THE COMPLETE NUCLEOTIDE SEQUENCE AND CHARACTERIZATION OF THE PSITTACID HERPESVIRUS 1 (PsHV-1) GENOME

by

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A thesis submitted to the Faculty of the University of Delaware
in partial fulfillment of the requirements for the degree of
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Without Light, there is only Darkness… But without Darkness, what is Light?

This manuscript is dedicated to my wife Sheri for her unwavering love, support and understanding, and to my girls, Madison and Sydney.
I would not be who I am without you.

Mom and Dad, thanks for believing.

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ABSTRACT

Dean Richard Thureen. The Complete Nucleotide Sequence and Characterization of the Psittacid Herpesvirus 1 (PsHV-1) Genome. (Under the direction of Dr. Calvin L. Keeler, Jr.)

Exotic birds, Psittaciformes in particular, continue to rise in popularity in North America, and such animals represent a small but growing and significant fraction of companion animal accessions to diagnostic laboratories (O'Toole *et al*., 1992). Consequently, Pacheco's disease, a psittacine herpesvirus infection, has become of increased concern to breeders and veterinarians. Mortality from Pacheco's disease can be very high and results in considerable economic loss to the pet bird industry. Often, captured young birds are quarantined together, under stressful conditions which promote the shedding of the virus from the pharynx and in the feces. Although the clinical signs are variable, infected birds are often lethargic, have inconstant diarrhea, and exhibit various lesions and hemorrhages of the liver and spleen. Secondary respiratory and renal complications may occur and mortality often approaches 100%.

Utilizing the recent advances in the expanding fields of bioinformatics and genomics technology, we have sequenced and characterized the causative agent for Pacheco's Disease virus (PDV), hereby designated PsHV-1 (Psittacid herpesvirus 1).

The PsHV-1 genome was determined to be 163,025 bp in length with a base composition of 60.95% G+C. The genome consists of two unique sequences, the long or

U_L (119,146 bp) and the short or U_S (16,405 bp), with the U_S region being flanked by inverted (I_R) and terminal (T_R) repeat elements 13,737 bp in length.

The PsHV-1 genome contains 73 putative genes and shares conserved homology with 70 of 77 genes identified within the genome of Infectious Laryngotracheitis virus (ILTV), an avian alphaherpesvirus. Similarities between PsHV-1 and ILTV can be seen in an inversion between U_L44 and U_L22 in the U_L region, the absence of repeats flanking the U_L region, the conserved structure of the U_S region, and a conserved cluster of 5 unique ORFs in the U_L region. PsHV-1 and ILTV also share positionally conserved genes in the U_S region that are novel to these virus genomes.

The similarity of the PsHV-1 (*psittacid*) and ILTV (*gallid*) genome structure and content, combined with phylogenetic analysis, suggests that PsHV-1 and ILTV represent a unique clade of avian alphaherpesviruses that are distinct from the Marek's family of alphaherpesviruses. We therefore recommend that PsHV-1 be classified as an avian alphaherpesvirus, genus *Iltovirus*.

Chapter 1

INTRODUCTION

1.1 Herpesviruses.

1.1.1 Classification of Herpesviruses.

The virus family *Herpesviridae* is divided into three subfamilies, the *Alpha-*, *Beta-*, and *Gammaherpesvirinae*, based upon their distinct biological properties, and more recently on the basis of their genomic content (Minson *et al.*, 2000). Herpesviruses are highly disseminated in nature, and have been identified in insects, reptiles, amphibia, invertebrates, and most species of birds and mammals (Comps and Cochennec, 1993; Rawlinson *et al.*, 1996).

The *Herpesviridae* vary in their biological characteristics. The *Alphaherpesvirinae* are classified on the basis of their variable host range, short replication cycle, rapid and efficient infection in culture, and their ability to establish latent infections within neural ganglia. Betaherpesvirus classification is also based on host specificity, length of the replication cycle, and cytopathic effects (CPE). *Betaherpesvirinae* replicate slowly in cell culture, disease develops slowly in the host, and some host cells are more susceptible to infection than others. The members of the

subfamily *Gammaherpesvirinae* are grouped based upon their preference for lymphoblastoid cells, and their ability to produce tumors (Rawlinson *et al.*, 1996).

The herpesviruses share several common properties, including a large array of conserved enzymes involved in nucleic acid metabolism, DNA synthesis, and protein processing. Herpesviruses also share the ability to remain latent in the infected host (Rawlinson *et al.*, 1996).

Phylogenetic studies using herpesvirus DNA sequences demonstrate a clear division of the subfamilies, and have resulted in the re-classification of some viruses from one subfamily to another (Buckmaster *et al.,* 1988; VanDevanter *et al*., 1996). The genomes of herpesviruses isolated from mammalian and avian hosts clearly descend from a common ancestor, but with significant variation in nucleotide sequence, gene content and genomic organization (McGeogh *et al*., 2000). An extensive amount of herpesvirus DNA sequence data is available, from single-gene analysis to genome-wide comparisons (McGeogh *et al*., 2000). The number of completed herpesvirus genomes now approaches 40 (www.ncbi.nlm.nih.gov/Genomes/VIRUSES/viruses.html).

1.1.2 Molecular Biology and Structure of Alphaherpesvirus Genomes.

Within the alphaherpesviruses, four genera have been established on the basis of genome sequence similarity: the genus *Varicellovirus* (prototype species varicella-zoster virus [VZV]), the genus *Simplexvirus* (prototype species herpes simplex virus type 1 [HSV-1]), the genus *Iltovirus*, or infectious laryngotracheitis-like viruses (prototype species infectious laryngotracheitis virus [ILTV]), and the genus *Mardivirus*, or Marek's

disease-like viruses (prototype species Marek's disease virus [MDV]) (International Committee on Taxonomy of Viruses; ICTVdb, 2004).

The genome of the prototype alphaherpesvirus, *herpes simplex,* is composed of two major segments, the unique long (U_L) and unique short (U_S), based upon their relative length. These segments are each flanked by inverted repeat sequence regions. The genes found in the unique regions are present in the genome as a single copy, but genes that are encoded in the repeat regions, such as ICP0 or ICP4, are present in the genome in two copies. Viral genes and their protein products are typically named for their relative position from left to right in the U_L or U_S region (e.i. U_L1, U_L2 or U_S1, U_S2). Unfortunately, many HSV-1 viral proteins have also been designated by alternate names, such as infected cell protein (ICP) number, virion protein (VP) number, or molecular weight, providing a somewhat confusing nomenclature for the herpesvirus researcher.

Pseudorabies virus (PRV) is a member of the *Alphaherpesvirinae* subfamily and is the causative agent of Aujeszky's disease. Based on genomic sequence information, PRV has been grouped in the genus *Varicellovirus* together with other important animal pathogens, such as bovine herpesvirus 1 (BHV-1) and equine herpesviruses 1 and 4 (EHV-1 and EHV-4). The PRV genome is similar in arrangement to the genomes of EHV-1, BHV-1, and VZV, consisting of U_L and U_S segments. The U_L region, unlike HSV, is not bracketed by repeat sequence. The U_S region is bracketed by inverted repeat sequences, similar to ILTV, which allows for the formation of two distinct isoforms of the genome. The biological relevance of this ability to invert the U_S region remains

unclear. The genomes of ILTV, MDV, PRV and HSV-1 are largely collinear, with the exception of a fairly conserved inversion of part of the U_L regions of PRV and ILTV as compared to HSV-1 and MDV. Again, the biological significance of this inversion is not known (Klupp *et al.*, 2004).

A *Herpesviridae* virion ranges from 180-200 nm in diameter and is composed of four major structural features; the envelope, tegument, capsid and core. The envelope is derived from the nuclear membrane and virally-encoded glycoproteins. A protein-filled tegument region lies between the capsid and the envelope. The capsid is icosahedral, 95-105 nm in diameter, and composed of 162 hexagonal capsomers, with a toroidal core about 75 nm in diameter. Herpesviruses have large and complex DNA genomes which are linear, double-stranded, and 130-230 kb in length (Roizman and Spears, 1996). The herpesviruses cannot be differentiated by virion morphology, or by their respective replication cycles. However, significant differences between subfamilies are apparent in their effect on host cells and in their genomic structure.

The genomes of the herpesvirus families are represented by six classes (designated A-F) based upon genome organization and sequence arrangement (Fields and Knipe, 1991). HSV-1 is classified as a class E genome structure, while PRV has a class D genome structure.

1.1.3 Lifecycle of the Herpes Simplex Virus (Prototype Alphaherpesvirus).

The lifecycle of the alphaherpesvirus HSV-1 has been extensively studied, and offers valuable insight into herpesviruses in general. Many of the stages identified within

HSV-1 infection and replication can be directly correlated to other members of the family. Below is an overview of the HSV-1 lifecycle.

1.1.3.1 Attachment.

Entry of HSV-1 into its host cell proceeds systematically, involving a number of viral envelope proteins, including glycoprotein B (gB), glycoprotein C (gC), glycoprotein D (gD), and the glycoprotein H and glycoprotein L (gH/gL) heterodimer. Virus attachment is initiated by interactions between virion gC and heparan sulfate moieties on the surface of the host cell (Herold *et al*., 1991). While this interaction enhances infection, it is not essential, as gC has been found to be dispensable for growth and replication *in vitro* (Herold *et al*., 1991), and cells that do not express heparan sulfate on their surfaces remain permissive to HSV-1 infection (Shieh *et al*., 1992; WuDunn and Spear, 1989). Virion attachment is stabilized by interactions between the gD protein and one of a number of cellular receptors, collectively referred to as the Herpesvirus entry (Hve) proteins (Spear *et al*., 2000). The Hve receptors are members of the tumor necrosis factor (TNF) family of proteins (including HveA) (Montgomery *et al*., 1996; Whitbeck, 1997; Roizman and Knipe, 2001), and the immunoglobulin superfamily, to which HveB, HveC, and HIgR are grouped (Warner *et al*., 1998; Geraghty *et al*., 1998; Cocchi, 1998).

1.1.3.2 Fusion and Penetration.

Following attachment, the HSV-1 envelope fuses with the host cellular membrane by a mechanism that requires gD (Ligas and Johnson, 1988), gB (Sarmiento *et al.*, 1979),

and the gH/gL heterodimer (Forrester *et al.*, 1992). Immediately following membrane fusion, the viral nucleocapsid and tegument proteins move into the host cytoplasm. Some of these tegument proteins (U_L48, U_L36), along with the nucleocapsid, are transported to the nucleus via microtubules, while other tegument proteins (U_L41, U_S11) remain in the cytoplasm (Sodeik *et al.*, 1997).

1.1.3.3 Transcription.

Herpesviruses utilize a number of host cellular functions to express the viral proteins necessary for their replication. The virus must express two major types of proteins, the non-structural proteins necessary for viral DNA replication, and the structural proteins needed to make up the virion. All herpesviruses characterized to date share the common characteristic of a regulated, temporal cascade of gene expression (Honess and Roizman, 1974; Roizman and Knipe, 2001).

In HSV-1, genes are expressed in 3 major temporal classes as immediate early (IE or α), early (E or β) and late (L or γ) genes. While these classes are convenient for use in literature as nomenclature, the actual progression of viral gene expression is more gradual, and somewhat overlapping, rather than in distinct stages. Immediate early (IE) genes are the first to be transcribed upon infection and activate transcription of the early (E) genes, whose gene products function to replicate viral DNA. The proteins involved in viral DNA replication in turn stimulate the expression of the late (L) genes, encoding the structural proteins.

After the viral DNA enters the nucleus via nuclear pores, transcription of the IE viral genes begins (Alwine *et al*., 1974; Costanzo *et al*., 1977). While cellular proteins are utilized for the synthesis of viral transcripts (e.g. host RNA polymerase II is responsible for synthesis of all viral mRNAs), viral proteins are necessary for the initiation and enhancement of transcription of some viral genes. There are a number of essential viral proteins which interact closely with specific cellular proteins to produce the viral proteins needed for a productive viral infection.

The first genes transcribed during viral infection are the immediate early (IE) or α genes. Transcription proceeds by recruitment of the host cellular transcriptional machinery to IE gene promoters that contain numerous regulatory sequences. IE gene expression does not require prior HSV protein synthesis. The virion protein U_L48 (VP16; α-TIF), however, plays an important role in enhancing the expression of IE proteins (Batterson and Roizman, 1983). U_L48 is known to interact with a number of cellular proteins, including HCF, Oct-1, TFII-B and TFII-D (Katan *et al*., 1990; Stern and Herr, 1991; LaBoissiere and O'Hare, 2000). The interaction between U_L48 and HCF within the cytoplasm is believed to result in the movement of U_L48 into the nucleus (LaBoissiere and O'Hare; 2000), where it binds to cellular Oct-1, which is bound to viral DNA at conserved consensus sites (TAATGArATT). U_L48/Oct-1 binding enhances the activity of the cellular transcriptional machinery assembled on the IE gene promoters. The IE proteins are composed of several multifunctional proteins that play essential roles in the regulation of later viral and host cell gene expression.

Within HSV-1, the immediate early proteins ICP4, U_L54 (ICP27), and ICP0 initiate the transcription of the early genes. ICP4 activates early gene expression by interacting with the promoter sites of several early gene cellular transcription factors (Smith *et al.*, 1993; Carrozza and DeLuca, 1996), and U_L54 is known to play a role in the expression of some E proteins (Samaniego *et al*., 1995; McGregor *et al*., 1996; Uprichard and Knipe, 1996;). ICP0 interacts with cellular proteins to activate transcription and to alter the host cell environment, making the host more conducive to viral protein synthesis and viral DNA replication (Uprichard and Knipe, 1996).

The early proteins are generally involved in viral DNA replication. The viral single-stranded DNA binding protein U_L29 (ICP8) is an early protein involved in transcriptional regulation. Beyond its role in DNA replication, U_L29 is also required for the transcription of late genes (Gao and Knipe, 1991; Chen and Knipe, 1996), and it is believed that U_L29 is involved in the rearrangement of the viral genome to allow specific late-transcription factors access to viral promoters (McNamee *et al*., 2000).

U_L29 also plays a role in limiting transcription from the parental genome. One major event between early and late gene transcription is believed to be the transfer of the transcription complex machinery from the parental to the progeny genomes. Therefore, if transcription of the parental genome is limited, immediate early and early gene expression is reduced (Godowski and Knipe; 1983, Godowski and Knipe, 1985). In addition to initiating the expression of its own genes, HSV-1 shuts down host cellular RNA, DNA and protein synthesis, disrupting host transcription, replication and

translation (Roizman and Tognon, 1983), and subsequently utilizes the cellular components for the synthesis of its own proteins.

The IE proteins initiate the transcription of not only the E genes involved in DNA replication, but also a subset of late (L) genes, (also known as early/late, leaky late, or γ1 genes). While the expression of these L proteins is not dependent on viral DNA replication, their levels are significantly increased upon initiation of DNA replication. A second subset of late genes (true/late or γ2 genes) is only transcribed after the initiation of viral DNA replication.

An important regulatory event in the kinetics of gene expression is the shutdown of IE and E gene expression. ICP4 represses itself by binding to repressor elements in its own promoter. The subsequent decreased levels of ICP4 therefore lead to a reduction of early and late gene expression (Kristie and Roizman, 1984; Faber and Wilcox, 1986; Muller, 1987; Leopardi *et al*., 1995).

HSV-1 disrupts the host cell in a number of ways. First, the virion host shut-off protein (vhs; U_L41) degrades existing cellular mRNAs early during infection (Oroskar and Read, 1987; Kwong *et al*., 1988; Zelus *et al*., 1996; Elgadi and Smiley, 1999; Karr and Read, 1999). Multiple viral gene products interfere with cellular transcription and translation (Wagner and Roizman, 1969; Hardwicke and Sandri-Goldin, 1994). For example, U_L54 (ICP27) inhibits cellular RNA maturation by redistributing splicing factors (Phelan *et al*., 1993; Sandri-Goldin *et al*., 1995). Given that very few HSV-1 viral transcripts are spliced, this probably gives viral mRNA a competitive advantage for translational machinery (Sandri-Goldin and Hibbard, 1996). U_S1 (ICP22), is required for

modification of host RNA polymerase II following infection (Spencer *et al*., 1997), altering its ability to transcribe from the host genome. HSV-1 also degrades a variety of cellular proteins involved in the regulation of the host cell cycle (Advani *et al*., 2000).

1.1.3.4 DNA Replication.

HSV-1 DNA replication occurs in specialized structures within the nucleus of the infected host cell, known as replication compartments (Roizman and Knipe, 2001). Viral DNA and replication proteins localize adjacent to cellular structures (ND10 sites), and the replication compartments form at these sites during viral DNA synthesis (Ishov and Maul, 1996). Upon entry into the nucleus, the linear viral DNA circularizes (Uprichard and Knipe, 1996). Viral DNA replication begins at one of the three origins of replication within the HSV-1 genome and initially proceeds via a theta (θ) replication mechanism. Once DNA synthesis begins, a rolling-circle replication mechanism produces progeny genomes in the infected cell as concatomers (Jacob *et al*., 1979). Seven herpes genes have been identified by genetic and biochemical methods as essential for viral DNA replication (Nishiyama, 2004). These genes encode for products that function as the origin binding protein (Ori; U_L9) (Elias *et al*., 1986), the single-stranded DNA binding protein U_L29 (ICP8) (Conley *et al*., 1981), a helicase-primase complex ($U_L5/U_L8/U_L52$), and a DNA polymerase complex (U_L30/U_L42) (Purifoy *et al*., 1977).

While HSV-1 encodes many of the proteins required for its own viral DNA synthesis, other cellular factors, such as DNA ligases and topoisomerases also function in viral DNA replication. The origin binding protein (U_L9) of HSV-1 provides the ATP-

binding and DNA helicase functions required for viral DNA replication (Elias and Lehman, 1988). U_L9 binds specifically as a homodimer to sites in the origin of replication which contain the sequence CGTTCGCACTT (Elias and Lehman, 1988; Koff and Tegtmeyer, 1988). The binding of U_L9 to this Ori sequence causes the formation of a single-stranded stem loop structure. The resulting ssDNA and U_L9 then recruit U_L29, a single-stranded DNA binding protein, which preferentially binds to single-stranded DNA and enhances the helicase activity of U_L9 (Ruyechan, 1983; Lee and Knipe, 1985; Boehmer *et al*., 1993). U_L29 is required for viral DNA synthesis and the efficient expression of late genes. In addition to its DNA binding activity, U_L29 has a DNA helix-destabilizing activity (Dutch and Lehman, 1993) and can also promote the re-naturation of complementary single DNA strands. U_L29 also promotes the helicase-primase activities of the helicase-primase core subunit U_L5/U_L52 in the presence of U_L8 (Falkenberg *et al*., 1997; Tanguy-LeGac *et al*., 1998) and modulates the polymerase activity of U_L30 (Chiou *et al*., 1985). The ability of U_L29 to interact with other viral replication and cellular proteins supports the idea that U_L29, acting as a scaffolding protein for viral DNA replication, functions to translocate replication proteins to the correct site in the nucleus. U_L29 is also required for the formation of viral replication compartments in the nucleus of infected host cells (de Bruyn Kops and Knipe, 1988; Bush *et al*., 1991).

The helicase-primase complex is a heterotrimer with 5'-3' helicase activity and is composed of the gene products of U_L5, U_L8, and U_L52 (Crute *et al*., 1988). Like U_L9, the U_L5 protein contains conserved ATP-binding and helicase motifs. The U_L52 protein has

a divalent metal binding motif similar to other DNA primases. The helicase, primase, and ATP-ase enzymatic activities are contained in a sub-complex of U_L5 and U_L52, and the presence of U_L8 (and U_L29) enhances the enzymatic activity of U_L5 and U_L52 (Crute *et al*., 1988).

The DNA polymerase holoenzyme consists of the U_L30 catalytic subunit and its accessory factor, U_L42 (Keir and Gold, 1963; Gibbs *et al*., 1988). U_L30 also has an intrinsic 3'-5'-exonuclease, proof-reading activity. The presence of U_L42 increases the processivity of U_L30, and the interaction between U_L30 and U_L42 is required for viral DNA replication (Nishiyama, 2004).

In addition to these essential viral replication proteins, HSV-1 expresses several other early viral proteins, such as thymidine kinase (U_L23) (Kit and Dubbs, 1965), ribonucleotide reductase (U_L39/U_L40) (Bacchetti *et al*., 1986), and uracil DNA-glycosylase (U_L2) (Caradonna *et al*., 1987). Although these proteins are considered nonessential for HSV viral DNA replication, many of these genes encode protein products that function in nucleotide metabolism (Roizman and Knipe, 2001).

The thymidine kinase (TK) protein has been identified as a target for the development of anti-viral therapies. In addition to its role of phosphorylating nucleosides for use in viral DNA synthesis, TK will phosphorylate and subsequently activate anti-viral nucleoside analog drugs, such as acyclovir (Fyfe *et al*., 1978). These analogs are then incorporated into viral DNA during replication, blocking chain elongation.

1.1.3.5 Assembly and Egress.

The assembly of the viral capsid of HSV-1 requires many of the late proteins. Assembly of these gene products occurs within the nucleus, but as several of these capsid proteins (U_L19 (VP5), U_L35 (VP26), U_L18 (VP23)) lack nuclear localization sequences (NLS), the proteins must form complexes with NLS-containing proteins (U_L38 (VP19C) or U_L26.5 (pre-VP22a)) in the cytoplasm for eventual transport to the nucleus (Newcomb *et al*., 1996).

The HSV-1 capsid is comprised of an outer shell which consists of penton-shaped subunits of the major capsid protein, U_L19, and hexons of U_L19 and U_L18 (Newcomb *et al*., 1993; Newcomb *et al*., 1996), connected by triplex structures formed by the two minor capsid proteins, U_L38 and U_L18. Two non-structural genes which encode multiple proteins, U_L26 (VP21/VP24) and U_L26.5 (pre-VP22a/VP22a), are also required for capsid maturation. The gene products of U_L26 and U_L26.5 are thought to perform a scaffolding function, similar to U_L29.

Viral DNA is loaded into empty capsids by a process that cleaves the concatemers and packages genome-length monomers within the capsid (Ladin *et al*., 1980; Ladin *et al*., 1982; Roizman and Knipe, 2001) to form a nucleocapsid. This capsid assembly process also requires other viral proteins, including U_L6, U_L15, U_L25, U_L28 (ICP18.5), U_L32, U_L33, U_L36 (ICP1/2), and U_L37 (Roizman and Knipe, 2001). Capsid assembly/maturation does not require any cellular factors (Newcomb *et al*., 1999).

The exact route of HSV-1 virion egress from an infected host cell remains unclear and is still under debate (Leuzinger *et al*., 2005; Mettenleiter and Minson, 2006). It has

been shown that the nucleocapsid buds through the inner nuclear membrane (Vlazny *et al*., 1985), acquiring some tegument proteins and the glycoprotein envelope during the process, but from this point, two egress pathways have been proposed. The re-envelopment model suggests that enveloped virions fuse with the outer nuclear membrane and release intact nucleocapsids into the cytoplasm that are then re-enveloped by budding into the Golgi. These re-enveloped particles are then secreted from the cell by a vesicular route (Siminoff and Menefee, 1966). More recently, Enquist *et al*. (1999) proposed a lumenal pathway model in which enveloped virions travel from the inner nuclear space to the Golgi in vesicles or within the lumen of the endoplasmic reticulum (ER) and are then released from the cell by the secretory route. Recent evidence using electron microscopy and glycoproteins isolated from the ER lends considerable support to the re-envelopment model as the major route of HSV egress (Granzow *et al*., 2001; Skepper *et al*., 2001), but the debate continues (Leuzinger *et al.*, 2005).

1.1.3.6 Latency.

One of the hallmarks of all herpesviruses is their ability to establish a latent infection that can persist for the lifetime of the host and which can, under certain conditions, reactivate as a productive lytic infection. "Latency" is defined as the retention of functional viral genomes in sensory neurons without the production of infectious virions and "reactivation" as the multi-step process leading from latency to virion assembly. It was believed that during HSV-1 latency, no viral progeny are produced and very limited viral transcription can be detected. In HSV-1 infections, the major site for

latent infection is sensory neurons in ganglion tissue (trigeminal ganglia for HSV-1 or sacral ganglia for HSV-2).

Following a primary HSV infection, usually at an oral or genital mucosal surface, the virus travels along the axon to the neuronal cell body. Once within the neuron, the virus enters a dormant state in which no lytic gene products are produced. During latency, the genome remains in the nucleus of the neuron as circular, extra-chromosomal DNA (Rock and Fraser, 1985; Mellerick and Fraser, 1987). In latently infected neuronal cells, the only major viral transcripts that can be detected are a family of RNAs referred to as the latency associated transcripts or LATs. A full-length 8.3-kilobase (kb) primary LAT transcript accumulates to low levels in infected neurons. Stable 2.0-and 1.5-kb introns derived from the full length LAT, however, are very abundant (Rock *et al*., 1987; Stevens *et al*., 1987; Krause *et al*., 1988). The LAT transcripts may also have other functions. It has been shown that LAT-negative viruses have increased immediate early gene expression in neurons, suggesting that LATs may limit viral gene expression (Garber *et al*., 1997) which would promote the latent state. It has also been hypothesized that LATs act by an antisense mechanism because a portion of the 8.3-kb LAT partially overlaps with the ICP0 and ICP4 mRNAs (Chen *et al*., 1997). More recently, it has also been suggested that these transcripts may protect infected neurons from apoptosis, allowing neuronal survival during latent infection (Perng *et al*., 2000).

It was believed that the lack of transcriptional activity allowed the virus to remain in the cell, thereby avoiding normal immune surveillance for the lifetime of the host, or until specific signals (such as stress, illness or exposure to UV light) reactivated the lytic

replication cycle and new progeny were produced (Roizman and Spears, 1996). Progeny virus then traveled back through the neuron axis to the site of the primary infection to re-initiate a new lytic replication cycle (Carton and Kilbourne, 1952).

But the popular concept that HSV-1 latency is characterized by a complete lack of lytic gene expression has been called into question by more recent studies. Decman *et al.* (2005) have demonstrated that HSV-1-specific $CD8^+$ T cells and the cytokine IFN-γ are persistently present in trigeminal ganglia harboring latent HSV-1, and that IFN-γ acts on latently infected neurons to inhibit HSV-1 reactivation, expression of the promoter activity of ICP0 (required for reactivation), gC promoter activity, and reactivation in neurons in which the ICP0 or gC promoter is active.

This suggests that HSV-1 latency is not necessarily characterized by a silent viral genome and a passive host immune system, but involves a more dynamic interaction in which viral genes are persistently expressed in latently infected neurons.

1.2 Avian Herpesviruses.

Among avian hosts, the herpesviruses are species-specific with regards to susceptibility, routes of infection, pathogenicity and virulence. To date, avian herpesviruses have been isolated from owl (*strigrid*), chicken and pheasant (*gallid*), turkey (*meleagrid*), pigeon (*columbid*), falcon (*raptor*), crane (*gruiformid*), duck (*anatid*), quail (*perdicid*), cardinal (*cardinalis*), finch (*telespyza*), starling (*aplonis*), vulture (*gyps*), canary (*passeriform*), and parrot (*psittacid*) (Aini *et al*., 1993; Günther *et al*., 1997**;** Cardoso *et al.*, 2005).

Similar to the difficulties observed with mammalian herpesviruses, avian herpesviruses are also difficult to classify based solely on their biological properties or host range. The application of more recent technologies, such as genome sequencing, has lead to the suggested reclassification of several of the avian herpesviruses.

Members of the avian subfamily *Gammaherpesvirinae* have a relatively narrow host range. All members replicate in lymphoid cells, and some may cause cytocidal infections in epithelial and fibroblastic cells (Fenner *et al*., 1987). Viruses within this subfamily are specific for either T or B cells, and transform these cells into tumors. Latent virus can be readily isolated from lymphoid tissue. Unclassified avian herpesviruses suspected to be *Gammaherpesvirinae* include strigrid herpesvirus 1 (owl hepatosplenitis herpesvirus) and perdicid herpesvirus 1 (bobwhite quail herpesvirus) (ICTV; http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/index.htm).

Avian *Betaherpesvirinae* have not been identified to date, but the crane herpesvirus (*gruid*) has been tentatively classified as a betaherpesvirus (Foerster *et al.,* 1989).

The avian alphaherpesviruses are characterized by a narrow host range and their ability to cause consistent hemorrhagic vascular and tissue damage. Marek's disease virus (MDV) was originally classified as a gammaherpesvirus based on the lymphotropic biology of the virus, but recent insight into MDV genome structure has resulted in the reclassification of MDV as a *Mardivirus* within the alphaherpesvirus subfamily (Buckmaster *et al*., 1988; ICTV http://www.ncbi.nlm.nih.gov/ICTVdb/index.htm).

Gallid herpesvirus 1 (GaHV-1; infectious laryngotracheitis virus; ILTV) is classified as an *Iltovirus* within the *Alphaherpesvirinae*, and causes acute, contagious infections in chickens, while anatid herpesvirus 1 (duck plague; duck enteritis) and columbid herpesvirus 1 (pigeon herpesvirus) also infect domestic and wild fowl, yet remain unclassified.

Psittacid herpesvirus 1 (PsHV-1) has been classified as either a beta- or a gammaherpesvirus, based on observations that the virus targets hepatocytes and lymphocytes and slowly forms syncytial plaques in tissue culture (Kaleta, 1990). More recently, phylogenetic studies of the viral U_L16 and U_L30 gene sequences have shown PsHV-1 to be most closely related to the alphaherpesvirus, ILTV (Günther *et al.*, 1997; Tomaszewski *et al.*, 2001; Tomaszewski *et al.*, 2003). ILTV is also a highly contagious and economically significant avian herpesvirus. Natural infections are limited to galliform birds and can be responsible for significant mortality and loss of productivity in commercial flocks.

1.3 Psittacid Herpesvirus (PsHV).

1.3.1 Historical Perspective.

Following a human pandemic of psittacosis (an infectious disease caused by *Chlamydia psittaci* which causes flu-like symptoms), which occurred in Europe and the United States during the winter of 1929-30, Pacheco and Bier sought to obtain evidence of the presence of psittacosis in Brazil. In 1930, they described a disease in parrots, caused by a filterable agent, which produced nuclear inclusions in affected host cells

(Gaskin *et al*., 1981). Rivers and Schwentker of the Rockefeller Institute obtained the agent from Pacheco and reported in 1932 that it was a virus unrelated to the agent of psittacosis (Rivers and Schwentker, 1932). The condition was coined Pacheco's parrot disease (PD) by Findlay (1933). The herpes group of viruses was among the PD agents suspected by Rivers and Schwentker, but it was the work of Simpson *et al*. (1974) and Simpson and Hanley (1976) that confirmed the cause of the disease as a herpesvirus. The virus was classified as a herpesvirus on the basis of electron microscopy, ether sensitivity, filtration, and the presence of intranuclear inclusion bodies in infected hepatocytes (Cho and McDonald, 1980). Today the virus is referred to in several ways; Pacheco's parrot disease virus, taken as a synonym for a herpesvirus-induced disease in psittacine birds, parrot herpesvirus, psittacid herpesvirus 1 (PsHV-1) and Pacheco's disease virus (PDV) are all accepted terms for the disease and the virus.

Most parrot species, originating from multiple geographic regions, are susceptible to Pacheco's disease. However, Amazon parrots (*Amazona* spp.) account for the majority of cases, followed by African grey parrots (*Psittacus erithacus*), macaws (*Ara* spp.), cockatoos (*Cacatua* spp.), and conures (*Aratinga* spp. and *Pyrrhura* spp.). Morbidity in Asian and Australian parakeets is usually only sporadic. Most conures are resistant, but they may be natural hosts in the wild and serve as asymptomatic shedders of the virus when stressed. Many other species may act as carriers (Gerlach, 1994).

Recent evidence also suggests that one or more PsHV genotypes are associated with internal mucosal papillomatosis of parrots (Styles *et al*., 2004). This debilitating

disease is common in imported and captive-raised Amazon parrots and macaws, in which it causes wart-like lesions in the oral cavity, upper digestive system, and vent.

1.3.2 Transmission and Host Range.

Stressful situations, such as exposure to sudden cold weather or relocation to unfamiliar locations, are predisposing factors for the appearance of PD clinical symptoms (Cheville, 1978). Therefore, disease outbreaks are commonly observed in recently imported birds (Hitchner and Hirai, 1979; Miller *et al*., 1979; Kaleta *et al.,* 1980). Outbreaks of PD may occur after the introduction of an infected psittacine bird (Martin *et al*., 1979; Miller *et al*., 1979; Gomez-Villamandos, 1991), but also may be seen in resident birds of a flock with no history of recent infection (Simpson and Hanley, 1977).

Known naturally susceptible hosts of PsHV include the following bird groups: macaws (*Ara*), Amazons (*Amazona*), conures (*Aratinga* and *Cyanoliseus*), African Grey parrots (*Psittacus*), lovebirds (*Agapornis*), lories (small Australasian parrots of the subfamily *Loriinae*) of the genus *Eos*, parakeets of the genus *Psittacula*, cockatoos (*Cacatua*), budgerigars (*Melopsittacus*), king parrots (*Alisterus*), cockatiels (*Nymphycus*), and superb starlings (*Lamprotornis superbus*) (Gerlach, 1994; Tomaszewski *et al*., 2004). Chick embryo fibroblasts are suitable hosts for propagating the virus (Findlay, 1933).

1.3.3 Clinical Diagnosis and Pathology.

The clinical picture of PsHV infection is non-specific and variable. Clinical signs may include somnolence, lethargy, anorexia, bristling or ruffled feathers, inconstant

diarrhea, bright yellow or green urates with scant feces (indicating liver damage), terminal anorexia, and in some macaws, visible jaundice and death (Panigraphy and Grumbles, 1984). Histology of post-mortem tissues may be necessary to confirm a diagnosis of PD.

Upon necropsy, infected birds demonstrate hepatomegaly (enlarged livers that may be mottled or have other color changes), splenomegaly, friability and diffuse patches of necrosis. PsHV may also cause liver lesions that mimic chlamydia infection. These lesions tend to be saucer-shaped and cause a faint yellow discoloration. Petechiae (pin-point hemorrhages) can be found on the coronary band of the heart, the ventriculus, and in mesenteric fat. Edema of the mesenteric fat and ascites may also be observed. Eosinophilic or acidophilic intranuclear inclusion bodies (Cowdry type A) are found in infected liver and spleen tissues. Secondary respiratory or renal complications are also common (Gerlach, 1984). Conjunctivitis, rhinitis, and sinusitis, as well as hemorrhagic enteritis, may also be observed. Watery diarrhea is commonly observed 10-12 days prior to death (Panigraphy and Grumbles, 1984; Gomez-Vilamandos *et al*., 1991).

Monolayers of primary chicken embryo fibroblast (CEF) cells inoculated with allantoic fluid from PsHV-infected specific pathogen free (SPF) eggs via the yolk sac show cytopathic effect (CPE) 48 to 72 hours post-inoculation. The CPE is characterized by large, refractile round cells with marked clumping (multinucleated giant cells; syncytial cells), which is consistent with other herpesvirus infections. Upon electron microscopic (EM) examination of cell-culture fluids, both enveloped and non-enveloped virus particles with a characteristic herpesvirus morphology, are observed (Panigraphy

and Grumbles, 1984). Hitchner and Hirai (1979) described the ability to grow PsHV on the chorioallantoic membranes (CAMs) of 10-day-old embryonated eggs, and the availability of a fluorescein-labeled antibody to PsHV allows the rapid diagnosis of PsHV infection by immunofluorescence (O'Toole *et al*., 1992).

1.3.4 Prevention and Control.

Control of PD is possible only by strict hygiene and the quarantine of newly imported birds. Since birds can asymptomatically carry PsHV with only intermittent shedding of the virus in the feces, the quarantine should include sentinel birds. The quarantine period should last at least six, and preferably twelve, weeks (Cho and McDonald, 1979; Randall *et al*., 1979). Concurrent infections with other disease agents may obscure the primary symptoms of PD. Since potential sources of PsHV in a susceptible population are asymptomatic carriers or recovered birds which shed the virus in their droppings (Vindevogel *et al*., 1982; Burgess and Yuill, 1983; Schuh and Yuill, 1985), all newly introduced birds should be isolated, observed and tested for the presence of PsHV. The most prudent course of preventive action is to immediately divide the flock into several smaller groups housed in separate rooms or buildings. Increased biosecurity measures during feeding and watering may also help retard the spread of infection (Simpson and Hanley, 1976).

An inactivated virus vaccine is available under conditional license in the USA (Biomune, Lenexa, KS). It is derived from inactivated whole virus suspended in an oil

emulsion and is recommended for use in susceptible parrot species, open breeding collections or flocks, and pet store birds as a series of two injections, 4-8 weeks apart.

Although the vaccine reduces primary virus excretion and clinical signs, as well as pathological lesions following challenge, it does not prevent infection or the establishment of asymptomatic carriers (O'Toole *et al*., 1992). Vaccination in the face of an outbreak is considered by some to be controversial, as handling may also enhance the spread of the virus.

1.3.5 Strain Variation.

Pacheco's disease once was thought to be caused by a single form of avian herpesvirus. However, Kaleta (1990) differentiated three plaque types which do not cross neutralize one another, suggesting at least three different serotypes. More recently, Tomaszewski *et al.* (2003) have determined that there are 4 unique genotypes of PsHV (genotypes 1-4). Genotype 1 is the most common cause of Pacheco's disease, followed by genotype 2 and, less frequently, genotypes 3 and 4. These findings demonstrate the potential difficulties in producing an effective vaccine that can protect psittacines, and suggests that the development of a subunit vaccine may be an effective approach.

1.4 Research Objectives.

While there is considerable literature pertaining to the diagnosis and pathology of Pacheco's disease in birds, previous studies have provided limited information regarding the molecular characteristics of the PsHV-1 virus, the causative agent of the disease.

Prior to this study, the only GenBank sequence accessions for PsHV were for U_L30, the DNA polymerase homolog, and U_L16, a scaffolding protein suspected to be a determinant of host range. Günther *et al*. (1997) reported restriction enzyme digestion patterns of PsHV and compared them to other avian herpesviruses.

Tomaszewski *et al.* (2003) reported the phylogenetic relationships of 4 different genotypes of psittacid herpesviruses based on an analysis of the U_L16 gene sequence. While they suggested that PsHV is an alphaherpesvirus, additional information is needed to determine the proper taxonomic placement of the PsHV-1 genotype into the *Alphaherpesvirinae* subfamily of *Herpesviridae*.

The primary objective of this research was to generate the complete nucleotide sequence of the PsHV-1 genome and to identify the major structural components and gene content of the genome. Preliminary comparative genome analysis of closely related herpesviruses offers some insight into the protein coding capacities of the viral genome, the regulation of gene expression, and may offer a means to identify PsHV-1 viral genes involved in pathogenicity, virulence, and host range specificity. A second objective of this study was to conduct a thorough bioinformatic and phylogenic analysis of the PsHV-1 genome and to compare those findings to other members of the avian *Herpesviridae*. This will allow for a clearer understanding of the proper classification of the virus, and may provide valuable insight into the evolution of the avian herpesviruses.

Chapter 2

MATERIALS AND METHODS

2.1 Virus and Cells.

2.1.1 Virus.

The reference Psittacid herpesvirus 1 (PsHV-1, isolate 97-0001) was kindly supplied by Dr. David Phalen of Texas A&M University. This isolate is from the liver of a male amazon parrot of unknown age, species unspecified (the animal's weight suggested a double yellow (*Amazona oratrix*) or a yellow-naped (*Ochrocephala auropalliata*) amazon), exhibiting hepatic and splenic lesions characteristic of PD. We were supplied with 0.5 ml of passage P1, harvested from infected CEF monolayers.

2.1.2 Cells.

Fibroblasts were isolated from 11-day-old chicken embryos by the warm trypsinization method (Freshney, 1983; Keeler *et al.*, 1991). Briefly, eggs were placed in a laminar flow hood, air sac up, and liberally sprayed with a 70% EtOH solution. The air sac was opened by striking sharply with forceps, and the surrounding shell was removed. Embryos were removed from the eggs, decapitated with scissors, and placed in a sterile 150 x 15 mm culture dish (on ice).

Wings, legs, and the internal organs were removed by gently scraping with the blunt side of the forceps. Eviscerated embryos were then placed into another sterile 150 x15 mm culture dish containing 10 ml of Dulbecco's modified essential media (DMEM/F-12; Life Technologies Inc., Gaithersburg, MD), supplemented with penicillin (50 µg/ml), and streptomycin (50 µg/ml).

The carcasses were finely minced by repeatedly snipping the tissues, then transferred to a sterile 200 ml trypsinizing flask with a stir bar. Minced tissue was washed 1X with 25 ml DMEM/F-12 (pre-warmed to 37ºC) by stirring for 5 min on a magnetic stir plate. After 5 min, tissues were allowed to settle out of solution and the media was decanted and discarded.

Trypsinization of the fibroblast-rich embryonic tissues began by adding 25 ml of pre-warmed (37ºC) 0.05% trypsin/EDTA (Sigma-Aldrich, St. Louis, MO) and gently stirring on the stir plate for 7 min. After 7 min, tissue was allowed to settle out of solution, and supernatant (cells and trypsin) was decanted into a cheesecloth-covered 500 ml beaker containing 10 ml of calf serum (Life Technologies Inc., Gaithersburg, MD) and placed on ice (addition of the calf serum deactivates trypsin activity). The process of adding warm trypsin, stirring and decanting cells to the serum-containing beaker was repeated 6 additional times.

After the final trypsinization, the gauze was removed from the top of the beaker, and equal volumes of the supernatant were decanted into 50 ml glass centrifuge tubes. Fibroblasts were pelleted by centrifugation for 15 min at 600 rpm, 4ºC.

Supernatants were aspirated from the cell pellet by utilizing a glass Pasteur pipet attached to an Erlenmeyer flask under vacuum. Fibroblast cells were gently drawn up into a 1 ml glass pipet and placed into a sterile, 500 ml, wide-mouth Corning bottle containing 10 ml of warm DMEM/F-12, supplemented with penicillin (50 µg/ml), streptomycin (50 µg/ml) and 10% fetal bovine serum (FBS; Life Technologies Inc., Gaithersburg, MD). Cells were swirled gently to assure complete resuspension in the fresh media. The final suspension ratio was 0.5 ml cells to 200 ml media. The cell suspension was passed through an additional cheesecloth filter.

The isolated fibroblast cells were plated unto 100 x 15 mm culture dishes by adding 10 ml of cell suspension to each plate. Cells were allowed to adhere to plates at 37ºC, 5% atmospheric CO_2 overnight.

2.2 Isolation of PsHV-1 Virions for Electron Microscopy.

Supernatants from PsHV-1 infected CEF monolayers were harvested and centrifuged for 10 minutes at 700 rpm in a clinical centrifuge to pellet the cellular debris. The infected CEF monolayers were then scraped, pooled and frozen at –80ºC.

Virions in the supernatants were precipitated by adding 5 M NaCl to a final concentration of 0.4 M and slowly stirring on a stir plate. PEG 8000 was added to a final concentration of 7% and the solution was allowed to continue stirring for 1 hr on ice. The solution was loaded into Nalgene ultratubes (P/N 3119-0050; 50 ml PPCO) and centrifuged for 30 min at 9,500 rpm, 4ºC in a HB-6 swinging bucket rotor (Sorvall

RC5C*plus* high speed centrifuge). The supernatant was discarded and viral pellets were resuspended in 1 ml TE (10mM TRIS, 1mM EDTA; pH 8.0), and stored at 4ºC.

To further purify virus particles from nuclear debris, the resuspended pellets were passed through a sucrose cushion. A 30% sucrose solution was prepared by adding 3g of sucrose to 7 ml of TE. The solution was loaded into a 5 ml Nalgene ultratube (P/N 3410-1351; 13 x 51 mm). One milliliter of the resuspended virions was layered onto the sucrose cushion and loaded into an AH650 rotor on the Sorvall OTD75B ultracentrifuge. The sample was centrifuged for 1 hr at 100,000 xg, 4ºC. The supernatant was decanted and discarded and the pellet was resuspended in 100 µl TE, gently mixed by swirling, and stored at –20ºC prior to use.

2.2.1 Electron Microscopy.

Evaluation of virion morphology was performed via transmission electron microscopy to investigate the surface view of mature virions by negative staining. Copper grids were cleaned in acetate, coated by dipping in a 2% collodion solution, and air-dried under a bench-top lamp for 10 min. Four wells of an optical microtiter plate were filled with 30 µl of a 15% solution of phosphotungstic acid (PTA) and 4 wells were filled with 30 µl of 2% saturated uryl acetate. Two additional wells were filled with 30 µl of the purified virion prep. The copper grids were floated upon the droplets containing the purified virions, allowing the virus particles to be attracted to the charged collodion. Grids were removed with forceps, blotted dry on Whatman paper and floated on the wells

containing 15% PTA for 2 min. Additional grids were removed from the virus solution, blotted on Whatman paper, and floated on the wells containing 2% uryl acetate for 2 min. Grids were washed in sterile dH_2O several times, and dried by carefully wicking onto a sterile patch of Whatman paper after each wash. Grids were thoroughly dried for 45 min under a bench-top lamp prior to loading.

Specimens were analyzed with a Zeiss CEM 902 electron microscope and Soft Imaging System Mega View II, equipped with a spectrometer to enhance image contrast, at an accelerating voltage of 80 kV with a magnification between ×16,000 and ×25,000. A liquid nitrogen-cooling trap for the specimen holder was used throughout.

2.3 Isolation of PsHV-1 Virions and DNA Extraction.

Infected monolayers were scraped into 2 ml/plate of SPGA buffer (0.218 M sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM sodium glutamate, 1% BSA; pH 7.0), and collected in 50 ml conical centrifuge tubes (Corning P/N 430828). Infected cell pellets from 20 plates were pooled and stored at –80ºC.

Tubes containing scraped and pooled PsHV-1 infected monolayers were removed from –80ºC and subjected to a 3X freeze/thaw lysis step. Pellets were thawed at 37ºC in a heated water bath for 5 min and re-frozen in an EtOH/dry ice bath for 5 min. The tubes were then briefly sonicated and vortexed. Infected cell pellets were resuspended in 9 volumes of lysis solution (0.25% Triton X100, 10 mM EDTA, 10 mM Tris HCl; pH 7.9) and gently rocked for 10 min at 26ºC. 5 M NaCl was added to each tube to a final

concentration of 0.2 M, and tubes were inverted 10X. Tubes were then centrifuged in a clinical centrifuge for 10 min at 1,400 rpm, 26ºC. Supernatants were decanted and set aside on ice. The lysis steps were repeated 3 additional times, and the supernatants were pooled.

Proteinase K was added at a concentration of 100 mg/ml to the pooled supernatants and allowed to digest for 3 hrs at 45ºC. The solution was extracted twice with an equal volume of phenol, then extracted once with an equal volume of chloroform:isoamyl alcohol (24:1). The upper, aqueous, layer was transferred to glass 30 ml Corning tubes. A one-tenth volume of 3M NaOAc and two volumes of 100% EtOH were added and the tubes sealed with parafilm and inverted 10X. Nucleic acids were allowed to precipitate overnight at -20ºC and then pelleted at 10,000 rpm for 30 min at 4ºC in a Sorvall R28 centrifuge rotor. Supernatants were decanted and discarded and the remaining pellet was dried for 20 min under vacuum. Pellets were resuspended in 0.5 ml dH_2O and transferred to 1.5 ml microfuge tubes.

Sucrose gradients were prepared by combining 25g of sucrose (Sigma-Aldrich, St Louis, MO) to 100 ml of 0% buffer (500 mM NaCl, 5 mM EDTA, 20 mM Tris; pH 7.8). Thirty-five milliliters of the sucrose solution was added to 38.5 ml Nalgene thin-walled ultratubes (P/N 3410-2539; 25 x 89 mm) and frozen to -20ºC. As the solution thaws, a 0-40% gradient forms in the tube. One milliliter of the resuspended viral DNA was loaded on top of the thawed sucrose gradient and centrifuged at 100,000 xg for 15 hr, 45 min, at 4ºC in a Sorvall S20/36 rotor.

The gradient was removed from the rotor and placed in a fixed position clamp, attached to an upright glassware support. An 18-gauge needle was passed through a No. 4 rubber stopper, and the stopper was carefully inserted into the top of the gradient tube. A second 18-gauge needle was gently inserted into the bottom of the tube and 1 ml fractions were collected in sterile 1.5 ml microfuge tubes. Twenty microliters of every other fraction were analyzed on a 0.8 % agarose gel, and appropriate fractions were pooled and viral DNA precipitated overnight in equal volumes of TE and 2 volumes of 100% EtOH at –80ºC.

Precipitates were centrifuged for 2 hr at 100,000 xg, supernatants decanted and discarded, and the pellets dried under vacuum for 10 min. Pellets were resuspended in 50 µl of TE and DNA concentrations were determined by spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE) and visualized by agarose gel electrophoresis. Purified viral DNA was examined by restriction endonuclease digestion (data not shown) and compared to previously published results (Günther *et al*., 1997).

2.4 Genomic Library Construction.

Purified genomic PsHV-1 DNA was sheared into random fragment lengths by use of a nebulizer and N_2 supply. Ten µg of genomic DNA was suspended in 2 ml of TMG buffer (50 mM Tris; pH 8.0, 15 mM $MgCl_2$, 25% glycerol), and placed in a modified nebulizer (Invitrogen, Carlsbad, CA; P/N 45-0072). The sample was nebulized at 30 psi for 30 sec for a total of 5 times (2.5 min total). The entire nebulization chamber was placed into a modified Sorvall rotor bucket and centrifuged for 1 min at 2,500 rpm, 4ºC.

The nebulized sample was then transferred to a sterile 15 ml conical tube (Corning, NY; P/N 430052). Cold 1-butanol was added at 1/10th volume and mixed by inversion to reduce the aqueous fraction. The phases were allowed to separate for 5 min, and the upper layer was removed and discarded. The 1-butanol extraction was repeated 5X, reducing the final volume to ~0.5 ml, which was transferred to a sterile microfuge tube. The nebulized DNA was precipitated overnight at –70ºC by the addition of 1/10th volume 3M NaOAc (pH 5.2) and 3 volumes of 100% EtOH.

To pellet the sheared DNA, the sample was centrifuged for 20 min at 14,000 rpm in a microcentrifuge, the supernatant decanted and discarded, and the pellet washed in cold 70% EtOH. The pellet was air-dried under vacuum for 5 min and resuspended in 100 µl of TE. A 2 µl sample was analyzed on a 1.0% pre-cast agarose gel (Reliant™ gel; FMC Bioproducts, Rockland, ME) to determine the size of the nebulized fragments.

The ends of the nebulized DNA fragments were repaired using the End-It DNA end-repair kit (Epicenter, Madison, WI) as per the manufacturer's instruction. This kit was used to fill-in of the 5' overhangs, generating blunt ends. Fragmented DNA was combined with a 10X polishing buffer, 10 mM dNTP, and 0.5 U of cloned *Pfu* DNA polymerase and incubated for 30 min at 72ºC.

Nebulized DNA fragments 1.5 to 2.5 kb in length were separated by agarose gel electrophoresis and isolated by carefully excising the desired bands from the gel with a sterile scalpel. The selected 'smear' of DNA fragments were gel purified using the Qiaquick™ gel extraction kit (Qiagen, Valencia, CA) (P/N 28704). Gel slices were weighed and mixed with appropriate volumes of binding buffer, heat solubilized at 50ºC

for 15 min, added to a silica-gel membrane mounted in a spin column, and bound by centrifugation for 20 min at 14,000 rpm. Impurities were removed with an 80% ethanol wash. Purified DNA fragments were eluted from the column with water and the concentration of the recovered DNA was determined by spectrometer as previously described. DNA fragments were ligated into the E*coR*V site of pBlueScript SK+ (Stratagene, La Jolla, CA).

Transformation of naïve DH10B *E. coli* cells (Life Technologies, P/N 15544-018) was via electroporation on a Cell-Porator™ electroporation system (Life Technologies P/N 71600-019). One (1) µl of the ligation mix was gently added to 24 µl of electro-competent cells in a microfuge tube on ice. The mixture was transferred to an ice-cold, sterile 1 cm gap electroporation chamber (Whatman/BioMetra, Florham Park, NJ; P/N 11608-031). Care was taken to make sure that the sample was placed directly between the chamber electrodes, and the chamber was placed into the Cell-Porator™. The capacitance parameters were set for 330 µF, and the shunt resistor was set at 4 kΩ. The DNA/cell mix was then electroporated with a final pulse of 2.38 kV. Using a sterile, disposable Pasteur pipette, the electroporated DNA/cell mixture was gently washed from the electrodes with 900 µl of pre-warmed SOC media and transferred to a 15 ml Falcon tube (Becton Dickinson, P/N 352059). Tubes were placed in an orbital shaker and gently agitated for 60 min at 200 rpm, 37ºC, to allow for cell recovery and expression of the resistance proteins. Transformed cells were spread onto agar selection plates containing ampicillin and incubated overnight at 37ºC. Glycerol archive stocks of transformed cells

were prepared using a Genetix Ltd. Q-Bot™ (Dorset, UK) robot to pick bacterial colonies. Stocks were stored at -70ºC and assigned a library designation.

Deep-well culture plates containing Terrific Broth (TB) media supplemented with 50 µg/ml of ampicillin were inoculated from the archive stocks by a Flexsys™ (Genomic Solutions, MI) robotic system, and the cultures were amplified for 22 hr at 37ºC with 250 rpm agitation. Individual plasmid clones, containing fragments of the PsHV-1 genome, were purified by a rapid extraction alkaline lysis protocol, performed by the E.I. DuPont de Nemours Agricultural Biotechnology, Gene Discovery Group, using a combination of Qiagen™ R.E.A.L. prep plasmid purification kits in a high-throughput 96-well format, as well as a Perfectprep-96 Robotic Workstation (Brinkmann-Eppendorf 5 Prime, Boulder, CO). Freshly cultured cells were pelleted at 3000 rpm for 10 min, 26ºC, and the media decanted. Cell pellets were resuspended in 300 µl of suspension buffer and lysed for 3 min in an alkaline buffer. After neutralization of the lysis solution, supernatants were purified by vacuum filtration. Purified plasmid DNA was then precipitated with isopropanol and pelleted at 3,000 rpm for 15 min. DNA pellets were washed with 70% ethanol, dried for 7 min in a speed vacuum, resuspended in low TE (0.5 mM Tris HCl, 0.01 mM EDTA; pH 8.0) and stored at -20ºC prior to sequencing.

2.5 DNA Sequencing.

Sequence reactions were prepared by the E.I. du Pont de Nemours Agricultural Biotechnology, Gene Discovery Group. At the time of sequencing, the facility utilized

24 Applied Biosystems PRISM™ 377XL automated DNA sequencers and 4 ABI PRISM 3700XL automated DNA sequencers (PE Biosystems, Foster City, CA).

ABI PRISM™ Ready Reaction Dye Terminator Cycle Sequencing kits (PE BioSystems, Foster City, CA) and the BigDye dideoxy-chain terminator sequencing chemistry v3.0 (PE Biosystems, Foster City, CA) were used to sequence genomic fragment inserts. These kits are designed for use in a high-throughput 96- and 384-well format, using *Taq* polymerase-FS and dichlororodamine dyes. Vector-primed M13-28 Reverse (5'-GGA-AAC-AGC-TAT-GAC-CAT-G-3') and M13-20 Forward (5'-TGT-AAA-ACG-ACG-GCC-AGT-3') oligonucleotide primers were used to sequence the first 500-600 nucleotides from the 3' and 5' ends of the inserts. A cocktail of the commercial reaction mix, one of the two primers, and dH_2O was aliquoted into each well of a microtiter PCR reaction plate (Axygen, Union City, CA; P/N PCR-96-AB-C). Approximately 600 ng of DNA template was added to the cocktail and cycle-sequenced using Perkin-Elmer 9700 thermocyclers and MJ Research PTC-100 DNA engines.

The following reaction parameters were used for template sequencing. An initial denaturation step of 96°C for 10 min, followed by 25 cycles of 96°C for 10 sec, 50°C for 30 sec, and 60°C for 4 min. Completed reactions were held at 4°C until the plates were removed prior to precipitation with 0.2 M $MgCl_2$ and100% ice-cold EtOH. DNA was immediately pelleted by centrifugation at 4,000 rpm for 30 min. Pellets were dried for 7 min under vacuum and resuspended in 4 µl loading buffer containing dye. Completed plates were frozen at -20ºC until loading onto 377XL or 3700XL automated sequencers.

Verified sequences were initially analyzed by ABI PRISM™ 377XL and ABI3700XL Collection software, and the raw sequence data was transferred to a remote analysis computer, where automatic gel lane tracking was confirmed manually. The raw chromatogram traces were converted to FASTA formats in order to compare to the GenBank DNA database using Blast and FASTA search algorithms. The primary tool used to query the GenBank database was BLAST - Basic Local Alignment Search Tool (Altschul *et al*., 1990). BLAST hits were sorted by hit into separate mail folders and compiled onto Excel spreadsheets.

2.6 Sequence Assembly.

Sequence data was separately analyzed by *Phred*™ , and contiguous DNA sequences were assembled utilizing the *Phrap*™ data analysis and consensus sequence assembly software suite (Ewing and Green, 1998), using the quality files and default settings (cross_match – minmatch 12 – penalty -2 – minscore 20) to produce the consensus sequence, which was manually edited with *Consed*™ (Gordon *et al*., 1998).

Phred™ is a public domain software program, developed by Green and Ewing at the University of Washington, which re-reads original ABI sequence trace data, re-calls bases, assigns quality values, and writes the base calls and quality values to editable output files. The quality value is a log-transformed error probability, specifically $Q = -10 \log_{10}(P_e)$, where Q and P_e are respectively the quality value and error probability of a particular base call. *Phred*™ achieves 40-50% lower error rates than original ABI software on large test data sets (Ewing *et al*., 1998). The major benefits of

using *Phred*™ are accuracy in base calling and highly accurate error probabilities calculated for each base, enabling a significant increase in the automation of the sequencing process.

The *Phrap*™ DNA sequence assembly program uses the *Phred*-assigned quality values to increase the accuracy of the assembled sequence *contigs* (individual sequence traces which align within the project and are *contiguous*, or touching), and examines all individual sequences at a given position, selecting the highest quality sequence to build the consensus. *Phrap*™ also uses input information about sequencing chemistry (dye terminator or dye primer) and confirmation by complementary strand data in estimating consensus quality.

Consensus assemblies are then accessed by the *Consed*™ sequence editor (Gordon *et al.*, 1998), which was designed for viewing and editing the contiguous sequences assembled by the *Phrap*™ program. It was written specifically to *Phrap*™, and it takes advantage of quality values assigned by *Phred/Phrap* and the consensus sequence created by *Phrap*™. In addition to a full set of standard features (view traces, edit reads by inserting a base, deleting a base, substituting a base, etc.), it supports an efficient editing procedure designed for use by *Phrap*™ in subsequent re-assemblies of the same data set. Homology searches to the consensus contigs were conducted using BLAST (Altschul *et al*., 1990), ORF Finder (Wheeler *et al*., 2004) and PSI-BLAST (Altschul *et al*., 1997).

2.7 Gap Closure and Junction Confirmation.

Smaller gaps in the consensus sequence were closed by primer-walking gap-spanning clones identified by clone-mate sequences. Larger gaps were closed by PCR amplification using viral genomic DNA as template. Genome-specific primers were also designed for PCR reactions to confirm the junctions of the internal and terminal repeat regions. Each 20 µl PCR reaction mix contained 50 ng of genomic PsHV-1 DNA, 25 pmol of each primer, 0.1 mM concentrations of each of the four deoxynucleotide triphosphates, 2.5 mM magnesium chloride, 0.75 U of *Taq*, and 1X buffer A (Promega, Madison, WI). Amplifications were performed on a PE Biosystems 9700 thermal cycler using an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C for 15 sec, 55°C for 30 sec, and 60°C for 4 min. Amplified templates were purified by application to Qiagen PCR Purification columns as per the manufacturer's specifications and used as templates in cycle sequencing reactions as described above.

2.8 Identification of Open Reading Frames in the PsHV-1 Genome.

The ORFinder analysis program is a graphical analysis tool that identifies all open reading frames (ORFs) of a user-determined size from within a defined nucleotide sequence. It can also deduce the amino acid sequence for BLAST against the NCBI databases. ORF size of >100 amino acids was chosen for analysis of the PsHV-1 genome. Subsequent analysis with smaller minimum values (50 aa; 20 aa) was used to identify additional ORFs. To corroborate ORFinder predictions, the complete PsHV-1 nucleotide sequence was used to generate a Vector NTI file and analyzed for open

reading frames. The predicted genes from the Vector NTI analysis were submitted to the NCBI BLAST server and results were compared to the ORFinder analysis. Putative functions were assigned based upon BLAST score.

2.8.1 Specific ORF Analysis.

The amino acid sequence of sORF2, the gJ homolog in PsHV-1, was aligned to gJ (U_S5) of ILTV, using the *AlignX* function within the Vector NTI™ suite of DNA sequence analysis programs. The PsHV-1 sORF1 amino acid sequence was also aligned with U_L47 of ILTV. The individual amino acid sequences for PsHV-1 sORF1 and sORF2, and ILTV U_S5 and U_L47 were also submitted separately to the NetOGlyc 3.1 server (www.cbs.dtu.dk/services/NetOGlyc) and the NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc) servers at the University of Denmark's Center for Biological Sequence Analysis. The NetOGlyc 3.1 server produces neural network predictions of mucin type GalNAc O-glycosylation sites in mammalian proteins, and the NetNGlyc 1.0 server predicts N-glycosylation sites in proteins using artificial neural networks that examine the sequence context for Asn-X-Ser/Thr motifs

2.9 Search for Transcriptional Control Elements in the PsHV-1 Genome

Since most genes in the HSV-1 genome are transcribed as capped and polyadenylated mRNAs by host RNA polymerase II (Roizman and Spears, 1996), it was assumed that the homologs in PsHV-1 would be similarly transcribed. The search for transcriptional control elements in the PsHV-1 genome utilized computerized, web-based

prediction tools, designed to identify putative promoters, TATA boxes, and polyadenylation sites. The successful outcome of utilizing these programs relied on two assumptions: (i) that the primary transcriptional elements between avian and human would be somewhat conserved, since the programs were designed based on human elements, and (ii) that the transcriptional elements of the virus and avian host would be similar. We closely followed the systematic approach taken by Klupp *et al.* (2004) to identify these elements in the PRV genome.

2.9.1 Promoter Searches.

The PsHV-1 genomic sequence was submitted to the Berkeley Drosophila Genome Project's Neural Network Promoter Prediction program, (http://www.fruitfly.org/seq_tools/promoter.html), a eukaryotic core promoter search engine. The promoter search was performed at a high stringency cutoff score of 0.99 (out of 1.00). The program returns predicted promoters sequences (50 bp in length) along with a predicted transcription start site (TSS). All of the promoters identified by the neural network prediction program were examined for the presence of a TATA box consensus using the TRANSFACFind search engine (http://motif.genome.jp/), a motif identifying software program available from the Kyoto University Bioinformatics Center. A low stringency was used for the TATA box searches (cutoff score of 65 out of 100).

2.9.2 Identification of Polyadenylation Sites.

Polyadenylation is the non-templated addition of a 50 to 200 nt chain (or 'tail') of polyadenylic acid [poly(A)] to an RNA transcript. Poly(A) tails are thought to function in mRNA stability and in the initiation of translation. The nucleotide sequences found in the genome that initiate this function are known as polyadenylation sites.

The genomic sequence of PsHV-1 was submitted to PolyADQ, a eukaryotic polyadenylation signal search engine at Cold Spring Harbor Laboratory (http://argon.cshl.org/tabaska/polyadq_form.html), to identify all potential polyadenylation signals within the nucleotide sequence. This program is designed to detect poly(A) sites in human DNA using weight matrices for base composition and position in the downstream element (Tabaska and Zhang, 1999). The default parameters were set to zero to identify the location of all AATAAA and ATTAAA consensus signals, which are returned with an associated score between 0 and 1. For each predicted poly(A) signal, the corresponding upstream genes were identified in the PsHV-1 Vector NTI file.

2.10 Phylogenetic Analysis Based on Protein Sequence Alignments.

Multiple sequence alignments of select *Herpesviridae* genes representing the 3 subfamilies were created using the ClustalW algorithm (Thompson *et al.*, 1994), a multiple alignment algorithm within *Phylogenator*, the proprietary software program (Pioneer Hi-Bred Int'l., Johnston, IA) that was used to determine the phylogenetic

relationship between the PsHV-1 protein sequences and existing amino acid sequences collected from GenBank. ClustalW is the same algorithm used in the Vector NTI *AlignX* program. ClustalW functions by first performing pairwise comparisons of each pair of sequences, using the pairwise alignments to create a guide tree, and then uses the guide tree to create a multiple alignment. For the analysis of PsHV-1 relationships, gap opening and gap penalty scores were set to the default score of -2 for both the pairwise and multiple alignment steps. Gap separation distance decreases the chance of gaps being too close to each other. Gaps that are less than the selected distance apart are penalized more than others. This does not prevent close gaps; it makes them less frequent, promoting a block-like appearance of the alignment. Decreasing gap penalties allows more gaps in the alignment (and fewer mismatches) but may allow matches that don't reflect actual sequence homology. Increasing gap penalties has the opposite effect; the alignment is more rigorous, but might miss matches that reflect sequence homology.

Default settings for the protein weight matrices were selected for both the pairwise and multiple alignment steps. Matrices allow the program to take the biochemical similarity of amino acid residues into account when calculating the score of an alignment. The Gonnet PAM 250 substitution matrix is an updated version of the PAM matrix. A default gap penalty of 10.0 and a gap extension penalty of 0.10 were selected for this analysis.

The 'delay divergent sequences' parameter determines how different two sequences must be for their incorporation into the multiple alignment to be delayed. The program delays the alignment of the most distantly related sequences until after the

more closely related sequences have been aligned. The setting shows the percent identity required to delay the addition of a sequence; sequences that are less identical than this value to other sequences will be aligned later. A default value of 30% was selected for this analysis.

Once the protein sequences were aligned, truncated sequences were manually removed to improve the alignments. This did not affect the validity of the alignments, as several GenBank accessions from each of the herpesvirus subfamilies were available for the desired genes. The most complete sequences were included in this analysis. The *Phylogenator* program also allows for the ends of the alignment to be trimmed by specifying coordinates of the desired sections, but no trimming was performed on any of the sequences prior to alignment.

Tree-building was done by the neighbor-joining procedure of the PHYLIP package of phylogeny programs (http://evolution.genetics.washington.edu/phylip.html), running in *Phylogenator*. In the process of building a tree, *Phylogenator* utilizes 5 separate PHYLIP programs. Initially, the alignment is processed by *Seqboot,* which generates the requested number of re-sampled alignments for bootstrapping. In our experience, re-samplings of 100 typically give very similar results to runs of 1000. The output from *Seqboot* is a file of re-sampled alignments and is saved as *.sb.out (A numeric name that is unique to each set of genes is generated after each run). Bootstrapping can be done by several methods. *Phylogenator* utilizes the half-delete jackknife method (Felsenstein, 1997), in which one half of the positions in the alignment

are randomly chosen and deleted. *Seqboot* supports other methods, but jackknife has the advantage of generating smaller dataset sizes.

The re-sampled alignments are then processed by *Protdist* (http://evolution.genetics.washington.edu/phylip/doc/protdist.html), which creates distance matrices for each alignment. The output from this program is *.pd.out. Each of these matrices is then used by *Neighbor* to create a neighbor-joining tree. Neighbor-joining has several advantages over parsimony. It is faster than parsimony and not affected by long-branch attraction, a problem caused by very distantly related sequences having similar amino acids in non-conserved positions due to chance. For parsimony, which counts the number of changes between two sequences, these positions are more of a problem than for distance methods which simply score the number of identical and similar amino acids. The other methods, maximum likelihood and Bayesian methods are not practical for subsequent bootstrapping applications because they are computationally very slow. The trees from this process are computer readable (NH format) file *.nj.tree.
(Newick format: http://evolution.genetics.washington.edu/phylip/newicktree.html)

These trees are collapsed into a single consensus tree by the program *Consense* (http://evolution.genetics.washington.edu/phylip/doc/consense.html), which yields two files, *.cons.out (human readable tree) and *.cons.tree (also NH format).

In the final step in the process, the consensus tree is processed along with the initial alignment by *ProtML*, the maximum likelihood program, to assign branch lengths and likelihoods to the consensus tree. The file from this program is *.ml.tree.

The *.ml.tree and the consensus tree with bootstrap values are then combined into the "merged" tree (*.merg.tree) which is in an extended NH tree format.

Final consensus trees were imported to *TreeView* (Page, 1996) for formatting the display as an unrooted tree. The image was captured from the desktop and imported into Microsoft PowerPoint for final editing and figure construction.

Subsequently, the complete genome sequence was entered into Vector NTI (InfoMax/Invitrogen, Carlsbad, CA), and the amino acid translations for specific gene products were compiled and used to construct additional trees using *Phylogenator*.

2.11 Nucleotide Sequence Accession Numbers.

The complete Psittacid herpesvirus 1 genome sequence has been deposited in GenBank under accession numbers AY372243 and NC_005264 (National Center for Biotechnology Information, Bethesda, MD).

Additionally, and specifically for comparison to PsHV-1, the complete ILTV genome has been assembled from previous GenBank accessions and the complete consensus sequence has also been submitted to the GenBank database under accession no. NC_006623 (Thureen and Keeler, 2006).

Chapter 3

RESULTS

3.1 PsHV-1 Virion Morphology.

PsHV-1 isolate 97-0001 was received as passage 1 on chicken embryo fibroblasts (CEFs). Subsequent propagation of the virus isolate on CEF monolayers revealed the rate of replication of PsHV-1 to be considerably slower than that observed with ILTV, yet consistent with previously observed results (Kaleta, 1990). While ILTV infection can result in almost complete CPE in a matter of hours (16 hrs), PsHV-1 infections took several days (~144 hrs) to reach >90% CPE.

The virions of the family *Herpesviridae* ranges from 180-200 nm in size and are composed of four major structural features; the envelope, tegument, capsid and core. Upon examination by electron microscopy as described in the Materials and Methods, PsHV-1 virions partially purified from virus-infected CEF monolayers seen in Figure 1 are about 200 nm in size, and are consistent with herpesvirus morphology. The PsHV-1 virions seen in Figure 2 are untreated with chloroform, and remain enveloped, with associated virally-encoded glycoproteins. A protein-filled tegument region lies between the capsid and the envelope. Although the herpesviruses cannot be differentiated by virion morphology alone, or by their respective replication cycles, this information, taken in context with other data, can aid

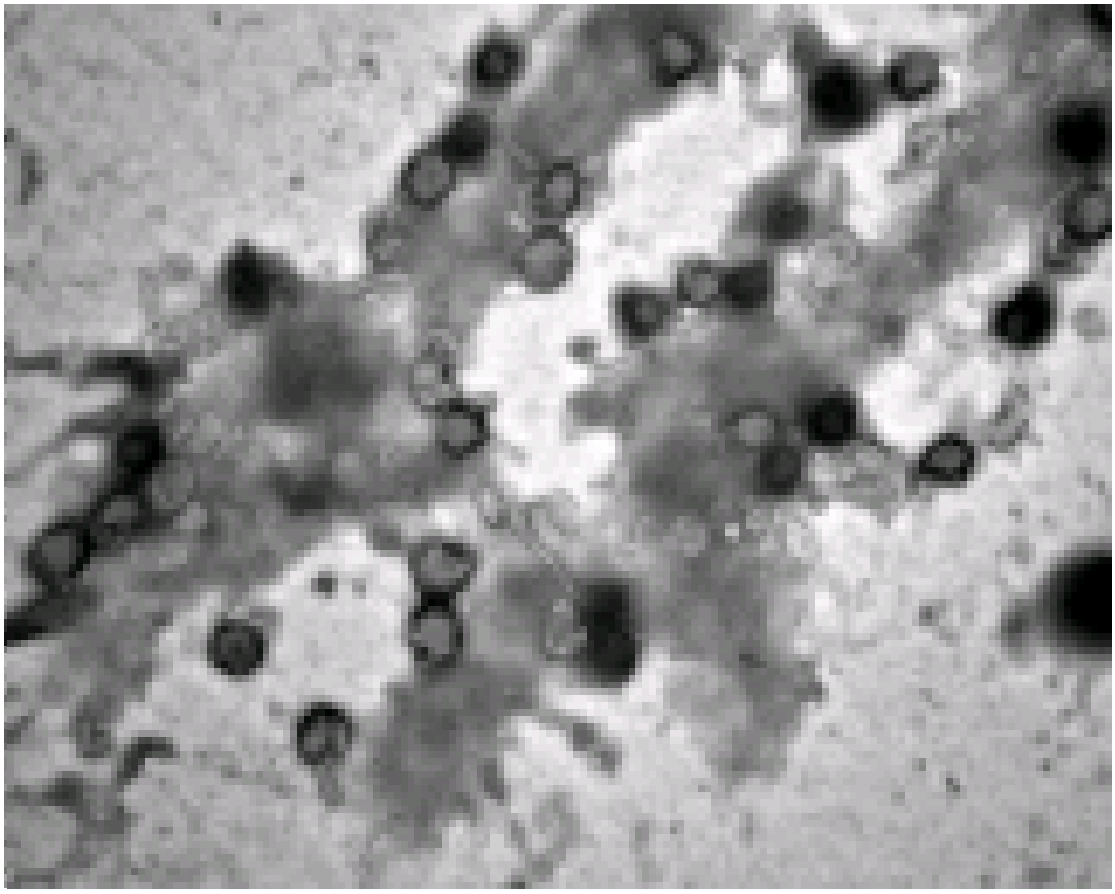


Figure 1. Electron micrograph of primary chicken embryo fibroblast monolayers infected with PsHV-1. Negative stain electron microscopy of PsHV-1 mature virions from an infected CEF monolayer. Viruses were stained with phosphotungstic acid and examined with a Zeiss CEM 902 electron microscope at 80 kV, between ×16,000 and ×25,000 magnification. Mature virions are negatively stained as dark hexagons with lighter capsid core. Surrounding cellular debris is seen as amorphous grey patches.

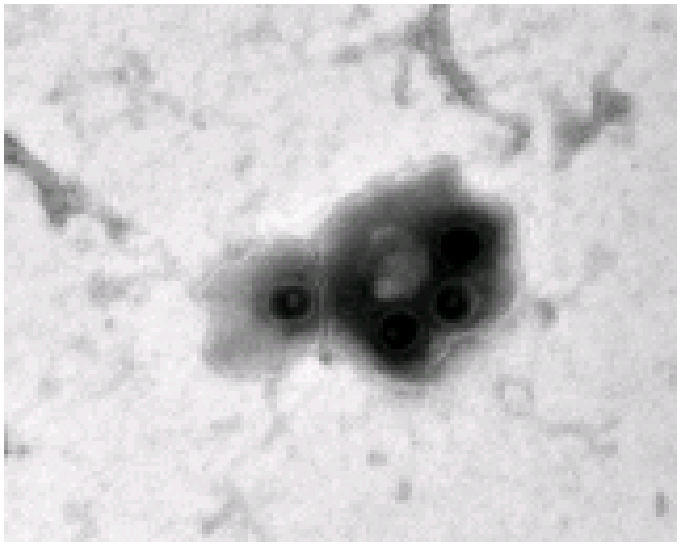


Figure 2. Electron micrograph of mature PsHV-1 virions.

Negative stain electron microscopy of PsHV-1 mature virions from an infected CEF monolayer. Viruses were stained with phosphotungstic acid and examined with a Zeiss CEM 902 electron microscope at 80 kV, between ×16,000 and ×25,000 magnification.

differentiation, since significant differences between subfamilies are apparent in their effect on host cells and in their genomic structure.

Genomic DNA was purified by sucrose gradient and used for restriction enzyme digestion analysis and genomic library construction. Restriction enzyme digestion of

purified PsHV-1 genomic DNA revealed patterns similar to those previously reported by Günther *et al.* (1997; data not shown).

Genomic subclone libraries were constructed to provide DNA templates for cycle sequencing reactions and amplification by polymerase chain reaction (PCR). Library PDV13.dt resulted in the glycerol archive library *vab3g* which was used to generate over ~90% of the sequence data used in the final nucleotide assembly of the genome.

Over 3,000 subclones were sequenced bi-directionally to generate a consensus sequence of 2,022 lanes of sequence data, which assembled in to one contiguous sequence and 126 (6%) singlet sequences (lanes of data not included in the assembly, but also not identified as vector sequence). Depth of coverage was ~8X with an average read length of 620 bp. A total of 1,253,640 bases were determined.

3.2 Genome Organization of PsHV-1.

The PsHV-1 genome was determined to be 163,025 bp in length with a base composition of 60.95% G+C. The genome consists of two unique sequences, the long or U_L (119,146 bp) and the short or U_S (16,405 bp), with the U_S region being flanked by inverted (I_R) and terminal (T_R) repeat elements 13,737 bp in length (Table 1and Figure 3).

Figure 3 is a visual summary of the data contained in Table 2, and illustrates the genome organization and gene content of the PsHV-1 genome.

Table 1. Comparison of Avian Alpha Herpesvirus Genome Organization

Virus	TR_L	U_L	IR_L	IR_S	U_S	TR_S	Total	Accession no.
PsHV-1	-	119,146	-	13,737	16,405	13,737	163,025	AY372243
ILTV	-	113,039	-	11,202	13,232	11,202	148,665	NC_006623
MDV-1	12,584	113,563	12,584	12,120	10,847	12,120	173,818	AF147806
MDV-2	11,951	109,932	11,951	9,164	12,109	9,164	164,271	AB049735
HVT	7,072	110,694	7,072	13,610	8,615	13,610	160,673	AF282130

PsHV-1, Psittacid herpesvirus 1

ILTV, infectious laryngotracheitis virus

MDV-1, Marek's disease virus, serotype 1

MDV-2, Marek's disease virus, serotype 2

HVT, herpesvirus of turkeys

TR_L – terminal repeat, long region

U_L – unique long region

IR_L – internal repeat, long region

IR_S – internal repeat, short region

U_S – unique short region

TR_S – terminal repeat, short region

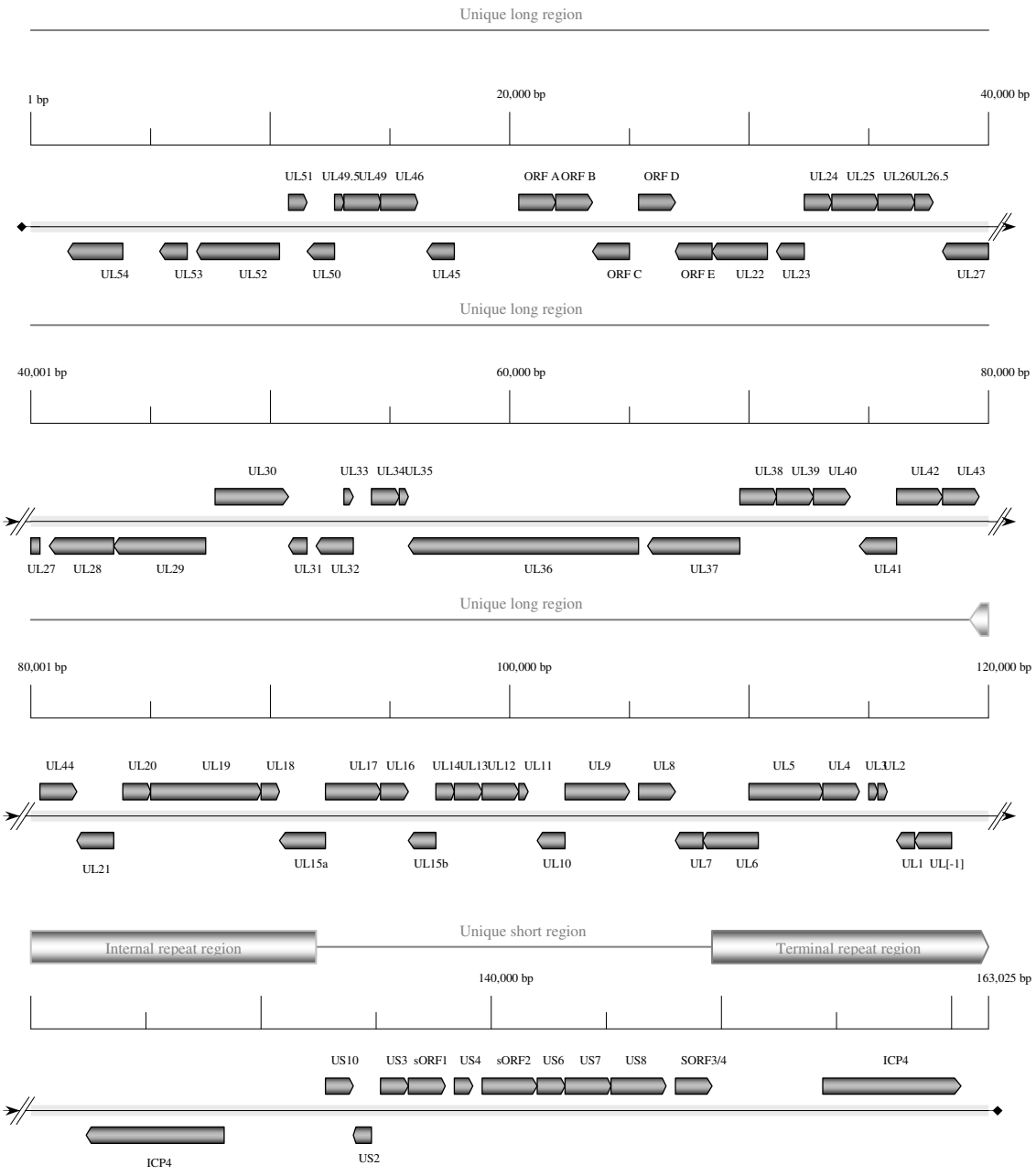


Figure 3. Organization of the PsHV-1 genome. This map of the PsHV-1 genome shows the locations and sizes of predicted ORFs. Predicted PsHV-1 genes are labeled according to homology with characterized HSV-1 genes. Thick gray bars flanking the unique short region indicate the internal and terminal repeat regions. A "ruler" designates distances in 5,000-bp increments. Arrows on the ORF boxes indicate orientations.

Table 2. Open Reading Frames Identified within the PsHV-1 and ILTV Genomes

PsHV1 ORF ID	Nucleotide Position (PsHV1)	ILTV ORF ID	Nucleotide Position (ILTV)	Length (aa)[α]		% Identity to ILTV	Putative Function
				PsHV1	ILTV		
UL54	4295-2376	UL54	12082-10787	639	420	38	Post translational regulator of gene expression
UL53	6461-5385	UL53	13916-12855	358	337	31	Glycoprotein K; exocytosis
UL52	10335-6454	UL52	17176-13847	1293	1110	42	DNA helicase-primase
UL51	10319-11101	UL51	17189 -17875	260	229	55	Unknown
UL50	12476-11232	UL50	19182-17935	414	416	46	Deoxyuridine triphosphatase
UL49.5	12667-13014	UL49.5	19336 - 19686	140	266	50	Putative viral membrane protein
UL49	13184-14038	UL49	19749 - 20546	283	117	50	Viral tegument protein
-	Not present	UL48	20695 - 21882	-	396	-	Viral tegument protein (a-TIF)
UL46	14500-16323	UL46	21888 - 23558	606	539	44	Tegument phosphoprotein; a-TIF modulation
UL45	17426-16521	UL45	24559-23663	301	281	26	Tegument/envelope protein
ORF A	21070-22260	ORF A	25275-26405	396	376	27	Hypothetical protein
ORF B	22726-23829	ORF B	26448-27467	367	340	30	Hypothetical protein
ORF C	25174-24056	ORF C	28529-27528	372	334	33	Hypothetical protein
ORF D	25554-26654	ORF D	28639 - 29760	366	374	38	Hypothetical protein
ORF E	28424-27054	ORF E	31067-29838	456	410	28	Hypothetical protein
UL22	31254-28834	UL22	33539-31128	828	779	34	Glycoprotein H; fusion -complexes with gL
UL23	32569-31550	UL23	34667-33576	339	363	40	Thymidine kinase
UL24	32533-33489	UL24	34556 - 35416	318	287	41	Unknown
UL25	33628-35511	UL25	35392 - 37107	627	572	47	DNA packaging protein
UL26	35709-37382	UL26	37288 - 39045	557	586	33	Capsid protein p40
UL26.5	37199-37891	-	Not present	230	-	-	Capsid protein
UL27	40995-38260	UL27	41747-39099	911	873	59	Glycoprotein B
UL28	43743-41164	UL28	44013-41722	859	537	47	ICP18.5; cleavage/packaging
UL29	47426-43860	UL29	47094-44098	1188	999	59	Major single strand DNA binding protein
UL30	47858-51103	UL30	47271 - 50291	1081	1007	54	DNA polymerase
UL31	52171-51134	UL31	51483-50467	345	339	68	Nuclear phosphoprotein

Table 2. Continued

PsHV1 ORF ID	Nucleotide Position (PsHV1)	ILTV ORF ID	Nucleotide Position (ILTV)	Length (aa)[α]		% Identity to ILTV	Putative Function
				PsHV1	ILTV		
UL32	54020-52164	UL32	53233-51479	618	582	48	Envelope glycoprotein
UL33	54019-54393	UL33	53190 - 53579	138	119	58	DNA packaging
UL34	54616-55440	UL34	53611 - 54486	300	290	62	Membrane associated phosphoprotein
UL35	55567-55992	UL35	54515 - 54886	141	124	50	Capsid protein
UL36	65672-56085	UL36	62584-54917	3209	2556	36	Major tegument protein
UL37	69349-66437	UL37	65881-63212	970	890	34	Tegument protein
UL38	69618-71078	UL38	65973 - 67280	486	412	41	DNA binding; capsid protein
UL39	71339-73774	UL39	67618 - 69972	818	785	50	Large ribonucleotide reductase
UL40	73851-74792	UL40	69827 - 70915	313	310	69	Small ribonucleotide reductase
UL41	76206-74884	UL41	72176-70983	440	398	73	Virion host shutoff
UL42	76680-78182	UL42	72398 - 73693	519	432	34	Processivity factor for DNA polymerase
UL43	78100-79626	UL43	73756 - 74970	508	300	11	Unknown
UL44	80418-81806	UL44	75683 - 76924	462	414	29	Glycoprotein C
UL21	83761-82052	UL21	78611-77016	569	532	28	Nuceocapsid protein
UL20	84089-84835	UL20	78782 - 79477	248	232	30	Membrane protein
UL19	85082-89323	UL19	79664 - 83872	1413	1403	65	Major capsid protein
UL18	89684-90649	UL18	84059 - 85015	321	319	64	capsid protein
UL15a	92293-90773	UL15a	86212-85103	513	764	66	Terminase; DNA packaging
UL17	92187-94634	UL17	86355 - 88505	815	341	38	Tegument protein
UL16	94538-95605	-	Not present	355	-	-	Tegument protein; host range
UL15b	96940-95588	UL15b	89761-88598	450	764	43	Terminase; DNA packaging
UL14	96939-97529	UL14	89595 - 90353	196	196	45	Unknown
UL13	97403-98785	UL13	90212 - 91606	486	465	45	Serine/Threonine protein kinase
UL12	99023-100585	UL12	91750 - 93366	566	526	58	Alkaline deoxynuclease
UL11	100585-100728	UL11	93259-93502	48	80	42	Myristylated tegument protein
UL10	102288-101047	UL10	94758-93580	413	393	46	Glycoprotein M
UL9	102386-105028	UL9	94653 - 97382	880	892	53	Ori-binding protein

Table 2. Continued

PsHV1 ORF ID	Nucleotide Position (PsHV1)	ILTV ORF ID	Nucleotide Position (ILTV)	Length (aa)[a]		% Identity to ILTV	Putative Function
				PsHV1	ILTV		
UL8	105636-107582	UL8	97378 - 99762	648	795	34	Helicase-primase component
UL7	108856-108690	UL7	100889-99816	388	358	41	Unknown
UL6	110909-108561	UL6	102807-100669	782	713	47	Minor capsid protein
UL5	110993-113560	UL5	102795 - 105314	855	840	58	Helicase-primase component
UL4	113781-114539	UL4	105403 - 105936	252	178	81	Unknown
UL3	114921-115523	UL3	106948-106349	200	196	66	Unknown
UL2	115671-116759	UL2	107950-107060	265	297	50	Uracil DNA glycosylase
UL1	117253-116705	UL1	107920 - 108279	182	131	32	Glycoprotein L
-	Not present	UL0	111514-110171	-	447	-	Unknown
UL[-1]	118632-117331	UL[-1]	111670 - 112026	463	501	23	Unknown
ICP4a	127595-121494	ICP4	118888-114500	2033	1463	35	Gene regulation
US10	133103-133948	US10	122103 - 122936	281	278	32	Unknown
-	Not present	SORF4/3	124190-123309	-	293	-	Unknown
US2	134374-134634	US2	125011-124325	85	118	34	Protein kinase
US3	136263-134785	US3	125100 - 126527	498	471	48	Protein kinase
sORF1	136535-138352	gp67	126616 - 128484	605	623	45	67kDa glycoprotein
US4	138546-139388	US4	128651 - 129526	280	292	24	Glycoprotein G
sORF2	139667-142642	gp60	129739 - 132693	991	985	18	60kDa glycoprotein
US6	142740-143891	US6	132441 - 133805	383	434	28	Glycoprotein D
US7	144089-145315	US7	133916 - 135001	492	362	34	Glycoprotein I
US8	145663-147369	US8	135198 - 136694	568	499	27	Glycoprotein E
-	Not present	US9	136704 - 137483	-	259	-	Unknown
SORF4/3	148377-149249	SORF4/3	137535 - 138416	290	322	34	Unknown
-	Not present	US10	138704 - 139402	-	232	-	Unknown
ICP4b	154577-160678	ICP4	142837 - 147225	2033	1463	35	Gene regulation

[a] aa, amino acids

The overall genome organization is consistent with other alphaherpesviruses and resembles a class D herpesvirus in genome structure (Roizman and Knipe, 2001). In the class D genomes, only the unique short component inverts relative to the long segment and forms two distinct isomers.

The PsHV-1 genome contains 73 putative genes as predicted by ORFinder (NCBI) and Vector NTI. Both prediction programs identified the same open reading frames and amino acid translations within the PsHV-1 genome ORFs that range from 48 to 3209 amino acids in length.

Sixty-one genes are located within the unique long region, 10 genes are in the unique short region, and a copy of the ICP4 gene is located within each of the repeats. BLAST results indicate PsHV-1 proteins share the strongest similarity (42.5% on average) to proteins from ILTV. Homology to PsHV-1 genes previously submitted to GenBank (U_L16 and U_L30) share 99-100% identity (VanDevanter *et al*., 1996; Günther *et al.*, 1997; Tomaszewski *et al.*, 2001). Overall, ILTV (77 total genes) and PsHV-1 (73 total genes) share homology between 70 conserved genes, which are collinear with the exception of the repeat regions. They also share 11 positionally conserved glycoproteins. The predicted PsHV-1 genes range from 13 to 73% identical at the amino acid level to ILTV.

3.2.1 U_L Region of PsHV-1.

All of the PsHV-1 open reading frames with significant homology to HSV were given the same U_L designations as the HSV-1 counterpart (Table 2, column 1). Genes without significant homology to HSV-1 were named as ORF plus a letter designation in accordance to their position. The unique long region of PsHV-1 extends from nucleotide 1 to 119,148 and contains 61 ORFs. The gene arrangement of the unique long region is similar to that found in ILTV, including an inversion of the segment from U_L22 to U_L44. A similar inversion is also found in the genome of PRV, extending from U_L27 (gB) to U_L44 (gC) (Ben-Porat *et al.*, 1984; Klupp *et al.*, 2005). We have examined the nucleotide sequence flanking the inversion in PsHV-1 in some detail. The non-coding sequence between U_L44 and U_L21 is 251 bp in length, and the intergenic space between U_L22 and ORF E is 413 bp long. Analysis of the regions between the PsHV-1 U_L44 and U_L21 and the U_L22 and ORF E genes for conserved motifs reveals little clue as to the specific site of the inversion.

Five ORFs (designated A-E), previously unique to ILTV (Ziemann *et al*., 1998; Veits *et al*., 2003) are also found in a co-linear arrangement in the U_L region of PsHV-1, adjacent to the 5-prime end of the inverted region (Figure 3). Open reading frames with homology to U_L48 (α-TIF), and U_L0 were absent from the PsHV-1 genome. The PsHV-1 genome also lacks a positional U_L homolog to HSV-1 U_L47, but a putative homolog with 18% identity to HSV-1 U_L47, previously described by Wild *et al.* (1996), is located in the U_S region.

3.2.2 U_S Region of PsHV-1.

The unique short region of PsHV-1 extends from nucleotide 132,886 to 149,290. There are ten predicted ORFs within the U_S region of PsHV-1: U_S10, U_S2, U_S3, sORF1 (gp67), U_S4, sORF2 (gp60), U_S6, U_S7, U_S8 and sORF3/4. Gene arrangement within the PsHV-1 U_S region is quite similar to the corresponding region of ILTV (Figure 4), however, a homolog to ILTV U_S9 is not present in PsHV-1, nor does PsHV-1 contain the duplication of the U_S10 and sORF3/4 genes within the inverted repeat region as observed in ILTV. Although the organization of the ILTV and PsHV-1 U_S regions are very similar, they differ greatly from the corresponding region of the three serotypes of MDV (Figure 4). Interestingly, neither avian herpesvirus U_L region contains a homolog to U_L47, a tegument phosphoprotein. However both ILTV (U_L47) and PsHV-1 (sORF1) encode an open reading frame in the U_S region with weak (18%) identity to the HSV U_L47 gene.

The U_S regions of both ILTV and PsHV-1 are predicted to encode five structural glycoproteins. One of these genes was initially designated gp60 (Kongsuwan *et al.*, 1993). Recent studies have adopted the HSV-1 designation of gJ (U_S5) for this open reading frame (Fuchs *et al.*, 2005). The PsHV-1 U_S region contains a collinear open reading frame. The predicted translation product of this gene is a glycoprotein that shares only 18% amino acid identity to the gJ glycoprotein of ILTV, although nine of the 10 cysteine residues are conserved (Figure 5). The PsHV-1 open reading frame has been designated sORF2.

3.2.3 Internal and Terminal Repeat Regions.

The I_R and T_R regions of PsHV-1 (located at nucleotide positions119,149 to 132,886 and 149,288 to 163,025, respectively) are 13,737 bp in length. Each repeat contains one copy of the ICP4 gene. A major structural difference between the PsHV-1 and ILTV genomes is observed in the size and genetic make up of their respective inverted repeat regions. The ILTV inverted repeat regions are 2,535 bp (18.5%) shorter than the PsHV-1 inverted repeats and contain two copies of three genes, ICP4, U_S10 and a homolog to the MDV sORF3/4 gene (Figure 4).

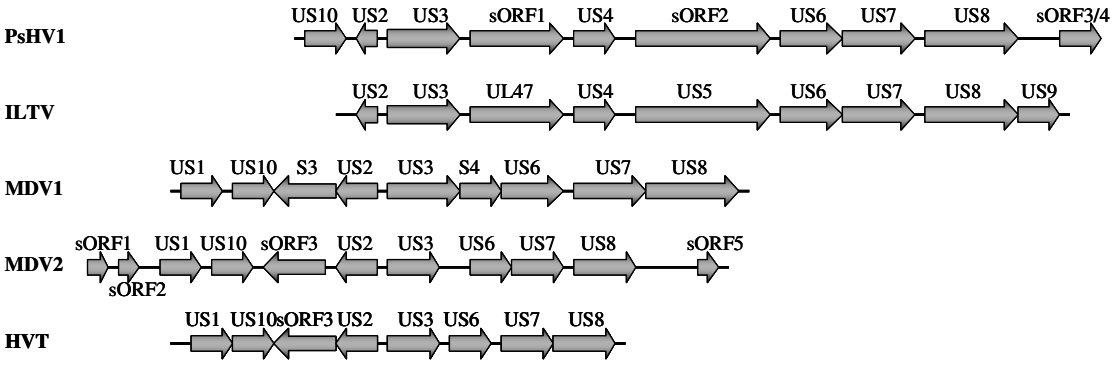


Figure 4. Comparison of the unique short regions of five avian alphaherpesviruses.

ORFs identified within the unique short regions of five avian alphaherpesviruses are indicated. Regions are aligned with respect to the conserved US2 and US3 genes. ORFs are labeled according to homology with characterized HSV-1 genes. Arrows on the ORF boxes indicate the direction of transcription.

3.3 Identification of Core Elements of Transcriptional Control.

3.3.1 Search for Promoter Elements and Polyadenylation Signals.

Of the 73 predicted ORFs in PsHV-1, 33 promoters were identified with the Berkeley Neural Network Promoter Prediction program at high stringency (0.99 out of 100) (Table 3). Using the TRANSFACFind search engine (http://motif.genome.jp/) at low stringency (65 out of 100), searches for TATA sequences were conducted in the PsHV-1 genome. For each of the 33 predicted promoters, the predicted transcription start site (TSS) location was also identified. The PolyADQ program was used to identify potential polyadenylation signals within the PsHV-1 genome. The program predicted 58 poly(A) sites within the PsHV-1 genome at the parameters specified. Thirty-seven (37) of these predicted sites matched to predicted ORFs in the genome (data not shown).

3.4 Phylogenetic Analysis of PsHV-1 Genes.

Phylogenetic trees generated from the analysis of herpesvirus DNA polymerase (U_L30), glycoprotein B (U_L27; gB), glycoprotein C (U_L44; gC), and ICP4 amino acid sequences are shown in Figures 6-9. These proteins represent those with the highest levels of conservation between *Herpesviridae* family members, and exhibit a distinct division of the subfamilies *Alpha-*, *Beta-*, and *Gammaherpesvirinae*. Tree constructions were based on homologies derived from amino acid sequence alignments and generated using a distance matrix method. Phylogenetic tree calculations were based on the

sequence distance method, which utilizes the Neighbor Joining (NJ) algorithm of Saitou and Nei (1987). Initially, the alignment was processed by *Seqboot*, which re-sampled the alignments 100 times for bootstrapping. The re-sampled alignments were then processed by *Protdist*, which created distance matrices for each alignment. Each of these matrices were then used by *Neighbor* to create a neighbor-joining tree. Without exception, PsHV-1 was most closely related to the avian alphaherpesvirus, ILTV.

Table 3. **Promoter Element Predictions within PsHV-1**

Start (nt)	End (nt)	Score	Promoter Sequence
6651	6701	0.99	tgcgccccgacatatagccggcgccgccacgccgcaaagc**cgcgcatatg** [a]
16073	16123	0.99	ggagcggctatatgacctgcgaacacactgcgagcacttc**aacgaaactg**
19066	19116	1.00	tccaggtgcataaataccgcgggaagcgcgcttcccgcg**catagggagc**
24440	24490	1.00	gcgcggcgcgttaaacacgcccgcccggattggcttgccg**aaacagctgc**
25347	25397	1.00	cgacctagcaaaaaatccggcggccggacgagttgggggt**agcggtgcgt**
28079	28129	0.99	gcgacgccgggtaaaagcgcgcggaagcgaacacgggctt**actcgcgtca**
28882	28932	0.99	gtagaacttgtatatggaccgcgcggcgaacgccaccgcg**aagaccccgg**
31363	31413	1.00	ctgatgtgcctataaacccccccaggtgctcgggcacgctt**gccgtatcca**
41310	41360	1.00	tcccgtctgtcaaaagcggcccgcccgcgtcgacttccac**gctatcggcc**
41633	41683	0.99	acgtggcgtctaaacatgggcccgctgacgagctcgtaga**agcggctggc**
45996	46046	0.99	gcgctctaggtaaaacagcgcgcgagcagtaagctctggg**ctgaacccgc**
55421	55471	1.00	tcacagcgtatattaaatgagcctgcgccaagcaaccctg**cataatcaca**
65549	65599	1.00	cgcatgcacgcaaaaaagcggcggacgtctccacgcacat**ggcgtagcac**
69442	69492	0.99	gtgtcgcgtataaatcctctccgcggcgaccacctgcgcc**acaaaagcgt**

Table 3. Continued

Start (nt)	End (nt)	Score	Promoter Sequence
80362	80412	1.00	tcagcggagattaaagaggcggccgcaaagaagcctatccagatccgtgc
94541	94591	0.99	cgcgtatccgtatctaaaatcggcacctcgtgcgccgagcgacttctcaa
97121	97171	1.00	cgaagaggcggcaaaaatcgccgcgcaagtcgataagcgcatggtctccg
109143	109193	0.99	tcgatgcatcttaaacacgcggagcagctcccgctcccacaacgcagaca
114533	114583	0.99	catgtaataacaaaaatgcccccacccgctcctggccgccgcccgacgcc
119979	120029	1.00	gggggggggcaaaaaaacggcctttccccctccgaggcgttgccgcgccc
130349	130399	1.00	atatactatatatatatgtacaggggtgcgaatgtttgtttaaggggcct
130784	130834	1.00	tactattatatatatatgtacaggggtgcgaatgtgtacttaagcggcct
131601	131651	0.99	cgcggtgcggtctaaaggcccggcaggcgtcagcgagttcttgcgtggcc
136477	136527	1.00	cggggagagatattatacgggcgctgggcgctgccggcgcgaatagcgct
142555	142605	1.00	cctcgcattacaaaaaaacgccgcggcgcggtctactttcgtggttgcgg
147900	147950	1.00	tcgccgatattataagcgcgcgacataggtgcgatcggccgcatatcgtg
151386	151436	1.00	gtatatactatatatatgtacaggggtgcgaatgtgtacttaagcggcct
151823	151873	1.00	tactaatatatatatatgtacgagggtgcgaatgtgtacttaagagggcc
152133	152183	1.00	cgcagcagcataaaaactgcggagacacgcgggctcgcgcatatcgccat
152331	152381	1.00	atgttgtgtatatatatatgactcatgactaaccacaggttttctcccca
152638	152688	1.00	ctcgcgtgtaaaaaaaggggggagcattttcctcgttccgcgcttgctcag
153744	153794	1.00	cggggcctagaaaaaaaaccccggacggaattgcctgtttctattgtttt
161481	161531	1.00	ccgcggggccccaaaaaacggcgcagccagcggcgtgtgtgtttttttat

[a] Transcription start site (TSS) shown in larger font.

Figure 5. Amino acid alignment of the predicted PsHV-1 sORF2 and ILTV gJ proteins. Amino acid sequences were aligned with the AlignX program (Vector NTI). Gaps in the alignment are represented as dashes. The consensus sequence is shown below the alignment. Potential glycosylation sites are indicated by shaded boxes, with asparagines (**N**) indicated in boldface type.

```
                1                                                   50
     PsHV1    (1) MLWPAALVAMFALAARAREIDNVTCSVVYGSNVARISKDYWLAQEGSLVS
     ILTV     (1) MGTMLVLRLFLLAVADAALPTGRFCRVWKVPPGGTIQENLAVLAESPVTG
Consensus     (1) M    L       A A       C V        I        E
                51                                                  100
     PsHV1   (51) FMTFENDDPVYFFMGRALGTGASG--GKYLYRVTRDNNTNVSHVQTSLFI
     ILTV    (51) HATYPPPEGAVSFQIFADTPTLRIRYGATEDELALERGTSASDADNVTFS
Consensus    (51)   T         F   A         G           T  S      F
                101                                                 150
     PsHV1   (99) PEHFNG----GILLDMGSNYTHDAETDPEFLNARYLVVNDTEDSVREETL
     ILTV   (101) LSYRPRPEIHGAYFTIGVFATGQSTESSYSVISRVLVNASLERSVRLETP
Consensus   (101)           G     G   T            R LV    E SVR ET
                151                                                 200
     PsHV1  (145) GPCPEMLRTLDVSGRTTLLYPSRYLHARDRMQVEKTMDTAKCVSTSVNLD
     ILTV   (151) CDENFLQNEPTWGSKRWLGPPSPYVRDNDVAVLTKAQYIGECYSNSAAQT
Consensus   (151)                 L  PS Y    D     K      C S S
                201                                                 250
     PsHV1  (195) ATVVFSVDFPFSERAILRVTQTFYPDDRPQKHLLYLASQGGK---TVHYS
     ILTV   (201) GLTSLNMTFFYSPKRIVNVTWTTGGPS-PSRITVYSSRENGQPVLRNVSD
Consensus   (201)         F  S   I  VT T      P     Y     G
                251                                                 300
     PsHV1  (242) EVSATVTPLPGGNASFNVTIEFTDQPDDTTIHLWVSPKGINDDPDAVSAY
     ILTV   (250) GFLVKYTPDIDGRAMINVIANYSPADSGSVLAFTAFRE--GKLPSAIQLH
Consensus   (251)       TP   G A  NV                          P A
                301                                                 350
     PsHV1  (292) YILNSLSSYDDPYAHEEISCDSVYQNIVMVLGSVVNITSPDPLPRAPDYS
     ILTV   (298) RID--MSGTEPPGTETTFDCQKMIETPYRALGSNV----PRDDSIRPGAT
Consensus   (301)  I    S    P       C          LGS V    P      P
                351                                                 400
     PsHV1  (342) YPTDDPAGETTESAVTWLEATAQTADEATTPLAAHATDGYEPTTAAATLS
     ILTV   (342) LPPFDTAAPDFDTGTSPTPTTVPEPAITTLIPRSTSDMGFFSTARATGSE
Consensus   (351)  P  D A            T       T         G   T  A
                401                                                 450
     PsHV1  (392) TTEDLTQETSTPTVTTVIDPTSGAVTTESRTTEGTAANVATTEAAGTEGQ
     ILTV   (392) TLSVPVQETDRTLSTTPLTLPLTPGESENTLFPTTAPGISTETPSAAHET
Consensus   (401) T     QET     TT           E      TA    T
                451                                                 500
     PsHV1  (442) NQEATTAGGPTNAATTLGHQTIEAATVEDSTTTARAAEYPTPTTTTVEPR
     ILTV   (442) TQ--------TQSAETVVFTQSPSTESETARSQSQEPWYFTQTPSTEQ--
Consensus   (451)  Q        T  A T           E          Y T T  T
                501                                                 550
     PsHV1  (492) PAGGTTIEDPTDSPAIEDTTTPTTTAAKVTALKTAAAEGPTPCVTTYTGS
     ILTV   (482) -----AALTQTQIAETEALFTQTPSAEQMTFTQTPGAETEAPAQTPSTIP
Consensus   (501)           T     E   T T  A   T   T  AE   P  T  T
                551                                                 600
     PsHV1  (542) DTEAAQSATSISDAVTPEDLTPETTTPVDWSVTSTYISVADGTSMPAPTP
     ILTV   (527) EIFTQSRSTPPETARAPSAAPEVFTQ---SSSTVTEVFTQTPSTVPKTTL
Consensus   (551)         T    A  P       T     S T T          P  T
                601                                                 650
     PsHV1  (592) SAVAHEASTTPAPTATAVPTSHTPKPQESTSTPSRAPTTGVAPVTANSLS
     ILTV   (574) SSSTEPAIFTRTQSAGTEAFTQTSSAEPDTMRTQSTETHFFTQAPSTVPK
Consensus   (601) S     A  T    A       T      T       T
                651                                                 700
     PsHV1  (642) PAATSSERIVILNTATASSGPGASTGATTAPISPPWSASPAGDGVTTSAA
     ILTV   (624) ATQTPSTEPEVLTQSPS-TEPVPFTRTLGAEPEITQTPSAAPEVYTRSSS
Consensus   (651)    T S     L        P   T    A        S A    T S
                701                                                 750
     PsHV1  (692) RTLEPSSTRKAVAAESTTAADDVATSELGSGDYGDHATEPPRIVITNPPG
     ILTV   (673) TMPETAQSTPLASQNPTSSGTGTHNTEPRTYPVQTTPHTQKLYTENKTLS
Consensus   (701)    E           T         E
                751                                                 800
     PsHV1  (742) ITTLHDADAAEEDPWPTRPLYSVNIVNATLTANGMLTATCMAAAKAKHAI
     ILTV   (723) FPTVVSEFHEMSTAESQTPLLDVKIVEVKFSNDGEVTATCVSTVKSPYRV
Consensus   (751)   T              PL  V IV       G  TATC    K
```

```
                801                                                 850
     PsHV1  (792) TFTWHVGSNMVPAITGPPEPGLMFNGNRAWSSRLQTVEYGISPSARLACM
     ILTV   (773) ETNWKVDLVDVMDEISGNSPAGVFNSNEKWQKQLYYRVTDGRTSVQLMCL
Consensus   (801)    W V    V        P   FN N  W   L         S  L C
                851                                                 900
     PsHV1  (842) ACTVPPAQRYCAHDVAVVARHDRLELDMQVDVATVSVVCSGLDGVESEPY
     ILTV   (823) SCTSHSPEPYCLFDTSLIAREKDIAPELYFTSDPQTAYCTITLPSGVVPR
Consensus   (851)  CT      YC  D    AR                 C         P
                901                                                 950
     PsHV1  (892) FVWTANGRPVPLGSVRTKRIPNDYGTPARWQSAIHISRFFVPAGHRDVYE
     ILTV   (873) FEWSLNNVSLPEYLTATTVVSHTAGQSTVWKSSARAGEAWISGRGGNIYE
Consensus   (901) F W  N    P     T       G    W S               YE
                951                                                1000
     PsHV1  (942) CTATLASGETIKATKN-WSNTDYLALQKNAAARSTFVVAGGITAFAVAEL
     ILTV   (923) CTVLISDGTRVTTRKERCLTNTWIAVENGAAQAQLYSLFSGLVSGLCGSI
Consensus   (951) CT     G      K         A    AA         G
                1001     1013
     PsHV1  (991) L------------
     ILTV   (973) SALYATLWTAIYF
Consensus  (1001)
```

Figure 6. Phylogenetic tree constructed from U_L30 (DNA polymerase) gene homologs within the *Herpesviridae*. The alignment is created using the ClustalW algorithm (Thompson *et. al.,* 1994). Trees were initially drawn with the *TreeView* program. Bootstrap analysis was performed within the *Phylogenator* program. Phylogenetic tree calculation is based on a sequence distance method and utilizes the Neighbor Joining (NJ) algorithm (Saitou and Nei, 1987). The measure of divergence is presented as a scale in the lower left.

Accession numbers of protein sequences used in tree assembly: AY372243 (PsHV-1), NC006623 (ILTV), AB024414.1 (MDV-2; HPRS24 strain), AF282130.1 (HVT), P04293 (HSV-1), P07918 (HSV-2), P09252 (VZV), NC006151 (PRV), AJ004801 (BHV-1.1), P08546 (HCMV), P27172 (MCMV), P03198 (HHV-4; EBV), P52367 (EHV-4), AAT79466 (VHV; Vulture HV), AAF66765 (MDV-1; GA strain).

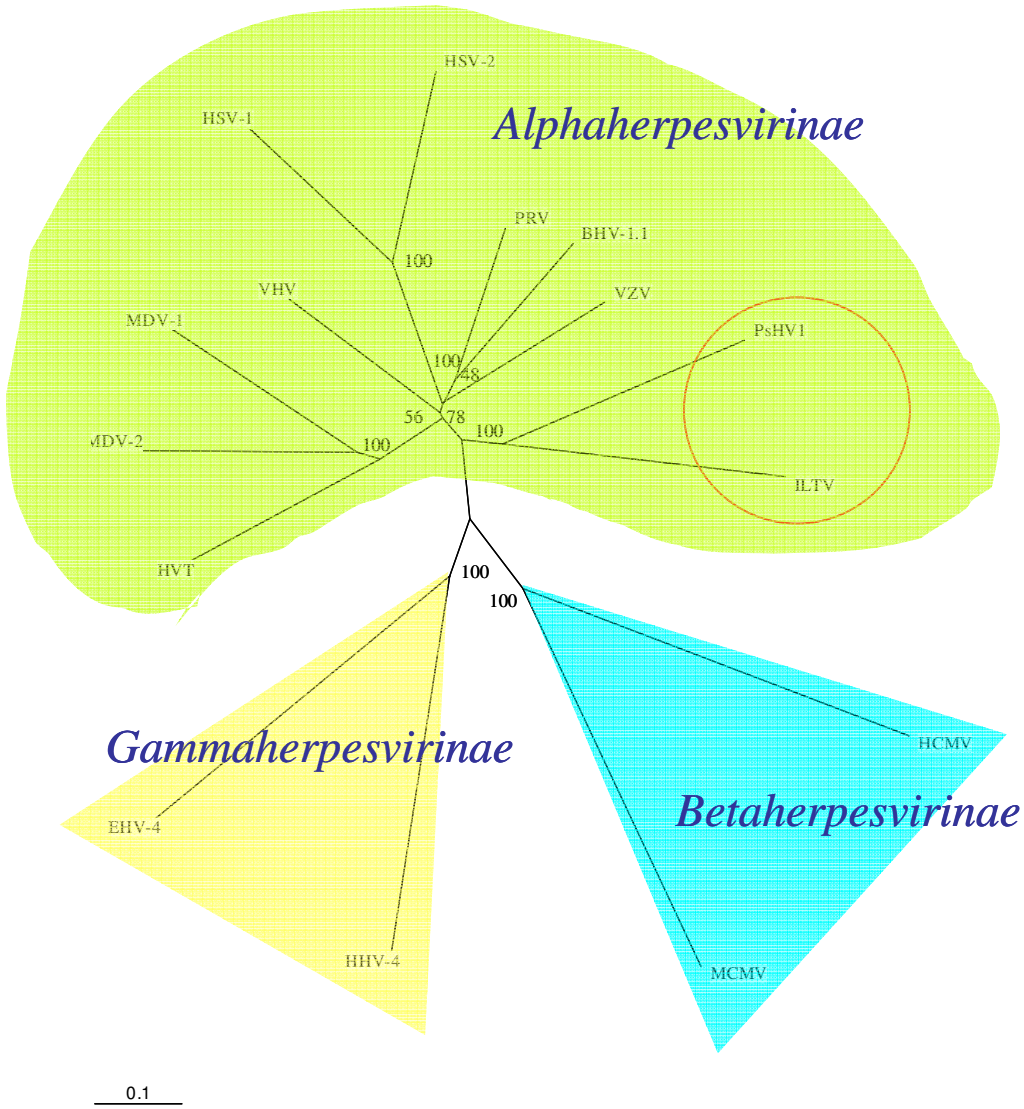

HSV-2
HSV-1
Alphaherpesvirinae
PRV
BHV-1.1
100
VHV
VZV
MDV-1
PsHV1
100
48
56
78
MDV-2
100
100
ILTV
HVT
100
100
Gammaherpesvirinae
HCMV
EHV-4
Betaherpesvirinae
HHV-4
MCMV
0.1

Figure 7. Phylogenetic tree constructed from U_L27 (gB) gene homologs within the *Alphaherpesvirinae*. The amino acid alignment was created using the ClustalW algorithm (Thompson *et. al.,* 1994). Trees were initially drawn with the *TreeView* program. Bootstrap analysis was performed within the *Phylogenator* program. Phylogenetic tree calculation is based on a sequence distance method and utilizes the Neighbor Joining (NJ) algorithm (Saitou and Nei, 1987). The measure of divergence is presented as a scale in the lower left.

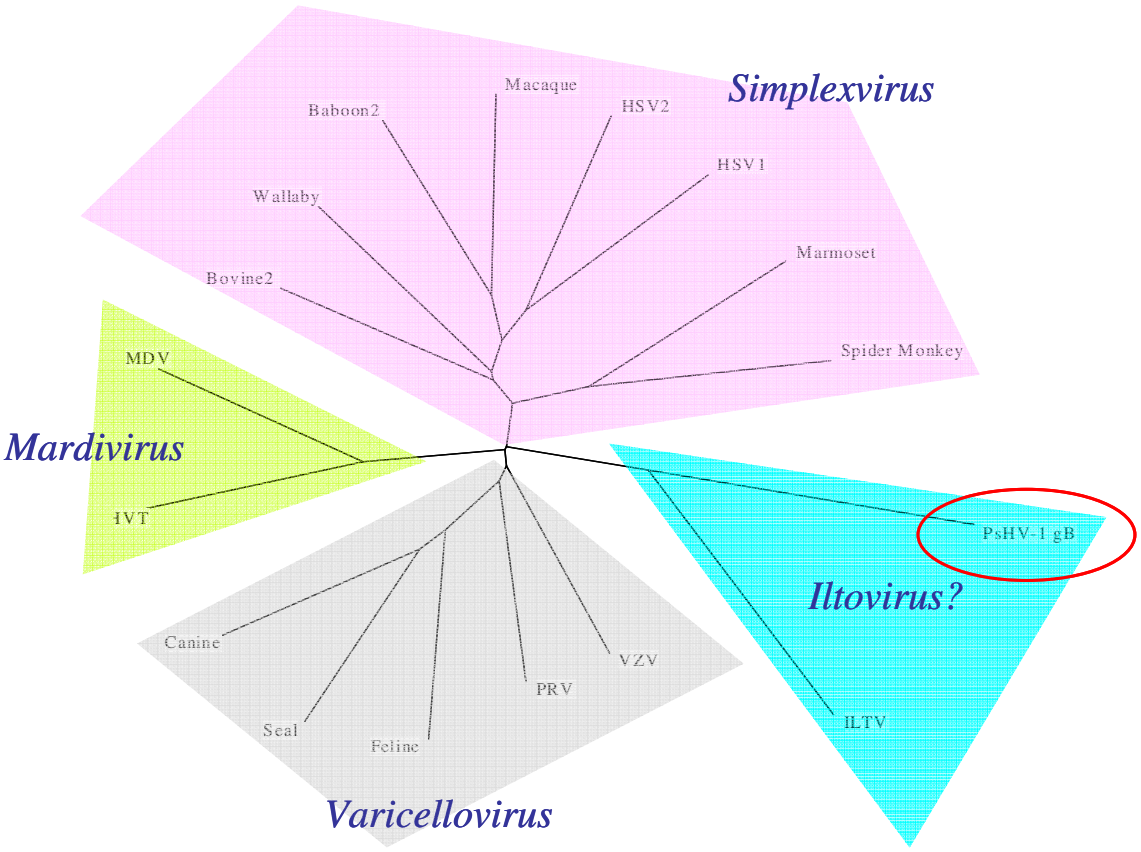

Simplexvirus
Macaque
Baboon2
HSV2
HSV1
Wallaby
Marmoset
Bovine2
Spider Monkey
MDV
Mardivirus
HVT
PsHV-1 gB
Iltovirus?
Canine
VZV
PRV
Seal
Feline
ILTV
Varicellovirus
0.1

Figure 8. Phylogenetic tree constructed from U_L44 (gC) gene homologs within the *Alphaherpesvirinae*. The amino acid alignment was created using the ClustalW algorithm (Thompson *et. al.,* 1994). Trees were initially drawn with the *TreeView* program. Bootstrap analysis was performed within the *Phylogenator* program. Phylogenetic tree calculation is based on a sequence distance method and utilizes the Neighbor Joining (NJ) algorithm (Saitou and Nei, 1987). The measure of divergence is presented as a scale in the lower left.

Accession numbers of protein sequences used in tree assembly: AY372243 (PsHV-1); U06635 (ILTV); AB012572 (MDV2); AAG30084 (HVT); AY129979 (MDV1); AJ133757 (HSV-1); Z49225 (Caprine); AF275348 (CeHV-7) X90446 (Canine); D86616 (FHV-1); U35883 (BHV-5); Z49223 (BHV-1); AY101384 (BHV-2).

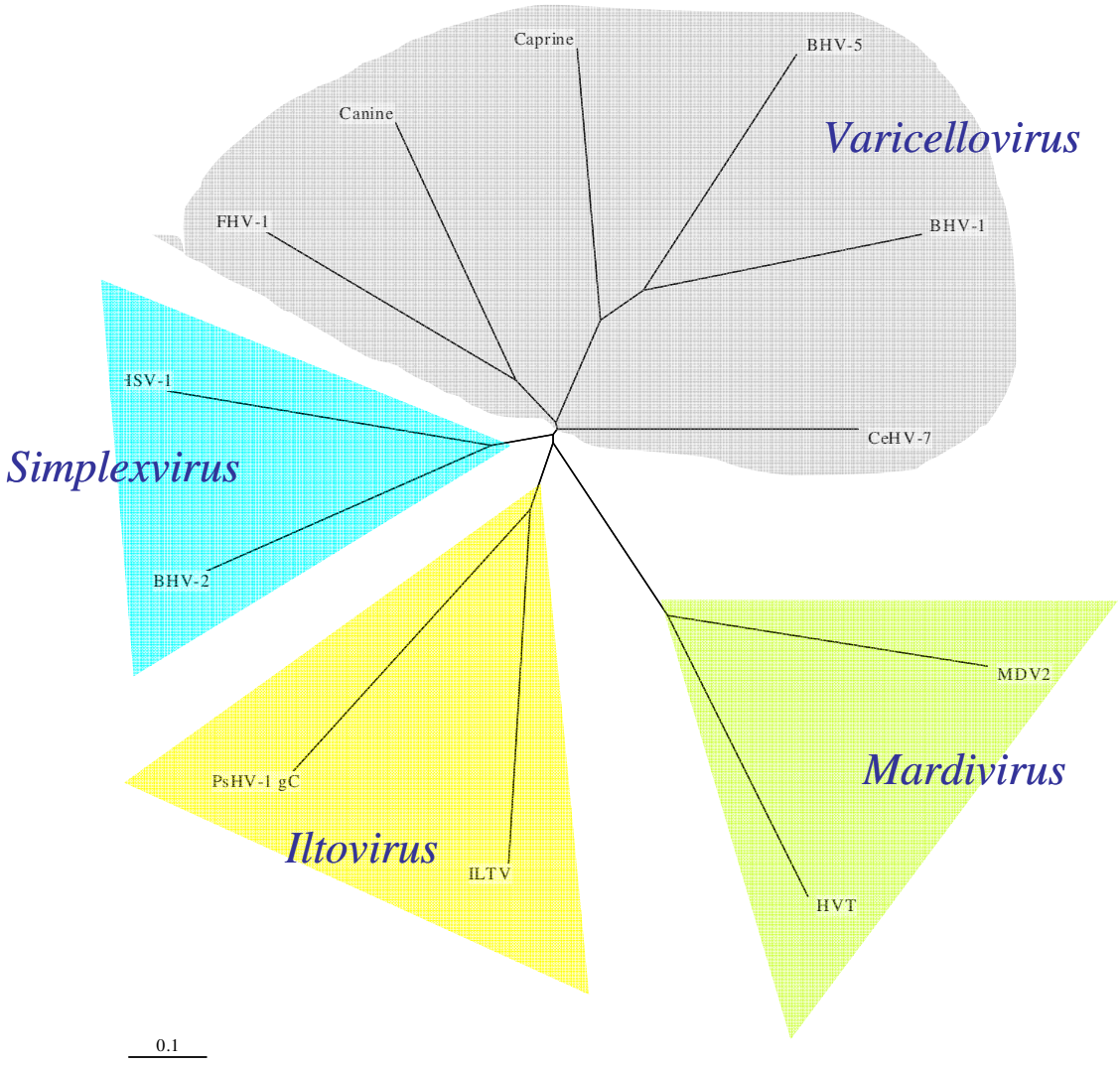
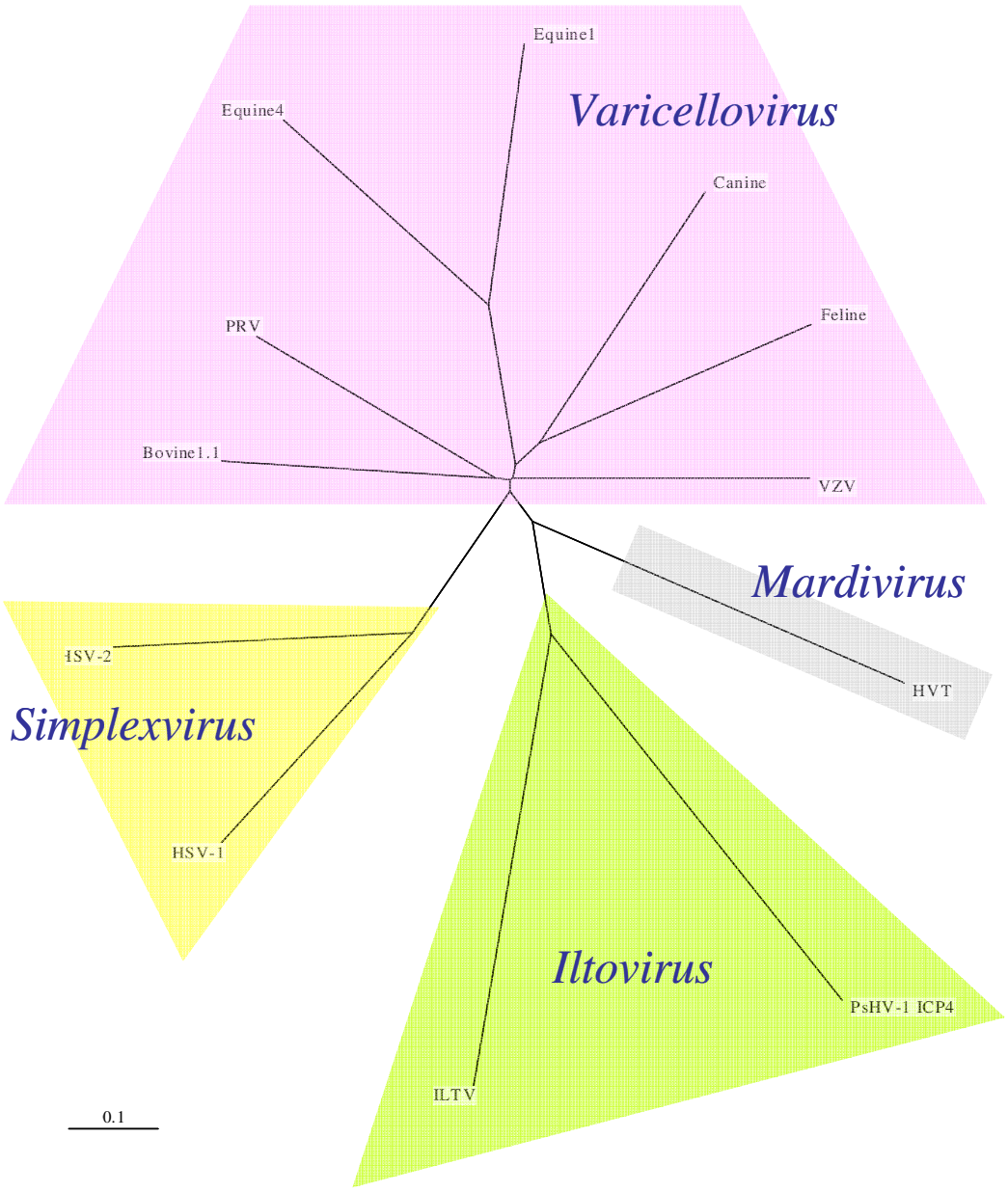

Caprine
BHV-5
Canine
Varicellovirus
FHV-1
BHV-1
ISV-1
CeHV-7
Simplexvirus
BHV-2
MDV2
Mardivirus
PsHV-1 gC
Iltovirus
ILTV
HVT
0.1

Figure 9. Phylogenetic tree constructed from ICP4 gene homologs within the *Alphaherpesvirinae*. The amino acid alignment was created using the ClustalW algorithm (Thompson *et. al.,* 1994). Trees were initially drawn with the *TreeView* program. Bootstrap analysis was performed within the *Phylogenator* program. Phylogenetic tree calculation is based on a sequence distance method and utilizes the Neighbor Joining (NJ) algorithm (Saitou and Nei, 1987). The measure of divergence is presented as a scale in the lower left.


Equine1
Equine4
Varicellovirus
Canine
Feline
PRV
Bovine1.1
VZV
Mardivirus
HVT
ISV-2
Simplexvirus
HSV-1
Iltovirus
PsHV-1 ICP4
ILTV
0.1

Chapter 4

DISCUSSION

4.1 Classification of the Avian Herpesvirus PsHV-1.

Herpesviruses are highly disseminated in nature, and have been identified in insects, reptiles, amphibia, invertebrates and many species of birds and mammals (Roizman, 1996). Herpesviruses vary greatly in their biological properties; some have wide host ranges, while others have very narrow ranges. Multiplication rates also vary from the efficient, rapid destruction of host cells, to slow, less destructive infections, while some herpesviruses can cause malignancy in their hosts (Roizman, 1996). Phylogenetic studies using mammalian herpesvirus sequences have helped to demonstrate the division of the *Herpesviridae* into the three subfamilies, and in some cases, patterns of divergence consistent with co-speciation of virus and host.

Herpesviruses of mammals and birds clearly descend from a common ancestor, but their genomes exhibit significant variation with respect to nucleotide sequence, gene content and genomic organization (McGeogh and Davison, 1999). Amongst avian hosts, the herpesviruses also tend to be species-specific with respect to their susceptibility, pathogenicity and virulence. Although they differ significantly in G-C content, 61% for PsHV-1 and 48% for ILTV, phylogenetically, PsHV-1 and ILTV are closely related. While their genomic structure, content, and organization is similar to other

alphaherpesviruses, they represent a unique class of avian alphaherpesviruses. Similarities between PsHV-1 and ILTV can be seen in the U_L inversion, the absence of repeats flanking the U_L region, the conserved structure of the U_S region, and a conserved cluster of 5 unique ORFs in the U_L region. The similarity of the PsHV-1 and ILTV genomes suggests that PsHV-1 (*psittacid*) and ILTV (*gallid*) represent a class of avian alphaherpesviruses that have diverged early from a common ancestor and are distinct from the Marek's family of alphaherpesviruses, as previously suggested by other researchers (Kingham *et al.*, 2001).

Based on this evidence we propose that PsHV-1 be formally assigned to the *Iltovirus* genus of the *Alphaherpesvirinae*.

4.1.1 Characterization of Isolate PsHV-1 97-0001.

The isolate provided by Dr. Phalen (PsHV-1, isolate 97-0001) was from the liver of a male Amazon parrot of unknown age and unspecified species. Details were not provided on the exact protocol used to isolate the virus, but presumably it was via liver homogenation and inoculation onto naïve CEFs. We were supplied with passage P1, harvested from infected CEF monolayers. Actual titer was unknown, as the MOI was difficult to determine due to the inability to identify specific plaques on the infected CEF monolayers. Attempts in our lab to determine titer using an immuno-fluorescent antibody were unsuccessful. Analysis of virion morphology and restriction enzyme digestions of purified viral DNA were consistent with previous results, suggesting that the isolate was a psittacine herpesvirus.

4.1.2 Structure of the PsHV-1 Genome.

Unlike the MDV family of avian alphaherpesviruses, which exhibit genome structures similar to HSV-1 and HSV-2, the PsHV-1 genome exhibits the gross structural characteristics of class D herpesvirus genomes, similar to PRV, ILTV, and VZV, which contain two domains of unique sequences. In this class of herpesvirus only the shorter unique sequence (U_S) is flanked by inverted repeats (I_R and T_R). The U_S region can then invert relative to the larger unique domain (U_L) allowing the genome to exist in two equimolar isomeric forms. The biological significance of this ability remains unclear, but is has been suggested (Thiry *et al.*, 2004) that it may provide an evolutionary advantage through recombination of the inverted segments during replication.

As evidenced by the overall co-linearity of their genomes, genomic architecture is well conserved among the alphaherpesviruses and can be somewhat compartmentalized into conserved blocks of genes that show homology in their protein-coding sequence and their position relative to each other. Herpesviruses share several common properties, including a large number of conserved enzymes involved in nucleic acid metabolism, DNA synthesis, and protein processing (Roizman, 1993). PsHV-1 is no exception. Consistent with other herpesviruses, the PsHV-1 genome encodes conserved DNA replication genes that may represent an evolutionary advantage for herpesvirus genome replication, allowing for persistence of infection and a broad host range for viral infection. These conserved genes might have been acquired from a host or from infection of a common host by an ancient herpesviruses. The acquired cellular genes could have

remained as essential genes for virus replication or could represent nonessential genes which were lost or replaced by other genes in the herpesviruses during evolution.

The conservation of these gene blocks suggests that transcript arrangement is critical in the viral life cycle, possibly by ensuring proper regulation of viral gene expression. Further investigation into the actual expression kinetics of the PsHV-1 transcripts would help define the specific function of these putative genes in PsHV-1. Searches for transcriptional elements within the PsHV-1 genome revealed little information of value. More structured analysis with additional parameters is needed to make any significant conclusions about these elements in the PsHV-1 genome.

4.2 Phylogeny and Phylogenetic Analysis of PsHV-1.

Because nucleotide sequence is often the primary determining factor of function, sequence similarity is often taken to imply similarity of function. Therefore, many researchers use the highest scoring BLAST homolog as the basis for assigning gene function, and in most cases, this assumption is valid (Eisen, 1998). Genes can become similar in sequence either as a result of *convergence* (similarities without a common evolutionary history) or *homology* (descent from a common ancestor, but modified by evolution).

One of the methodologies used to determine the degree of relatedness is the generation of phylogenetic trees, derived from homologous nucleotide sequences or amino acid sequences, to graphically represent relationships, which may also indicate the evolutionary history of the various organisms.

Gene function changes as a result of evolution, therefore reconstructing an evolutionary history can help predict function in uncharacterized genes. The generation of a phylogenetic tree representing the history of a gene and its homologs is the first step in functional prediction, and may add valuable insight into the proper classification of the organism as a whole (Swofford *et al*., 1996; Eisen, 1998).

Three common methods for generating phylogenetic trees are *parsimony* (fewest functional changes over time), *distance* (evolutionary distance between sequences to determine branch patterns and lengths), and *maximum likelihood* (scoring and alignment based on probability). Bootstrapping analysis can assign confidence levels to groupings within the tree (Eisen, 1998).

The strategy most commonly used to generate a phylogenetic prediction of gene function is to first choose a gene of interest and identify its homologs, then align the amino acid sequences. A gene tree can then be generated, the known functions of the homologs overlayed, and the most probable function of the gene of interest can then be inferred (Weisburg *et al*, 1989).

McGeoch and Cook (1994) established a relational pattern between mammalian herpesviruses and their hosts, which follows a co-evolution model. They derived a herpesvirus evolutionary rate in relation to the timescale for host evolution as derived from paleontology. Davidson (2002) extensively reviewed the evolution of the herpesviruses, and states that the co-evolutionary estimates for avian alphaherpesviruses of ~80-120 million years ago (mya) is too recent, as the ancestors of mammals and birds are believed to have diverged ~310 mya (Kumar and Hedges, 1998). Drake and Hwang

(2005) calculate the evolutionary mutation rate in the laboratory of pathogenic mammalian herpesviruses to be ~0.003 mutations per genome replication. Given this relatively low rate of nucleotide substitution, it is possible that recombination plays an important role in herpesvirus evolution. The presence of the inverted repeats in the D and E class genomes of the alphaherpesviruses may allow for recombination to occur during genome replication (Thiry *et al.*, 2004).

Figures 6 through 9 represent phylogenetic trees based upon amino acid sequence homologies. In our analysis, PsHV-1 consistently forms a unique clade with ILTV, suggesting that PsHV-1 be classified within the *Iltoviruses* and the subfamily *Alphaherpesvirinae*. As more lineages of the avian herpesviruses are identified and characterized, the proper placement of the avian viruses into the subfamilies of the *Herpesviridae* will be more accurately determined.

4.3 Comparison of the Genome Content of PsHV-1 and ILTV.

The PsHV-1 genome shares several characteristics with that of ILTV with respect to genome arrangement and content which may provide clues to differences and similarities in their tissue tropism, pathogenicity and host range. One characteristic which sets PsHV-1 and ILTV apart from the other alphaherpesviruses is the presence of five unique, conserved ORFs in the U_L region (ORF A – ORF E), which Veits *et al.* (2003) have found to be dispensable for ILTV replication in tissue culture. The HSV-1 genome lacks these conserved genes. Database searches return no significant homology to other cellular or viral proteins, nor are there any conserved motifs that would suggest

function (Veits *et al.*, 2003). Mutant viruses with deletions in each of the 5 ORFs were created and compared to wild-type virus. It has been postulated that these genes may play a role in immune evasion or species-specificity. This may reflect the host range of the avian alphaherpesviruses to their reptilian ancestors, and the adaptation of PsHV-1 and ILTV during host co-evolution. Given that neither PsHV-1 nor ILTV induce tumors, and the rapid replication of ILTV in the host, immune evasion strategies may be dispensable in these viruses. ILTV and PsHV-1, like MDV, also exhibit very narrow host ranges, so it is possible that these species-specific genes are adaptations to the avian hosts to benefit replication. Similar experiments with the ORF A-E homologs in PsHV-1 may help determine the role of these gene products in the PsHV-1 lifecycle.

Other gene homologs found in the mammalian herpesviruses (U_L55, U_L56, U_S9, U_S11, U_S12), which are not present in PsHV-1 may represent species- or host-specific evolutionary changes that the mammalian herpesviruses incorporated into their genomes as they co-evolved with their hosts.

Genomes of ILTV and PsHV-1 contain an inversion of the genome from U_L22 to U_L44, which is similar to an inversion found in the PRV genome. We have examined the genomic regions between the PsHV-1 U_L44 and U_L21 and the U_L22 and ORF E genes in detail, and analysis of these sequences for conserved motifs reveals little clue as to the specific site of the inversion. The biological significance of this conserved U_L inversion is remains unknown.

Additionally, both ILTV and PsHV-1 contain structurally similar glycoprotein C (gC) proteins, which appear to lack consensus alphaherpesvirus heparin binding sites

(Kingsley and Keeler, 1999). Experiments have shown that the initial attachment step of ILTV to susceptible cells does not involve interactions with heparan or chondroitin sulfate containing proteoglycans. These same observations have been confirmed for PsHV-1 (Kingsley, personal communication; data not shown).

Neither the PsHV-1 nor the ILTV avian herpesvirus genomes contains a positional U_L homolog to U_L47, a herpesvirus gene encoding a tegument phosphoprotein, but they have do have a putative homolog in the U_S region, previously described by Wild *et al.,* (1996).

The U_L regions of ILTV and PsHV-1 also exhibit some differences in gene content. PsHV-1 is predicted to contain a homolog to the U_L16 gene, a tegument protein predicted to be involved in determining host range, which is absent from the ILTV genome. Conversely, ILTV is predicted to contain homologs to the U_L48 and U_L0 ORFs, both of which are not found in the PsHV-1 genome.

The amino acid sequences of the ILTV genes U_L0 and $U_L[-1]$ share significant homology to one another, and Ziemann *et al.* (1998) suggest that these two genes represent an ancient replication event that occurred after divergence of the ILTV lineage from the *beta-* and *gamma-herpesviridae*, as well as the mammalian alphaherpesviruses. They speculate that U_L0 and $U_L[-1]$ of ILTV may be involved in the determination of host tropism and pathogenicity. The U_L region of the PsHV-1 genome contains a positional homolog to the ILTV $U_L[-1]$ gene.

Homology to α-TIF (U_L48), a viral transcription factor which functions together with components of the cellular transcriptase complex to mediate virus-specific

immediate early (IE) transcription, was absent in the PsHV-1 genome. In other herpesviruses this non-essential tegument protein (VP16) functions with components of the cellular transcriptase complex to enable virus-specific immediate early (IE) transcription. In certain isolates of HSV-1, lacking viral α-TIF, sufficient amounts of IE proteins are made to promote viral growth and replication, although CPE in tissue culture progresses more slowly (Rajcani and Durmanova, 2000).

Such may be the case with PsHV-1. In ILTV, which contains a homolog to U_L48, infection results in >90% CPE in primary cell culture after 16 hr. In our experience, PsHV-1 infections to >90% CPE are considerably slower (~144 hr), which may indicate a lower replication efficiency due to the lack of the α-TIF gene product. This slow growth in tissue culture might also account for some early confusion regarding the classification of PsHV-1.

There are ten predicted ORFs within the U_S region of PsHV-1, and gene arrangement within the U_S region is quite similar to the corresponding region of ILTV, however, a homolog to ILTV U_S9 is not present in PsHV-1. Brandimarti and Roizman (1997) reported that the U_S9 product of HSV-1 interacts with the ubiquitin-dependent pathway for degradation of proteins as the virus enters the cell, and is dispensable for growth *in vitro*. Nishiyama *et al.* (1994) identified the U_S9 tegument protein of HSV-1 as a possible target of the protein kinase encoded by U_S3. The U_S region of PsHV-1 also lacks the duplication of the U_S10 and sORF3/4 genes within the inverted repeat region as observed in ILTV.

In HSV and CMV, the U_S regions contain many of the viral genes affecting antigen presentation. In MDV, SORF3 is involved in down-regulating the expression of cell surface major histocompatibility complex (MHC) class I proteins. Marek's disease virus mutants with deletions in SORF3 are highly cell-associated, spread by cell-to-cell contact, and produce little or no cell-free virus. These characteristics also suggest a role in immune evasion of the host.

The evasion of acquired cellular immunity is critical for infection and the lifelong persistence of herpesviruses in their hosts. The importance of cellular immunity is emphasized by the considerable number of viral proteins herpesviruses use to alter the antigen-presenting machinery of the host cell. This interference with the MHC class I antigen-presenting pathway is a common mechanism used by mammalian herpesviruses to evade immune detection (Johnson and Hill, 1998).

Although the organization of the PsHV-1 and ILTV U_S regions are very similar, they differ greatly from the corresponding region of the three serotypes of MDV (Figure 4). The ILTV and PsHV-1 genomes contain two collinear ORFs in the U_S region; U_L47/sORF1 and U_S5/sORF2, respectively. Initially, U_S5 was described in ILTV to encode a 60kDa protein, and tentatively named gp60 (Kongsuwan *et al.*, 1993). More recent studies have adopted the HSV-1 designation of gJ (Fuchs *et al*., 2005). The ILTV gJ positional homolog in PsHV-1, which we have designated as sORF2, appears to be mainly conserved only by position, and shares only an 18% amino acid identity (Figure 5).

The gJ homolog in ILTV encodes a N- and O-linked modified glycoprotein with significant homology to EHV-1 gp2 (Fuchs *et al.*, 2005). Veits *et al*. (2003) have indicated that gJ may be a dominant antigen for the humoral immune response against ILTV in chickens. More recent studies conducted with gJ-negative ILTV mutants have demonstrated that the gJ gene plays a role in the severity of infection and suggest that gJ may be of interest as a target for live-virus vaccine development in ILTV (Fuchs *et al.,* 2005).

Another conserved ORF found to be positionally unique to these avian herpesviruses is PsHV-1 sORF1 and its ILTV homolog U_L47. Previous examination of the U_S region of ILTV reported the translocation of a U_L HSV-1 homolog to a position within the U_S of ILTV, and designated the ORF as U_L47 (Wild *et al.*, 1996; Ziemann *et al*., 1998). Other researchers have suggested that the 'U_L47' ORF of ILTV is not a homolog to HSV-1 U_L47, but rather a unique ORF which encodes for a 67kDa protein (Kongsuwan *et al.*, 1993). The U_L47 genes of HSV-1 and PRV act as major tegument proteins (VP13/14), and are closely associated with the product of U_L48 as modulators of VP16 (α-TIF) (Donnelly and Elliott, 2001; Kopp *et al.*, 2002). The U_L47 product in HSV-1 is post-translationally glycosylated (Meredith *et al*., 1991).

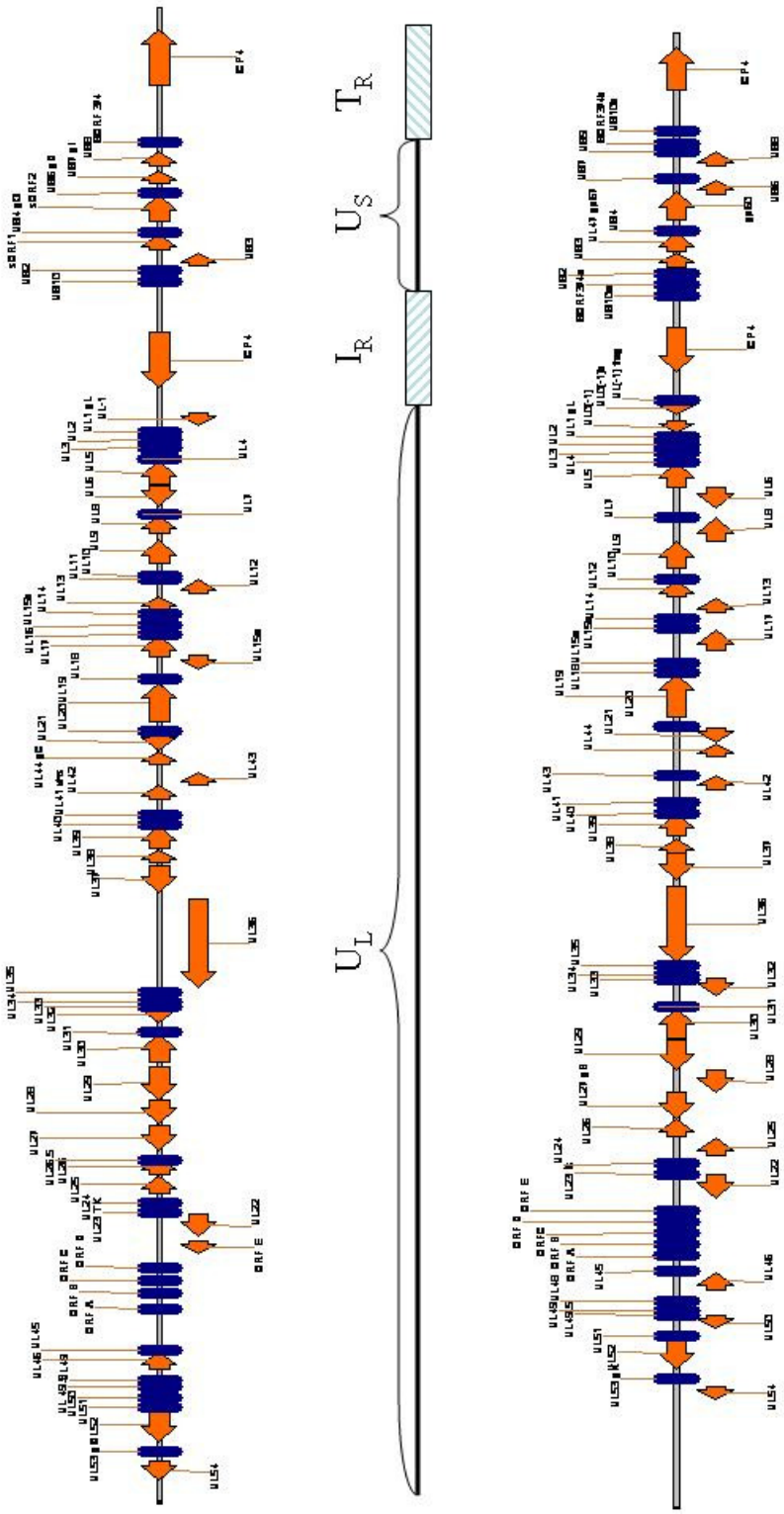
The predicted PsHV-1 sORF1 and ILTV U_L47 proteins share 42% amino acid identity, yet have limited identity to U_L47 of HSV-1 (18%). Sequence analysis shows no evidence of N- or O-linked glycosylation, or transmembrane domains (data not shown), so the potential function of these ORFs in PsHV-1 and ILTV remains unclear.

The proper designation for the gp67/U_L47 sORF1 and U_S5/gJ sORF2 open reading frames in PsHV-1 remains unclear, emphasizing the need for additional experiments to determine if these ORFs in PsHV-1 encode for proteins, and to identify the function of these putative gene products. Perhaps in PsHV-1, which lacks a U_L48 homolog, the U_L47 ORF is a residual, inactive gene. Further studies to confirm the presence of RNA transcripts from U_L47 are needed.

Herpesvirus-encoded structural glycoproteins play major roles in host range and pathogenicity. The gene products of sORF2/gJ may determine host and/or tissue preferences for PsHV-1 and ILTV. It is intriguing to speculate that differences in gJ may result in the uniquely different tropisms of ILTV and PsHV-1, while U_L47/sORF1 and the unique characteristics of gC attribute to some of their common biological properties.

One major structural difference between the PsHV-1 and ILTV genomes occurs in the size and genetic make up of the inverted repeat regions (Tables 1 and 2, Figure 10). The ILTV inverted repeat regions are 2,535 bp (18.5%) shorter than the PsHV-1 inverted repeats and contain two copies of three genes, ICP4, U_S10 and a homolog to the MDV sORF4/3 gene (Figure 4). The significance of the difference in the relative sizes of the repeat regions remains unclear, as is the redundancy of several genes in the ILTV repeats. It is possible that these are residual ORFs with no function.

Figure 10. Complete comparison maps of the PsHV-1 and ILTV avian alphaherpesviruses. Vector NTI-generated display of the genomic components of PsHV-1 (upper) and ILTV (lower). Open reading frames as depicted as orange arrows, with the direction of transcription indicated by arrowhead. Blue bands indicate smaller ORFs. (Transcriptional information can be found on Table 2). Center figure depicts the general structure of the genomes, which consist of the unique long (U_L), internal repeat (I_R), unique short (U_S), and terminal repeat (T_R) regions.


T_R
U_S
I_R
U_L

4.4 Future Work.

The well-characterized kinetics of the HSV-1 replication cycle were explained in detail in Chapter 1. Given the genomic data we have generated, it is assumed that the general replication kinetics of PsHV-1 would be similar to that of HSV-1. By utilizing microarray technology, it is feasible to determine the actual expression profile of the transcripts of PsHV-1 and ILTV infection, which may shed additional light on the differences observed between ILTV and PsHV-1 infections *in vitro*. DNA microarrays hybridized to cDNA generated from total RNA isolated over the course of cell culture infections would allow for the generation of transcriptional maps. The information gained using these approaches may then be used for a variety of purposes. From a control standpoint, one would to be able to identify unique characteristics from which additional diagnostic assays could be developed. Unique genes may also be identified as potential targets for the development of subunit vaccines or live attenuated recombinant vaccines. A complete transcription expression profile for PsHV-1 would also aid in the analysis of the other genotypes of PsHV, and may allow for more advanced diagnostic protocols.

Based on the phylogenetic analysis of U_L16, Tomaszewski *et al.* (2003) have identified four 'genotypes' of PsHV. Similarly, Sellers *et al.* (2004) have identified the presence of novel ILTV subgroups that may play a role in the propagation and subsequent outbreaks of ILTV. Comparative sequence analysis of additional genes would aid in understanding the apparent diversity within these related groups of avian alphaherpesvirus.

Current advances in bioinformatics tools would also allow for whole-genome comparisons of the sequenced avian herpesviruses, which would provide a great benefit in determining the proper taxonomic placement of these viruses within the *Herpesviridae* subfamilies, as well as aid in examining the evolutionary history of the herpesviruses as compared to their hosts.

4.5 Conclusions.

Utilizing recent advances in the expanding fields of bioinformatics and genomics technology, we sequenced and characterized the entire 160,025 bp genome of the causative agent for Pacheco's Disease virus (PDV), designated PsHV-1 (Psittacid herpesvirus 1).

Herpesviruses of mammals and birds clearly descend from a common ancestor, but their genomes exhibit significant variation with respect to nucleotide sequence, gene content and genomic organization (Hanson and Bagust, 1991). The similarity of the PsHV-1 (*psittacid*) and ILTV (*gallid*) genome structure and content suggests that PsHV-1 and ILTV represent a unique clade of avian alphaherpesviruses that have diverged early from a common ancestor and are distinct from the Marek's family of alpha-herpesviruses, as previously suggested by other researchers (Kingham *et al*., 2001). We therefore recommend that PsHV-1 be classified as an avian alphaherpesvirus, genus *Iltovirus*.

Although a close relationship to ILTV is evident by the high degree of gene conservation, differences in tissue tropism and host range may be due to small but significant differences in gene content or protein expression. A whole genome

comparison and nucleotide alignment of the PsHV-1 to the other avian alpha-herpesviruses, as well as the other members of the *Herpesviridae* may demonstrate a more complete picture of the true order of evolution within the family.

Similarities between PsHV-1 and ILTV can be seen in the U_L inversion, the absence of repeats flanking the U_L region, the conservation of genes within the U_S region, and a conserved cluster of 5 unique ORFs in the U_L region. It is also notable that PsHV-1 lacks a homolog to U_L48, yet is capable of efficient replication.

As more avian herpesvirus sequence becomes available, additional avian herpesvirus genera may be identified. With the advent of rapid and cost effective sequencing strategies, it is now feasible to sequence representative herpesvirus isolates from different avian genera in order to determine whether the PsHV-1/ILTV clade of alphaherpesviruses is broadly represented or if there are other evolutionarily distinct genera of avian alphaherpesviruses. The complete nucleotide sequence of the psittacid herpesvirus 1 (PsHV-1) genome offers researchers the opportunity to begin a comparative analysis of a unique class of avian herpesviruses that are biologically and phylogenetically unique from the *Mardivirus* genus of *Alphaherpesviridae*.

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