; Everett et al. 2014). Sumoylation has been found in LANA and two major KSHV encoded transcription factors, K-Rta (KSHV replication and transcriptional activator) and K-bZIP (KSHV basic leucine zipper) (Campbell and Izumiya 2012; Gunther et al. 2014). A unique SUMO-2-interacting motif within LANA was found to be essential for KHSV latency (Cai et al. 2013). SUMO-modification of LANA is connected to SUMO-binding histone sumoylation, gene silencing, and latency establishment. SUMO-modification has also been identified in HSV-1 ICP0, which can mediate degradation of both sumoylated and unmodified promyelocytic leukemia (PML or ND10) bodies. PML bodies promote intrinsic antiviral resistance against herpesvirus infections (Saffert and Kalejta 2006). The assembly and disassembly of PML (ND10) bodies at herpesvirus replication complexes are SUMO-dependent, and modulation of the SUMO modalities by viral proteins during the lytic replication cycle may help dictate whether viral replication will proceed or if latency will be established (Cuchet et al. 2011; Cuchet-Lourenco et al. 2011; Marcos-Villar et al. 2011; Cuchet-Lourenco et al. 2012). It is interesting to find ORF6 has 5 potential sumoylation sites, which suggests ORF6 may function as nuclear regulatory protein

via sumoylation during KHV infection. It will be interesting to find whether ORF6 shares a similar function as M2, LANA, or LMP1 during latency. Since ORF6 also has homology to conserved domains of EBNA-3B from EBV and ICP4 from HSV-1, it will be also interesting to know if ORF6 has any similar function to EBNA-3B and ICP4 during latency.

In summary, this is the first report of a latent protein detected for a member of the *Alloherpesviridae*. The ORF6 protein is present during productive infection *in vitro* as well as during latent infection in B cells of latently infected koi, and not in other peripheral WBC of latently infected koi. The protein appears to be around 140 kDa in molecular mass and may have post-translational modifications that are similar to transcription transactivator proteins of other herpesviruses. KHV ORF6 protein may hold the key to understanding herpesvirus latency. These results provide strong evidence supporting herpesvirus latency as conserved biology across *Herpesvirales*.

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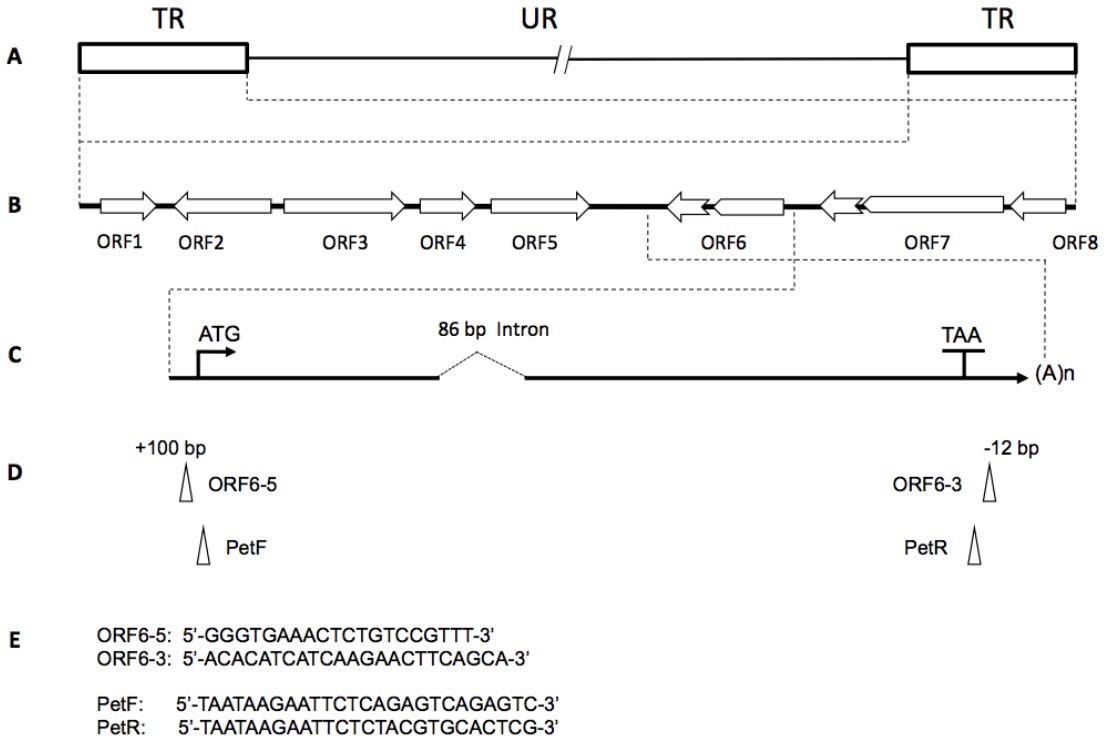

Figure 4.1. Schematic of KHV genome and location of primers. **A.** KHV genomic structure. TR indicates the terminal repeats containing ORFs 1-8; UR indicates unique regions containing ORFs 9-156. **B.** The expanded TR with ORF1-8; broken arrows indicate introns for ORF6 and ORF7. **C.** The expanded 5'-3' ORF6. The dashed line indicates the 86 bp intron. **D.** The relative location of the primers used in RT-PCR for full-length ORF6 mRNA amplification and the primers used for construct of expressin vector pet6XHN for ORF6 coding region amplification without the ATG but with addition of *Eco*RI sites. **E.** Primer sequences used in RT-PCR and PCR.

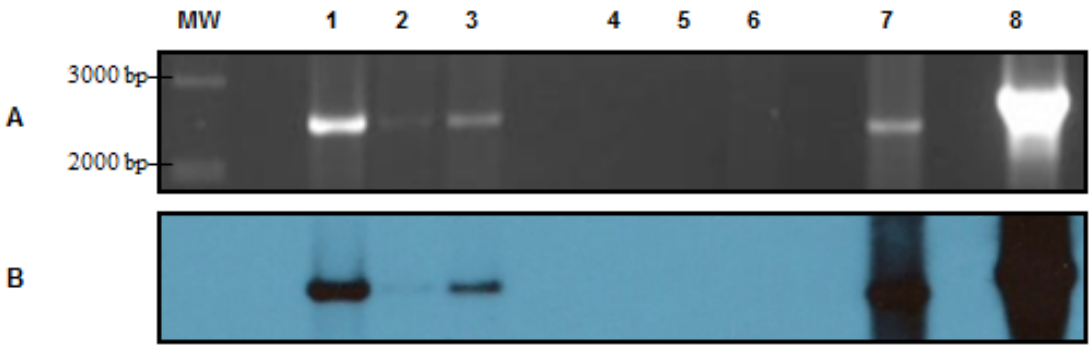

Figure 4.2. Detection of full-length ORF6 transcript. **A**. One-step RT-PCR with primers ORF6-5 and ORF6-3 that recognize the 5'- and 3'- untranslated region. Lanes 1-3: RNA extracted from B cells of koi with latent KHV infection at 150 ng, 300 ng, and 500 ng respectively. Lanes 4-6: RNA from KHV$^-$ koi at 150 ng, 300 ng, and 500 ng respectively. Lane 7: RNA extracted from KHV infected CCB cells at day 12 pi. Lane 8: genomic DNA. The spliced transcript is expected to be 2313 bp, the unspliced product is expected to be 2399 bp. MW: molecular weight marker 1 kb Plus (Invitrogen), shown are 2000 bp and 3000 bp. **B**. Autoradiogram of membrane after gel transfer from (A) by probing with DIG DNA probe, which is specific to KHV ORF6 as reported previously (Reed et al. 2014).

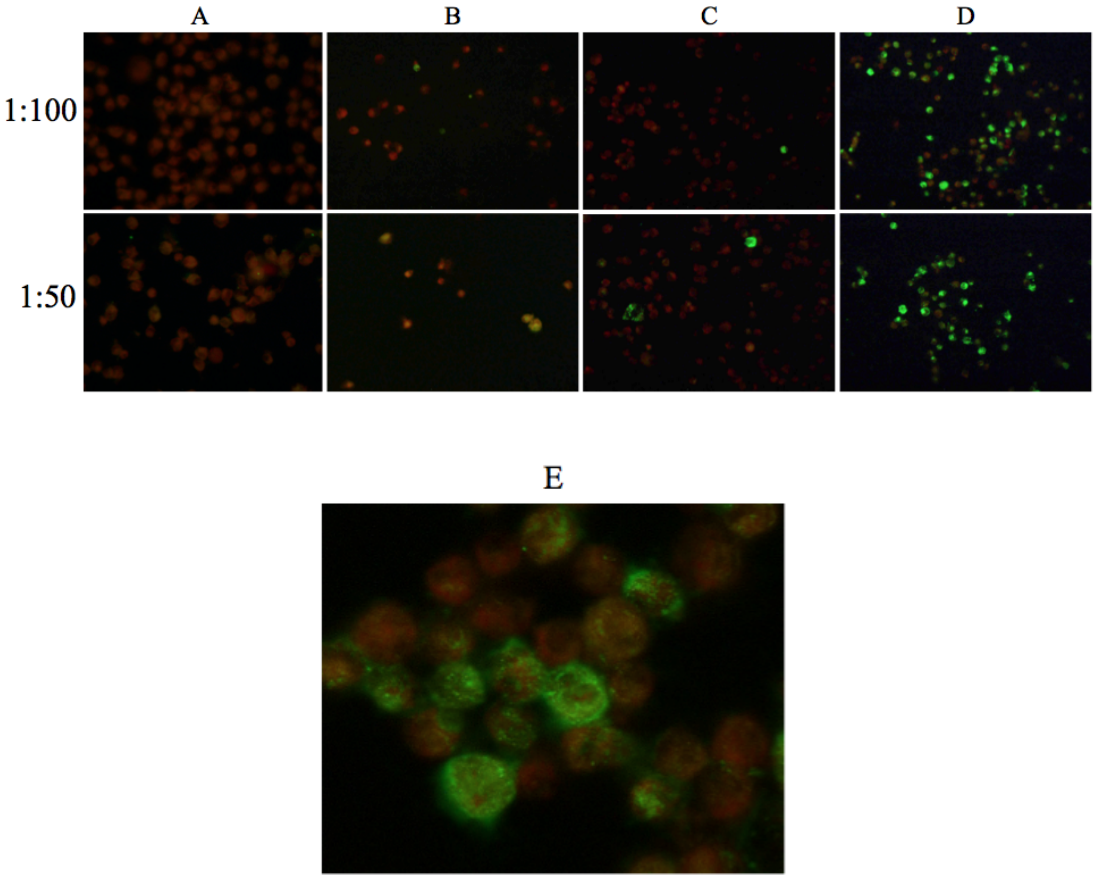

Figure 4.3. Indirect immunofluorescence assay for CCB cells. **A.–D.** Cells were infected with KHV and harvested on day (**A**) 0, (**B**) 1, (**C**) 5, and (**D**) 12 post infection (dpi). Cells were stained with anti-ORF6 peptide antibody at 1:00 dilution (top panels) or 1:50 dilution (bottom panels) then secondary donkey anti-rabbit IgG conjugated with FITC and counterstained with Evan's blue. Fluorescent images were captured at 20X magnification. **E.** Cells as in (**D**) image captured at 40X; cells demonstrate nuclear fluorescent signal of anti-ORF6 antibody staining.

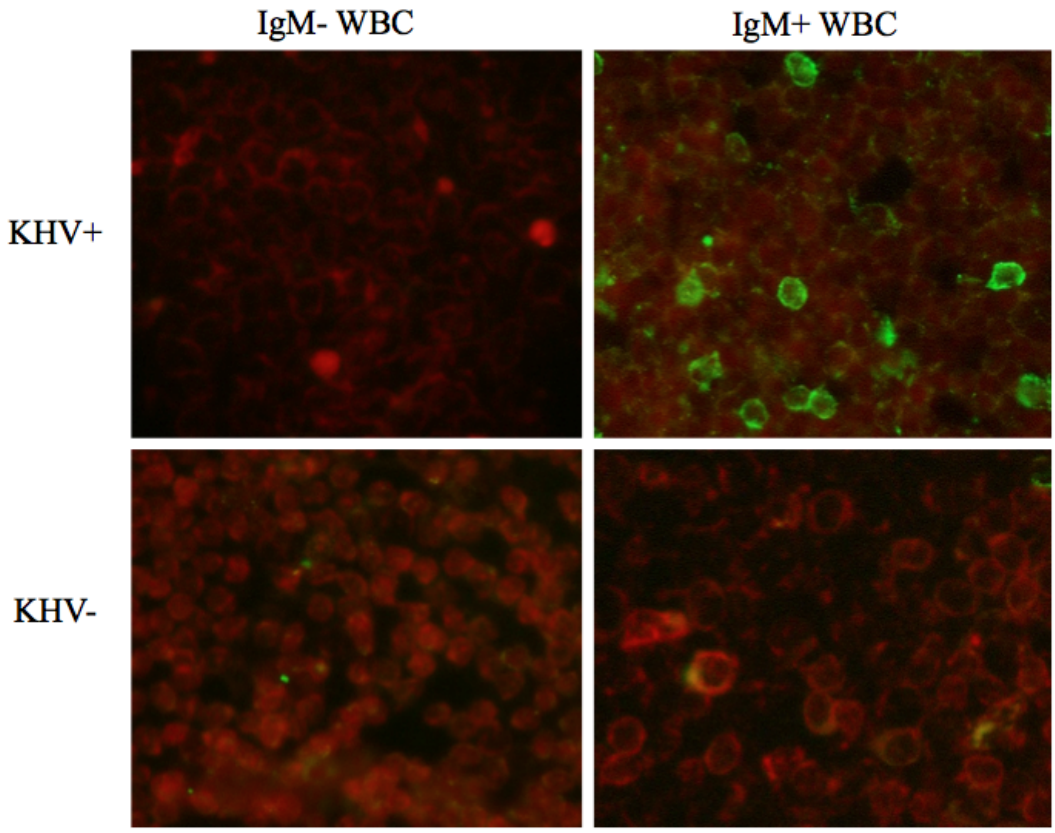

Figure 4.4. Indirect immunofluorescence assay for koi peripheral WBC. IgM^+ B cells or IgM^- WBC from both KHV^+ koi and KHV^- koi were stained by anti-ORF6 peptide antibody at 1:100 and then secondary donkey anti-rabbit IgG conjugated with FITC. Fluorescent images were captured at 40X magnification.

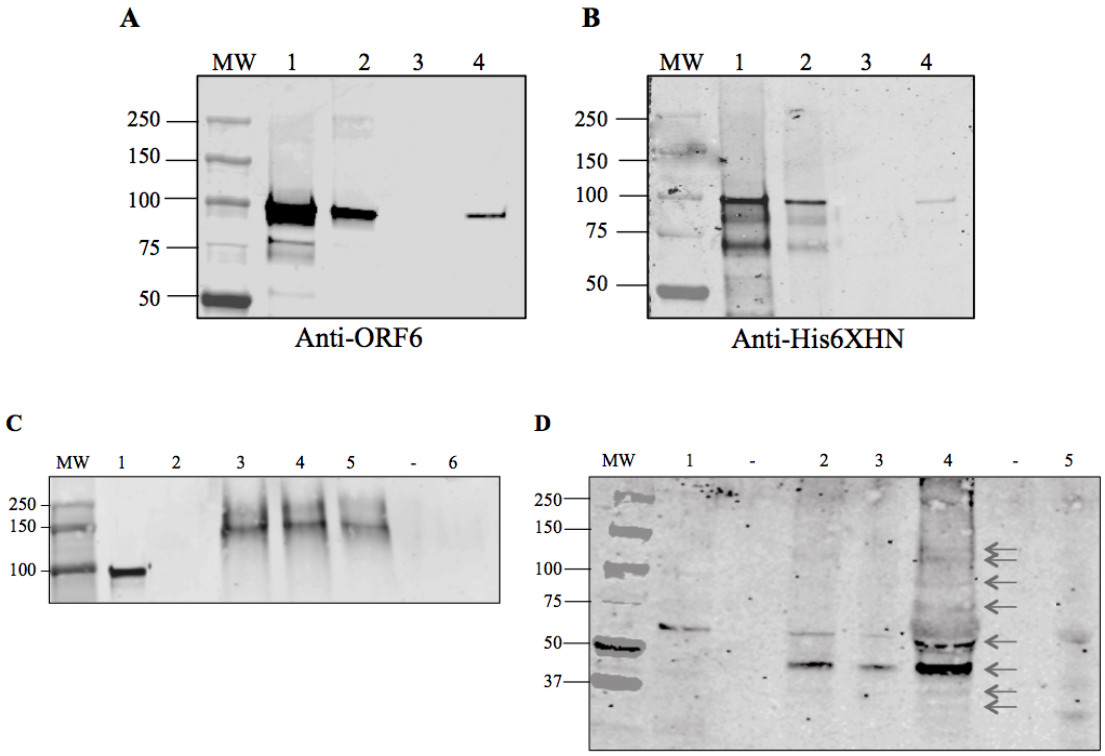

Figure 4.5. Detection of ORF6 protein by western blot. **A.** and **B.** Detection of ORF6 protein from pet6XHN ORF6 expression vector by (A) anti-ORF6 peptide antibody and (B) anti-6xHN polyclonal antibody. Lanes 1: BL21 cell lysates following IPTG induction; lanes 2: BL21 cell lysates following IPTG induction at 1:10 dilution; lane 3: BL21 cell lysate without IPTG induction; lanes 4: ORF6 protein purified by his-tag from the Ni-column. **C.** ORF6 protein purified from the column and CCB cell lysates harvested at day 12 post-infection stained with anti-ORF6 peptide antibody. Lane 1: BL21 cell lysates with IPTG induction; lane 2: BL21 cell lysates without IPTG induction; lane 3: KHV-infected CCB cell lysates in Laemelli SDS buffer with 5% β-ME; lane 4: KHV-infected CCB cell lysates in Laemelli SDS buffer with 1 mM DTT; lane 5: KHV infected CCB cell lysates in 2X 8M urea buffer; lane 6: uninfected CCB cell lysates in Laemelli SDS buffer with 5% β-ME. **D.** Detection of sumoylated proteins by anti-PanSUMO antibody. Lane 1 BL21 lysate following IPTG induction; lane 2, 3, and 4: KHV-infected CCB cell lysates in Laemelli SDS buffer with 5% β-ME; lane 5: uninfected CCB cell lysates in Laemelli SDS buffer with 5% β-ME. Arrows indicate bands detected by anti-panSUMO antibody. MW: Precision Plus Protein Dual Color Standard (BioRad).

Table 4.1. Predicted sumoylation sites for the 725 aa ORF6 protein from GPS-SUMO prediction software.

Position	Peptide	Score	Cutoff
373	AETEVPI**K**TEPQDTS	50.593	16
156	ELGTSPV**K**KPDPKLP	39.125	36.625
177	LKPIYRV**K**ADGAAFF	37.867	36.625
39	ARHRSTV**K**SFGPKLA	36.813	36.625
399	DLAKLTA**K**PEEKPSK	17.959	16

Chapter 5. Concluding Remarks

Aimee N. Reed

Koi herpesvirus is an important and deadly disease of ornamental koi and common carp, and it has devastated the aquatic community in the past 18 years since its emergence. The severity of disease and its swiftness upon which it spread around the globe has motivated the scientific community to discover ways to control the virus and alleviate disease in affected fish. The biggest blockade to finding a cure for this deadly herpesvirus is latency. A latent virus can be harbored in a host for years undetected with no clinical disease, then once reactivated can cause significant disease in its host as well as be transmitted to naïve hosts. Because the genome alone resides in very few host cells during latency, there is very little opportunity to interfere with latency or prevent reactivation. The pivotal focus on investigating latency and reactivation is identifying viral gene expression in order to determine a target for therapeutic intervention.

The goal of these studies was to identify and characterize viral genes expressed during latency for KHV in its natural host, the koi. We first identified the peripheral B lymphocyte as the main host cell in which KHV goes latent. By isolating IgM+ peripheral WBC or by using an internal RNA probe there was ample access to latently infected host material that was humanely acquired, as the fish were anesthetized for phlebotomy and then recovered. Because KHV has B cells as its main cell type for latency, it can be considered a lymphotropic herpesvirus, reminiscent of gammaherpesviruses such as KSHV and EBV of humans.

Next, I described the identification of the expression of viral gene ORF6 during KHV latency. ORF6 is a hypothetical protein that has never been investigated nor characterized in the KHV genome. Conserved domain searches of the ORF6 protein sequence identified homology to transcription transactivators that are important in the molecular latency programs of other herpesviruses. It would be interesting to discover the function of ORF6 and compare it to latency-associated transcripts of other herpesviruses. These studies also confirmed that ORF6 is translated to protein that is present in both productive infections *in vitro* as well as latent infections *in vivo*. Antibodies used in these studies were raised against

synthetic amino acids from the predicted C-termial region of ORF6. Additionally, a construct was designed to produce an *in vitro* recombinant full-length protein of ORF6; it would be interesting to raise antibodies to the entire native ORF6 protein for further characterization and investigation into KHV latency.

The transcriptome results from Chapter 4 suggest that ORF6 is the main viral gene transcribed during latency; this provides an optimistic possibility for working with a single target for intervention of latency for KHV. The next conceivable step in the continuation of this project is to characterize the function of ORF6 during KHV latency. There can be two approaches to this: the first is to block the transcription of ORF6 by inhibiting the promoter with PMO oligos and investigating latency phenotypes in infected fish. Our lab has previously worked with PMOs to block the transcription of genes causing disease for HSV-1. Blocking the promoter region near the start codon for ORF6 will prevent its transcription thereby blocking its ability to function. Fish with and without application of PMO would be infected with KHV and survivors would be latently infected. It is plausible, therefore, to test for latency by molecular methods described in this dissertation to see if treating with PMOs will prevent the establishment of latency. Additionally, fish treated with and without PMOs would be exposed to temperature stress by increasing water temperatures, encouraging reactivation of KHV from latency and assess if PMOs affect KHV's ability to reactivate from latency. The second method for investigating function of ORF6 during latency would be to construct a recombinant virus with a deletion for ORF6 and determine if the deletion mutant virus has a different latency and reactivation phenotype when compared to wild type virus. Because ORF6 has homology to Epstein Barr virus EBNA-3B, it would be interesting to see if replacing the ORF6 deletion with recombinant EBNA-3B would recover the wild-type latency and reactivation phenotype.

The works in this dissertation also highlight a novel natural host model for studying herpesvirus latency. To study herpesvirus latency and reactivation, an ideal host and virus model system would (1) harbor latent herpesvirus infection in an easily

accessible location, (2) enable non-terminal collection of an adequate supply of infected cells, (3) have a long host lifespan to facilitate longitudinal studies, (4) offer a highly sensitive and quantitative assay for latent infection, (5) be cost-effective for host infection studies, and (6) benefit from the well-established host genomics tools. KHV and its natural host, the koi, satisfy all these criteria. KHV latency in koi has great potential to serve as a novel and humane platform for exploring the treatment of latency in a natural host model, and could provide continued access to latently infected cells during latency and reactivation studies as well as collecting data from the same host at several different time points, such as gene expression changes before and after treatment, without sacrificing the animals.

The studies conducted in this dissertation have established a pathway to a new frontier in investigating herpesvirus latency. By continuing these works in ORF6 it is plausible that the complete elimination of KHV latency is just within reach.

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