PSITTACID HERPESVIRUSES ASSOCIATED WITH INTERNAL PAPILLOMATOUS DISEASE AND OTHER TUMORS IN PSITTACINE BIRDS

A Dissertation

by

DARREL KEITH STYLES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2005

Major Subject: Veterinary Microbiology

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Approved by:

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ABSTRACT

Psittacid Herpesviruses Associated with Internal Papillomatous Disease and Other Tumors in Psittacine Birds. (August 2005)

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Internal papillomatous disease (IPD) is characterized by mucosal papillomas occurring primarily in the oral cavity and cloaca of Neotropical parrots. These lesions can cause considerable morbidity, and in some cases result in mortality. Efforts to demonstrate papillomavirus DNA or proteins in the lesions have been largely unsuccessful. However, increasing evidence suggests that mucosal papillomas may contain psittacid herpesviruses (PsHVs). In this study, PsHV 1 genotype 1, 2, and 3 DNA was found in 100% of mucosal papillomas from 30 Neotropical parrots by PCR using PsHV specific primers. However, *Psittacus erithacus* papillomavirus and finch papillomavirus DNA were not detected. Additionally, a novel PsHV sequence related to, but phylogenetically distinct from PsHV 1, was identified in 4 African grey parrots (*Psittacus erithacus*), two of which exhibited papillomas. These findings suggest that mucosal papillomas may develop in parrots latently infected with PsHV. Tumors of the bile and pancreatic ducts have also been observed in parrots with IPD. Other mucosal tumors including carcinomas of the proventriculus and ventriculus may be coincident

with bile duct tumors, but cloacal carcinomas usually develop as solitary lesions. To test whether PsHV was associated with these tumors, the fresh tissues from 11 parrots and the formalin-fixed paraffin-embedded (FFPE) tissues of 5 parrots exhibiting mucosal tumors were examined by PCR. All tumors were found to contain PsHV 1 genotype 3 DNA except one bird with a cloacal carcinoma that contained genotype 4. Histologically normal tissues available from six parrots did not contain PsHV DNA. Experiments were performed using the FFPE tissues of 5 parrots with IPD related tumors known to contain PsHV by PCR, to show that the virus was in significantly higher concentration in the neoplastic tissue compared to adjacent histologically normal tissue. Neoplastic and adjacent unaffected cells were dissected from the tissues using laser capture microdissection and the DNA was examined by PCR. In situ hybridization using PsHV specific probes and direct in situ PCR were also performed on the tissues. A strong association was shown between infection by PsHV 1 genotype 3 and birds manifesting IPD related tumors and other neoplasms of the digestive tract.

DEDICATION

This dissertation is dedicated to the work of the veterinarians and researchers whose efforts have advanced the knowledge of avian medicine and biology. And to my family and friends who have supported me through this effort.

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CHAPTER I

INTRODUCTION

Overview

Internal papillomatous disease (IPD) affects predominately captive psittacine birds of new world distribution, with the greatest prevalence reported in macaws (*Ara* spp.), Amazon parrots (*Amazona* spp.), conures (*Aratinga* spp.), and hawk-headed parrots (*Deroptyus accipitrinus*) (Graham, 1991; Gibbons et al., 2002; Hillyer et al., 1991; Phalen et al., 1997; Van der Heyden, 1988). IPD is characterized by mucosal papillomas affecting primarily the epithelium of the oral cavity and cloaca, but are also observed in the esophagus, crop, proventriculus, ventriculus, conjunctiva, nasolacrimal duct, and bursa (Graham, 1991; Phalen et al., 1997). Grossly, mucosal papillomas are pink to grey-tan verrucous lesions that may cause increased morbidity, interfere with reproduction, and potentially result in death (Graham, 1991; McDonald, 1988; Phalen et al., 1997). Other tumors have also been associated with IPD including adenomas and carcinomas of the bile and pancreatic ducts (Graham, 1991; Elangbam and Panciera, 1988; Gibbons et al., 2002; Hillyer et al., 1991; Phalen et al., 1997; Potter et al., 1983), and perhaps carcinomas of the proventricular, ventricular, and cloacal mucosa. Because IPD and associated neoplasms posed a threat to the health of captive parrot populations, investigations into the cause of IPD and associated tumors were initiated.

Cutaneous and mucosal papillomas in mammals and cutaneous papillomas in two species of birds were known to be caused by species-specific papillomaviruses

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This dissertation follows the style of *Virology*.

(Brentjens et al., 2002; Moreno-Lopez et al., 1984; O'Banion et al., 1992). However, there was little evidence supporting papillomaviruses as the cause of mucosal papillomas in parrots (Johne et al., 2002; Latimer et al., 1997; Sundberg et al., 1986). Due to the paucity of evidence associating papillomaviruses with mucosal papillomas, Graham (1991) introduced the term IPD to differentiate this disease from those papillomas known to be caused by papillomaviruses. IPD and associated tumors appeared to have a common but undetermined etiology (Graham, 1991; Phalen et al., 1997).

Clinical observations suggested that mucosal papillomas developed in parrots that survived outbreaks of Pacheco's disease (PD) (Gaskin, 1989; Van der Heyden, 1988). PD is a generally fatal disease found predominately in Neotropical parrots, but also affects parrots from other geographical distributions (Tomaszewski et al., 2003). PD is caused by one of four genotypes of psittacid herpesvirus (PsHV) and its lethality depends on the species of parrot infected as well as undetermined viral factors (Tomaszewski et al., 2003). The possibility that a herpesvirus could be the cause of mucosal papillomas in parrots was supported by studies showing that in some cases, papillomas in other vertebrates were associated with herpesviruses rather than papillomaviruses (Calle et al., 1999; Goodwin and McGee, 1993; Herman et al., 1997; Lowenstine et al., 1983; Quackenbush et al., 2001). There were also specific reports associating PsHV infection with the presence of mucosal papillomas (Johne et al., 2002; Phalen et al., 1998).

The hypothesis of our investigation was that survivors of PsHV infection may develop IPD. We offer compelling evidence of a causal relationship between PsHV

infection and the lesions associated with IPD. In the first report, we present data demonstrating a strong correlation between PsHV infection and the presence of mucosal papillomas. In additional studies, we also show the association between PsHV infection and the other tumors associated with IPD. Finally, our investigation of mucosal papillomas coincidentally yielded the discovery of a new species of PsHV found in African grey parrots that is related to, but phylogenetically distinct from, those PsHVs known to cause PD.

This introduction will review the biology of both herpesviruses and papillomaviruses and their potential to induce tumors. The molecular biology of both PsHV and avian papillomaviruses is still under investigation, so much of the information on the biological properties of these viruses is lacking. Therefore, these two viral families will be broadly discussed and model viruses will be selected to describe more specific biological properties.

Herpesviridae

Herpesviruses are double-stranded DNA viruses with linear genomes ranging from 124-235 kbp in length. Herpesvirus genomes can be divided into six groups designated A-F based the number and location of internal and terminal repeat sequences separating the unique and quasi-unique sequences. The genome is packaged as a toroid in most herpesviruses, enclosed within an icosadeltahedral capsid surrounded by an amorphous material called the tegument. The tegument contains viral proteins essential for the efficient replication of the virus. Herpesviruses are enveloped viruses and the envelope appears to be derived from the cellular plasma membrane incorporating viral

glycoprotein spikes that project from the surface (Boehmer and Nimonkar, 2003; Herausgeber, 1992; Roizman and Pellet, 2001).

Herpesviruses are divided into three subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammherpesvirinae* that are differentiated according to their biological properties, but the subfamilies do share some common characteristics. All herpesviruses produce their own enzymes and other factors involved in viral nucleic acid synthesis. Viral DNA synthesis and assembly of capsids occurs in the nucleus of the cell, and capsids are enveloped as they transit through the nuclear membrane. Productive infection always results in the death of the cell. Importantly, all known herpesviruses can exist in a latent state within their natural hosts while expressing a small subset of viral genes. Latent infection appears to be favored by particular subsets of the trigeminal ganglion sensory neurons while productive infection is preferred by other types of sensory neurons. (Herausgeber, 1992; Roizman and Knipe, 2001).

Alphaherpesviruses exhibit a variable host range and engage in a relatively short reproductive cycle. They spread rapidly in culture and destroy infected cells efficiently. Alphaherpesviruses have the ability to penetrate both the apical and basilar aspects of epithelial cells as well as the dendrites of neurons. They also have the potential to establish latent infections in sensory ganglia (Roizman and Pellet, 2001).

Betaherpesviruses express a restricted host range and display a long reproductive cycle that results in slow spread in culture. These viruses often induce cytomegaly in infected cells. Betaherpesviruses establish latent infections in lymphoreticular cells and possibly secretory glands as well as other tissues (Roizman and Pellet, 2001).

Gammaherpesviruses are usually limited to members of the same order as their natural hosts. They replicate in lymphoblastoid cells and cause lysis in some types of epitheliod and fibroblastic cells in vitro. Gammaherpesviruses tend to be specific for T or B lymphocytes where they typically reside in a latent state in vivo (Roizman and Pellet, 2001).

Model alphaherpesvirus-human herpesvirus 1

Herpes simplex virus (HSV 1) was chosen as the model virus to describe the molecular properties of PsHVs because information regarding the molecular biology of the PsHVs and their nearest known avian alphaherpesvirus relatives is incomplete or lacking. PaHV 1 (passerid herpesvirus 1) appears to be phylogenetically the closest PsHV relative, but only 177 bps of UL30 is available for study and little is known about the molecular biology of this virus (Wellehan et al., 2003). This lack of information makes the placement of PsHV 1 questionable, but the complete sequence for the next nearest PsHV relative, GaHV 1 (gallid herpesvirus 1 or infectious laryngotracheitis virus), is available in the GenBank accession no. NC_006623), but again little is understood about the molecular biology of the virus and it has also diverged significantly from PsHV (Tomaszewski et al., 2001). The biological properties of another close PsHV relative, GaHV 2 (gallid herpesvirus 2 or Marek's disease, GenBank accession no. NC_002229) are better understood, but this virus is too dissimilar for direct comparison

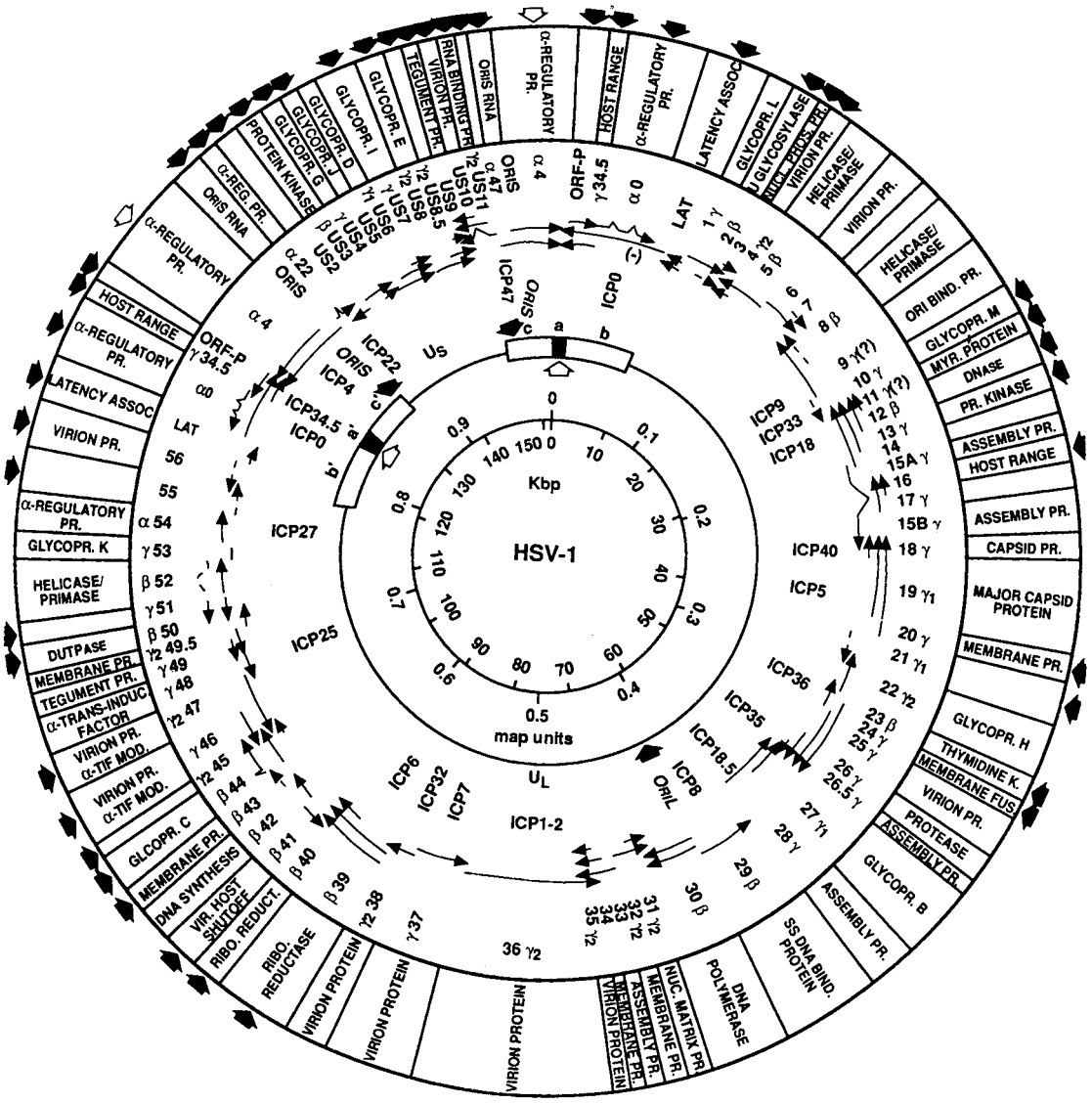

Fig. 1.1. Map of HSV-1 genome (from Roizman and Sears, 1996). A more complete gene map may be found at http://www.stdgen.lanl.gov/ (Roizman and Knipe, 2001). The outermost radial shows the protein names, the next radial shows the UL or US designation along with the gene type (α, β, or γ). The arrow radial designates the direction of transcription. The next radial inward shows the ICP protein designations. The next inward radial is a schematic showing the three origins of replication and the flanking LTR regions. The innermost radial shows the map units and kilobase pair measurement.

to PsHV. However, much is known about herpes simplex viruses that share structurally similar genomes to PsHV with some comparable biological activities.

HSV 1 is a 152 kbp alphaherpesvirus that causes productive lesions of oral mucocutaneous tissues of humans where the virus resides latently in the ganglia associated with the site of active replication. The linear double-stranded DNA genome arrangement of HSV-1 belongs to group type E, which is divided into unique long (UL) and unique short (US) coding sequences each flanked by their own individual inverted terminal repeat sequences (Fig. 1.1). The UL and US sequences can invert relative to each other creating 4 linear isomers based on restriction endonuclease analysis. There are currently 84 known open reading frames (excluding the two latency associated transcripts) within the viral genome (Rajčáni et al., 2004). Compared to other DNA viruses, there is relatively little overlap of the viral genes and only a few known instances of transcript splicing. There are three classes of ORFs in HSV; those with common initiation signals but different termination points; those with different initiation sites but common termination points; and those that are nested differing at both initiation and termination points (Rajčáni et al., 2004). The HSV genes are divided into five groups designated α, β_1, β_2, γ_1, and γ_2. These genes are transcribed in a relatively sequential fashion according to the needs of the replicating virus. Transcription of HSV genes will be described later in detail (Boehmer and Nimonkar, 2003; Roizman and Knipe, 2001).

Viral infection is initiated by attachment to cell surface receptors followed by fusion with the plasma membrane. HSV can utilize more than one attachment pathway so a number of viral glycoproteins may be involved in this attachment. This observation is

supported by the fact that in vivo HSVs attach not only to different cell types, both epithelial cells and neurons, but also to different polarized surfaces including the apical and basilar aspects of the epithelial cells and the axonal and dendritic portions of the neurons. Glycoprotein gB and/or gC binds with cellular heparin sulfate attached to cell-surface proteoglycans as the major binding factor for HSV (Shukla and Spear, 2001). Glycoprotein gC has also been shown to be required for attachment to the CD35 (CR-1) receptor on the apical portions of polarized cells but not for attachment of the virus to the basilar surface of the same cell (Roizman and Knipe, 2001). Glycoproteins gB, gD, gH, and gL are believed to be essential for entry (Spear and Longnecker, 2003). Glycoprotein gD is the primary ligand for three recognized cell surface receptors that mediate fusion with the plasma membrane. These receptors are HVEM (herpesvirus entry mediator, a member of the tumor necrosis family receptors), and nectins-1 & 2 (members of the immunoglobulin receptor group) (Spear et al., 2000). Any single receptor can bind gD and mediate fusion, and the receptor preference appears to vary with viral serotype. The exact mechanism by which gD mediates fusion is not well understood, but glycoproteins gB, gH, and gL are required for successful entry. HVEM is expressed on the exposed surface of a variety of cell types including epithelial cells. The nectins are located at the gap junctions of cells including epithelial cells and neurons and are not exposed unless there is cell disruption. It is speculated that initial attachment in polarized cells is accomplished via HVEM and that cell-to-cell transmission is mediated via the nectins, particularly in the case of neurons (Haarr et al., 2001).

After this fusion event, the proteins of the tegument are released. There are at least 15 recognized tegument proteins and some estimates are as high as 21 proteins that

perform a variety of functions to prepare the cell for viral replication (Bearer and Satpute-Krishnan, 2002; Mettenleiter, 2002). Two of the most notable tegument proteins are VHS and α-TIF. VHS (UL41 or viral host shut-off protein) is active in the cytoplasm where it terminates cellular and viral protein synthesis by destabilizing and degrading mRNA in a sequence specific fashion (Esclatine et al., 2004). Reasons for the destruction of host mRNA are obvious, but degradation of the viral mRNA seems less intuitive. However, it is believed that degradation of viral mRNA permits the rapid switching of one regulatory class of HSV genes to another. It is speculated that VHS degrades only select host cellular proteins, thus disabling specific functions of cellular metabolism while permitting activities conducive to viral replication (Smiley, 2004). The α-TIF protein (UL48 or alpha trans-initiating factor) is transported into the nucleus where it acts as a transcriptional activator of the early viral α-genes (Roizman and Knipe, 2001).

The de-enveloped virions are transported to the nuclear pores via the cellular cytoskeleton. Retrograde transport of the virions to the nucleus of neurons is accomplished by attachment of the tegument protein VP16 (US11) to the minus-end dynein-type microtubule motor that carries the virion to the nucleus (Bearer and Satpute-Krisnan, 2002; Tomishima et al., 2001). It is speculated that the protein coded by US9 may serve as the signal that directs transport to the nucleus (Tomishima et al., 2001). The virion then attaches to nuclear pore complexes by action of the cell protein β importin (Ojala et al., 2000). The viral DNA is then released into the nucleus through a virally mediated process where it immediately circularizes (Roizman and Knipe, 2001).

HSV-1 blocks the cell cycle at G1 to prevent cellular DNA synthesis by inhibiting cyclin-dependent kinase leading to redistribution of transcription factor E2F4 into the nucleus resulting in the complex formation of retinoblastoma tumor suppressor protein (pRb) and p107 thus arresting the cell cycle (Flemington, 2001; Ehmann et al., 2000). Additionally, the ubiquitin-ligase activity of ICP0 degrades centromeric proteins (LaMonte et al., 2001).

Viral gene transcription proceeds as a cascade in an orderly manner and is performed by cellular RNA polymerase II. The α-genes are the first to be transcribed and map near the UL and US termini and within the terminal repeat flanking these regions (Fig. 1.1). Transcription of the α-genes is activated by the γ-gene protein α-TIF (VP16) (alpha trans-inducing factor that is packaged in the tegument) in concert with the cellular transcription factor Oct-1 (LaBoissiere, S. and O'hare, P., 2000). The promoter for the α-genes is ICP4 (gene α4) and ICP0 (gene α0), which is required for efficient reactivation of latent virus (Halford and Schaffer, 2001; Rajčáni et al., 2004). ICP0 also functions as an ubiquitin-ligase targeting specific cellular proteins (Everett, 2000). The α0 product is a cosmopolitan trans-activator of other gene groups. The α4 product (ICP4) positively regulates β and γ-genes and down-regulates both itself and α0 (Grondin and Deluca, 2000). Hence, these alpha gene products peak early in infection although they continue to be produced throughout the replication cycle. The β-gene products are primarily involved in viral nucleic acid metabolism and DNA synthesis and their. The β_1 genes prepare the cell for viral DNA synthesis (typified by UL29, the major ssDNA binding protein that facilitates renaturation, complementarity, and strand transfer), and the β_2 genes are involved with nucleic acid metabolism and DNA synthesis

(typified by UL23, thymidine kinase, and UL30, DNA polymerase). The γ gene products are primarily the structural proteins of the virus and the proteins associated with DNA packaging and viral assembly. Without viral DNA synthesis, expression of γ gene products is poor. The transcripts are transported to the cytoplasm where they are translated on both free and bound polyribosomes. Most of the viral proteins are extensively processed after synthesis and are transported back into the nucleus (Roizman and Knipe, 2001).

HSV DNA replication is best understood during the lytic state of HSV. Replication proceeds bi-directionally from one or more origins of replication. There are 3 origins of DNA replication, 2 are found in the unique short region (*OriS*) and one in the unique long region (*OriL*) (Boehmer and Nimonkar, 2003). These multiple origins suggest that initiation of DNA synthesis may be trans-activated or the sites are individually responsive to factors in the cellular environment. The UL9 origin of binding protein recognizes each origin of replication and forms a complex with the DNA binding protein UL29 that anchors the DNA polymerase UL30 to the DNA template. UL9 and ICP9 then act to unwind the origin (He and Lehman, 2000). DNA replication then proceeds by rolling circle replication producing head-to-tail concatemers of viruses joined at their terminal repeat sections employing both viral and cellular enzymes and factors (Boehmer and Nimonkar, 2003).

One important viral enzyme that prepares purine nucleosides for incorporation into the DNA is UL23 thymidine kinase (TK). TK phosphorylates purine pentosides and other nucleoside analogs not recognized by analogous cellular kinases. This recognition of various nucleoside analogs by TK permits use of anti-viral drugs blocking its action.

Acyclovir, a common anti-herpesviral drug, is phosphorylated by TK but not cellular kinases and is incorporated into the nascent viral DNA chain. Acyclovir then acts as a chain terminator that halts viral DNA synthesis (Morfin and Thouvenot, 2003).

HSV DNA can isomerize by homologous recombination. This isomerization is not a property of all herpesvirus genomes. As previously described the UL and US sequences can invert relative to their terminal repeat sections creating four isomers. The purpose of this isomerization is unknown, yet it is not a requirement for replication in vitro. However, when single plaques infected with wild-type virus are sampled, each isomer is present in equimolar concentration. It has been speculated that isomerization may prevent stalling of DNA synthesis are collapsed replication forks (Boehmer and Nimonkar, 2003; Roizman and Knipe, 2001).

The γ genes are now transcribed and proteins necessary for construction and processing of the virion are produced during this part of the DNA replication cycle. The capsids are assembled in the nucleus although the specifics of their assembly are not well understood. The viral DNA concatemers are then cleaved and packaged into assembled capsids and data suggests this proceeds from the L terminus toward the S terminus (Houma and Brown, 1997). Models for the packaging process suggest that a receptor placed at an opening of the capsid complexes with a concatemer and viral DNA-cleaving/packaging proteins that are as yet not well understood. The capsid receptor then scans the DNA concatemer until a terminal repeat sequence is detected. This terminal repeat is secured to the capsid receptor continues to scan the DNA to locate the corresponding second terminal repeat sequence that encompasses a single genome complement. During this scanning process, the DNA appears to be “spooled”

into the icosodeltahedral capsid until the corresponding terminal repeat is located. The DNA is threaded into the capsid contacting the inner surface of the capsid shell, wrapping in an organized fashion around the inside of the shell concentrically one layer at a time (Zhou et al., 1999). The DNA appears to be organized along a definite axis but the ordering of the strand becomes more chaotic the further it is from the capsid wall. This spooling pattern gives the appearance of a toroid in cross-section. The densely packed DNA may be stabilized by the polyamines spermidine and spermine. When the second terminal repeat is encountered, the two terminal repeat sequences are then aligned by the capsid receptor, the DNA cleaving/packaging protein cuts the viral DNA concatemer at a specific recognition site within the terminal repeats and leaves the terminal repeat ends packaged within the capsid unjoined. The unjoined ends will be eventually repaired when the viral DNA circularizes after release into the host nucleus. Then the cleaving/packaging protein repairs the gap in the DNA concatemer so that the process can be repeated with another empty capsid (Houma and Brown, 1997; Roizman and Knipe, 2001).

The capsid is then surrounded by a visually amorphous structure called the tegument that contains up to 21 known proteins critical for viral transport and replication (Bearer and Satpute-Krishnan, 2002; Mettenleiter, 2002). Models for envelopment and release suggest that the capsid undergoes an envelopment/de-envelopment/re-envelopment process. The capsid appears to first bud through the inner lamella of the nuclear membrane acquiring an initial lipid envelope. This activity appears to be directed by the products of UL31 and UL34, which target the nuclear membrane and facilitate egress (Ye and Roizmann, 2000). There is some debate whether the capsid is fully tegumented

at this point or tegumentation is completed after nuclear budding. However, data supports the idea that the capsid is de-enveloped at the outer lamella of the nucleus and re-enveloped by the endoplasmic reticulum because the UL31 and UL34 proteins are absent from intracytoplasmic and extracellular enveloped virions and the major tegument proteins UL46 and UL49 are present in intracytoplasmic and extracellular virion envelops but not perinuclear envelopes (Mettenleiter, 2002). Therefore, most of the tegumentation process appears to occur in the cytoplasm. Studies of the budding of the avian alphaherpesvirus GaHV-1 support this pathway where tegumentation has been observed to occur primarily in cytoplasmic vesicles (Granzow et al., 2001). One important HSV protein mediating tegumentation is the product of UL48. The UL48 protein act not only as a transcription factor but also is a major component of the HSV tegument and is essential for viral assembly (Mettenleiter, 2002). UL48 also appears to anchor UL41 (VHS protein), which is vital for successful cell infection. The tegumented capsid is then targeted to the trans-Golgi network which is enriched with viral glycoproteins (Johnson and Huber, 2002).

Glycoproteins gD, gE/gI heterodimer, and gM have been proposed as mediators of final envelopment. The tegument proteins are believed to interact in a protein-protein specific action with the cytoplasmic tails of the viral glycoproteins to facilitate the final budding process (Chi et al., 2005; Mettenleiter, 2002). The gE/gI complex has been proposed to act as a signal directing the virion to the plasma membrane and both gD and gE/gI anchor the envelop to the tegument coated capsid (Farnsworth et al., 2003; Mettenleiter, 2002). Transport of the enveloped virion to the plasma membrane has been speculated to occur in a cytoplasmic vesicle. The anterograde motor kinesin has been

shown to bind tegument proteins VP16, VP22, and US11, with US11 being critical (Bearer and Satpute-Krishnan, 2002; Tomishima et al., 2001). The capsid is believed to be released into the extracellular environment by fusion of vesicles carrying enveloped virus at the plasma membrane.

Superinfection of cells infected with HSV is prevented by the action of glycoprotein gD. HSVs attempting to superinfect a cell will attach to the cell membrane, but are then endocytosed and destroyed (Roizman and Knipe, 2001). The mechanism by which gD conveys this protection is thought to be by blocking of the shared alphaherpesvirus receptor HveC (Geraghty et al., 2000). The gD glycoprotein competes with exogenous virus for HveC and blocks ligand binding sites.

A characteristic common to all herpesviruses is the ability to establish latency. After a cycle of active replication (not always necessary) in the epithelial cells at the site of entry, intact HSV virions enter a sensory neuron presumably via similar mechanisms used to enter epithelial cells. The capsid is then transported to the neuronal nucleus by retrograde axonal transport to the nucleus involving microtubules as previously described. A brief period of replication may occur in the sensory neurons, which is then followed by latency although replication is not a prerequisite for the establishment of latency in the ganglion cell body (Jones, 2003; Khanna et al., 2004). The genome of HSV persists as an episome associated with chromatin (Jones, 2003). A report by Cai et al. (2002) places the estimate of copies of HSV/neuron to be between 2-20 copies. What viral/cellular factors maintain the latent state are still being investigated. However, there is little evidence for viral DNA transcription occurring in the neuron except latency-associated transcript (LAT). LAT is a stable intron approximately 2 kb in length derived

by splicing of an 8.3 kb transcript (Roizman and Knipe, 2001). The promoters for LAT are neuron-specific and several candidate proteins have been studied but none confirmed to date (Jones, 2003). LAT run in part anti-sense to the 3' terminus of the critical transcriptional HSV pan-activator protein ICP0 (gene α0) and thus may act as an inhibitor of reactivation (Jones, 2003). No detectable protein is produced by LAT, yet LAT is abundant in the neuronal nucleus. The functions of LAT are still being investigated, and it appears to be important but not absolutely essential for establishing latency or re-activation of the virus. LAT has been shown to block the caspase-8 (death receptor pathway) in vitro, which may prevent apoptosis (Peng et al., 2004). This apoptotic blocking function may be essential during the reactivation phase.

Reactivation begins with viral replication in the neuronal nucleus in response to local or systemic host stimuli. Corticosteroids produced during stress can stimulate replication at (*OriL*) and may be one mechanism leading to reactivation (Hardwicke and Schaffer, 1997). Corticosteroids may also induce apoptotic pathways in neurons, which underscore the importance of potential LAT protection. HSV pan-transactivator ICP0 has been shown to be essential for reactivation, however the mechanisms up-regulating its expression or de-repression are not well understood (Halford and Schaffer, 2001). While host immunity may play a role in controlling latency, it does not appear that cells of the immune system directly inhibit reactivation (Sawtell, 2003). The nascent virions are then re-conveyed to the site of active replication (epithelial cells) by axonal transport (Roizman and Knipe, 2001).

Oncogenic herpesviruses

Oncogenesis is an important aspect of several animal herpesviruses. Representatives include members from alphaherpesviruses such as Mareks's disease herpesvirus (MDHV or GaHV-2) affecting gallinaceous birds and gammaherpesviruses such as Epstein-Barr virus (EBV or HHV-4) and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) affecting humans (Epstein, 2001). The relevance of discussing herpesvirus oncogenesis pertains to tumorgenic activity reported in this paper caused by PsHV. This section will briefly review the oncogenic mechanisms of MDHV, EBV, and KSHV.

MDHV is an alphaherpesvirus based on the genomic organization, but its biological behavior is more consistent with gammaherpesviruses (Biggs, 2001). Similar to EBV, MDHV is lymphotrophic and oncogenic. However, MDHV infects T lymphocytes rather than B lymphocytes and causes paralysis due to lymphocyte accumulation in the peripheral nerves and lymphomas found in a variety of organ systems in gallinaceous birds. Several proteins and gene regions have been proposed as cause of neoplasia in MDHV, but the Meq protein appears to be the most likely candidate to date (Lupiani et al., 2004; Liu and Kung, 2000; Venugopal, 2000). Meq is present in two copies in the MDHV genome located in the repeat regions flanking the UL sequence (Tulman et al., 2000). The leucine-zipper regions at the N-terminal region of Meq are closely related to the Jun/Fos oncoproteins, and the C-terminal region is similar to the WT-1 tumor suppressor gene (Lupiani et al., 2004). Meq can be found as a homodimer and a Meq/Jun heterodimer (Levy et al., 2003). The Meq homodimer represses the MDV origin of replication suggesting that is may be involved in latency and replication and the Meq/Jun heterodimer transactivates the Meq promoter (Levy et al., 2003; Lupiani et al.,

2004). It has been proposed that Meq transforms cells through several pathways affecting the cell cycle. Meq's transcriptional activation capacity up-regulates the anti-apoptotic protein Bcl-2 and suppresses the apoptosis inducing Bax protein (Levy et al., 2003). Meq also appears to sequester p53 either through direct binding or other interactions. Meq binds to and is also phosphorylated by CDK2. The phosphorylation state of Meq is thought to alter its function in different situations such as acting as a transactivator or suppressor. Meq also may translocate CDK2 into the nucleolar periphery where it activates cyclins that phosphorylates pRb (Liu and Kung, 2000). Meq's interaction with the Jun/Fos system promotes cell growth and enhances cell-transforming capacity.

Epstein-Barr virus has been associated with the development of the B lymphocyte origin tumors Burkitt's lymphoma and Hodgkin's disease, and also implicated in nasopharyngeal carcinomas and gastric carcinomas in humans (Epstein, 2001; Niller et al., 2004). Two viral proteins have been suggested as having transforming capacity, Epstein-Barr nuclear antigen 2 (EBNA2) and latent membrane protein 1 (LMP1) (Hammerschmidt and Sugden, 2004). EBNA2 enables the transcription of promoters of the Notch signaling pathway (Hammerschmidt and Sugden, 2004). The contributions of the proteins induced by the action of EBNA2 are not currently known, however it does activate LMP1. LMP1 interacts with and activates a variety of cellular adaptor proteins from the tumor necrosis family (Damania, 2004; Pagano et al., 2004). These include anti-apoptotic proteins such as Bcl-2, epidermal growth factor, and cell-adhesion molecules. Importantly, LMP1 activates the transcription factor NF-κB in both lymphocytes and epithelial cells (Pagano et al., 2004). Cofactors are speculated to be

involved with the development of EBV associated Burkitt's lymphoma and nasopharyngeal carcinoma. Chronic malaria has been associated with the development of Burkitt's lymphoma (Hammerschmidt and Sudgen, 2004). It is possible that immunosuppression coupled with the development of germinal centers of lymphocytes from the malarial infection increases the likelihood of EBV oncogenesis (Pagano et al., 2004). The specific cofactors for nasopharyngeal carcinoma have not been identified, but may be related to tumor-promoting compounds found in certain ethnic foodstuffs or perhaps development of these tumors may be more of a function of population genetics since they show population predilections (Pagano et al., 2004).

Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8) causes vascular endotheliosarcomas primarily in HIV-infected individuals and classically on the skin of men of Mediterranean descent (Damania, 2004). In HIV patients, the tumors are not restricted to the skin but disseminate to the viscera. KSHV establishes life-long infection in the B-lymphocytes of the host. KSHV infection is pervasive in the population, but KS lesions result only when the host is immunocompromised or individual host factors or permissive to tumorogenesis. Several viral proteins have been shown to transform cells, K1, vGPCR, Kaposin, and homologs of cellular cytokines and chemokines (Pagano et al., 2004). K1 can activate the transcription factor NF-κB, block Fas-induced apoptotic pathways, and stimulates B cell proliferation via a tyrosine-based activation motif (Damania, 2004; Pagano et al., 2004). KSHV protein vGPCR can activate phospholipase C and phosphatidylinositol 3 pathways. Kaposin interacts with GTPases that regulate integrin-mediated cell adhesion (Damania, 2004). KSHV also

encodes gene analogs for anti-apoptotic activity such as viral Bcl-2 and viral Inhibitor of Apoptosis (Pagano et al, 2004).

Papillomavirinae

The lesions of IPD, particularly mucosal papillomas, are both grossly and histologically similar to those lesions known to be caused by papillomaviruses in other animals. Species-specific papillomaviruses cause cutaneous papillomas in finches and African grey parrots (Jacobson et al., 1983; Lina et al., 1973). Therefore, the first investigations into the cause of mucosal papillomas focused on finding papillomaviruses in the lesions. Hence, a brief discussion of the molecular biology of papillomaviruses and their ability to transform cells is warranted.

Papillomavirinae is a subfamily of the larger family *Papovaviridae*. Over 70 different members of this subfamily have been described. All members of the subfamily tend to be host specific and possess the ability to transform cells and result in the formation of proliferative papillary lesions or papillomas of the squamous or mucosal epithelium depending on the particular virus. Papillomas are usually benign space-occupying lesions where cell proliferation is under viral rather than host control. Papillomaviruses work to establish a "regulated disorder" that permits greater viral amplification and dissemination of the virus (Doorbar, 2005; Lowy and Howley, 2001). However, when the transforming capacity of the virus becomes unrestricted then more serious neoplastic lesions (carcinomas) can result. The ability to cause serious disease depends upon the species of papillomavirus since some have greater transforming capacity than others. While many papillomaviruses can transform cells in culture, they

do not undergo productive replication to produce intact virions. This obstacle has impeded more complete understanding of papillomavirus biology. Advances have been made using organotype (raft) culture systems that establish polarized cell layers and permit some papillomaviruses to complete their life cycle, but more work needs to be done in this area (McLaughlin-Drubin et al., 2003).

Papillomaviruses have a circular double-stranded DNA genome packaged into a non-enveloped icosahedral capsid. The virions range from 52-55 nm in diameter and the icosahedral capsid is a 72 capsomere shell composed of 2 structural proteins, major capsid proteins L1 and L2 (Modis et al., 2002). The genome for all papillomaviruses is approximately 8 kbp in size and the open reading frames (ORF) are all located on one strand of the DNA indicating that all of the viral genes are confined to one strand. The genome is structured into early (E) and late (L) gene regions separated by a segment that contains no ORFs, the long control region (LCR), the upstream regulatory region (URR), and the non-coding region. There are eight early genes designated E1-E8 and two late genes, L1 and L2 (Fig. 1.2). Because of the small size of the genome, there is considerable overlap of the ORFs of the E genes but the L genes are virtually sequentially ordered with minimal overlap. There is great variability in the organizational order and even the number of the E genes varies with the individual papillomaviruses (Doorbar, 2005; Howley and Lowy, 2001).

Model papillomavirus-bovine papillomavirus 1

While the molecular biology of FPV and PePV is described to some detail, much more is known about bovine papillomavirus 1 (BPV 1) as a model virus for describing

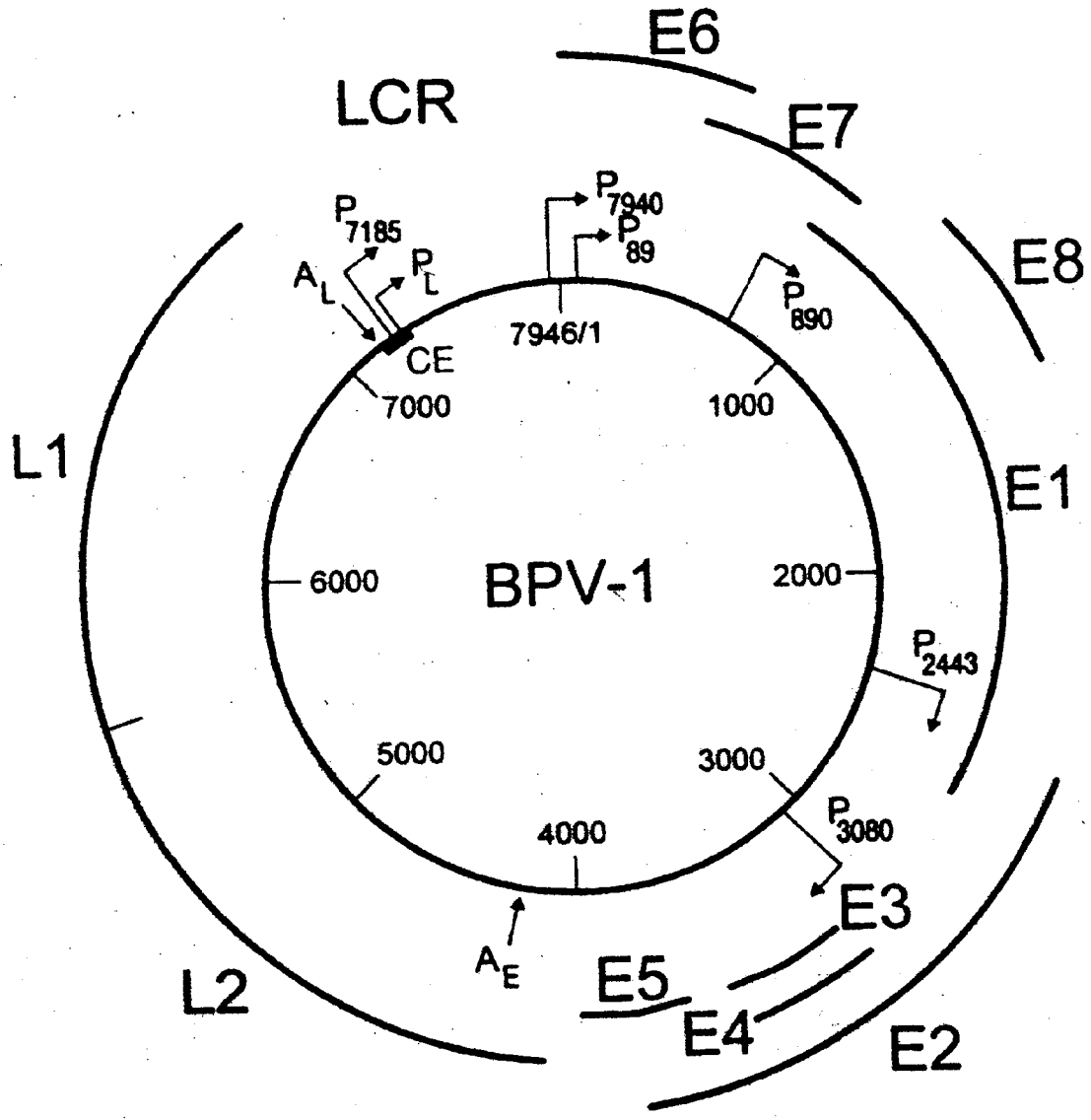

Fig. 1.2. Map of BPV-1 genome. The schematic shows the ORFs of the early and late genes. P_n designates individual early promoters where n is the approximate nucleotide position of the transcription initiation site. P_L is the promoter for the late genes. A_E and A_L are the polyadenylation sites for the early and late genes. CE is the constitutive transcriptional enhancer region. (from Lowy and Howley, 2001).

the biological behavior of all papillomaviruses. Where relevant, comparisons of FPV and PePV homologs of BPV structure and products will be made. Also, much is

understood about the molecular biology of HPVs, especially in regard to their oncogenic potential. Mechanisms involving HPVs will also be addressed when appropriate.

BPV is the cause of fibropapillomas in cattle and also demonstrates the ability to transform cells in culture making it a good model for understanding papillomavirus biology. BPV is approximately 8 kbp in size and the genome is organized into 8 early genes (E1-E8), two late genes (L1 and L2), co-joined by a non-coding regulatory region (LCR) (Fig. 1.2) (Howley and Lowy, 2001).

Like all papillomaviruses, BPV attains access to the cell by a break in the epithelial defense. The target of infection is the basal epithelium since it is the only cell capable of dividing (Egawa, 2003). The process of viral attachment and entry is poorly understood, however it has been shown that heparin sulfate mediates the initial attachment of some HPVs via the L1 protein (Joyce et al, 1999). It appears that BPV is cosmopolitan in its selection of cell types since it has been shown to infect a wide variety of cells *in vitro*. Therefore, the cell-tropism of BPV or HPV is not cell-receptor specific, but papillomaviruses overall do appear to bind as yet unidentified conserved cell-surface receptor and heparin sulfate is a likely candidate (Giroglou et al., 2001). The assumed sequence of events is that BPV binds to some widely expressed receptor on basal cells, is endocytosed, and then transported to the nucleus. Recent evidence suggests that this is mediated by endocytosis of clathrin-coated vesicles (Culp and Christensen, 2004). At the nucleus, the virion is uncoated with the assistance of the reducing environment found in the cell cytoplasm and the viral DNA is released into the nucleus (Li et al., 1998).

Papillomavirus transcription is tightly regulated because different products are required as the virus is carried with evolving cell types from basal cell to differentiated

cell. Transcriptional regulation must be carefully synchronized with cell differentiation for productive infection to occur. Because of the overlap in the ORFs, particularly in the E region, a variety of polycistronic mRNA species are produced and splicing of these species results in the production of the various viral proteins (Longworth and Laimins, 2004).

The LCR contains constitutive enhancer elements that are responsive to both cellular factors and viral regulatory elements (Howley and Lowy, 2001). These enhancers express some cell or tissue specific behavior and are thought to be essential for initial transcription of viral genes. There are 7 recognized promoters in BPV, six are associated with transcription in transformed cells and the seventh with transcription of late genes in productive cells. Likewise there are two poly-adenylation sites, one for the E genes and another for L genes. Several different cellular transcription factors have been identified as initiators of early transcription. Common binding sites to all papillomaviruses are those for TFIID binding to TATA boxes upstream from the early start sites (Longworth and Laimins, 2004). Upstream of these TATA sequences are found binding sites for factors AP1and Sp-1 found in all HPV types and there are additional transcription factor binding sites including Oct-1 and glucocorticoid responsive elements (Chang and Laimins, 2000; del Mar Pena and Laimins, 2001). As the name early would imply, the E genes are the first to be transcribed. The E genes are responsible for initiation of viral DNA replication and the transforming capacity of the virus. Control of E gene transcription is mediated by the various proteins produced by E2 via E2-repsonsive elements found in the LCR. There are 3 species of E2 protein, full length E2TA, and two shorted splice products E2TR and E8/E2 (Howley and Lowy,

2001; Ozbun and Meyers, 1998). The E2TA is a transcriptional activator for the E genes while E2TR and E8/E2 are transcriptional repressors that inhibit the transactivation function of E2TA. E2TA can activate almost all of the E promoters, although sensitivity to E2TA activation varies. The E2TR and E8/E2 repressors bind at sites in close proximity to the TATA box near the individual promoters. It is thought that these E2 repressors displace the transcriptional activator SP1 and also inhibit the formation of the transcriptional pre-initiation complex. It is important to note that mutations leading to the loss of function of the E2 repressors or loss of sensitivity at the E2 repressor binding site can permit the deregulation of cell transforming proteins such as E6 and E7 and lead to unrestricted cell growth and more serious neoplasia (Jeon et al., 1995).

One study reports that both FPV and PePV lack classical ORFs for E6 and E7 (Terai et al., 2002). However, these investigators did designate a region within the genome as E7 and X-ORF (E6-like region). While there is little overall homology between this new E7 region and E7's of other papillomaviruses, it does share classical binding motifs for pRB.

Transcription of the E genes is followed by either steady-state viral DNA replication or transition to productive infection. Steady-state replication occurs after initial infection and is divided into two phases, establishment and maintenance. During the establishment phase, the low viral copy number is amplified to 50-400 copies per basal cell. This amplified copy number remains relatively constant during the following maintenance phase of infection. Papillomavirus DNA can either exist as an extrachromosomal plasmid or integrate into the host genome. In basal cells, papillomavirus DNA is maintained as multi-copy episomes that replicate in concert with

the cell cycle (You et al., 2004; Wilson et al., 2002). The origin of DNA replication is in the LCR region and efficient replication requires the activity of both E1 and E2 proteins. E1 is a multi-functional protein that binds to the origin with E2 to form a stable complex. E1 forms a hexameric structure at the origin and acts as an ATP-dependent helicase to unwind the DNA and also recruits DNA polymerase α to induce replication (Wilson et al., 2002). Other cellular factors such as thymidine kinase and proliferating cell nuclear antigen are also required to complete papillomavirus DNA replication. The papillomavirus DNA is maintained then at a relatively constant copy number in a plasmid state. These newly replicated genomes then serve as templates for increased expression of E1 and E2, which facilitates additional expression of viral genome (Middleton et al., 2003). What mechanisms induce the change from steady state to vegetative replication with production of infectious virions is unknown. Presumably, these factors are related to the state of cell differentiation because only terminally differentiated cells produce infectious virions (Doorbar, 2005).

Cell transformation is part of the strategy papillomaviruses use to replicate. Transformation is dependent up expression of viral DNA and whether the papillomavirus DNA exists in an integrated state or as a plasmid is immaterial. Early proteins E5, E6, and E7 are primarily responsible for cell-transforming activities. The E5 protein is highly conserved among papillomaviruses that induce fibropapillomas and exists as a homodimer localized primarily to the Golgi and smooth ER. E5 has the ability to transform E5 does not exert any enzymatic activity but its primary target is the β receptor for platelet-derived growth factor (PDGF) in BPV (Howley and Lowy, 2001). However, the target in HPV appears to epidermal growth factor receptor (EGF) because

E5 increases phosphorylation of the receptor and decreases its degradation and its primary activity appears to be in differentiated cells (Fehrmann et al., 2003; Rodriguez et al., 2000). Each subunit of the E5 homodimer binds to a PDGF β receptor resulting in dimerization and activation of the receptors. The activated receptors then precipitate a cascade that leads to mitogenic stimulation. Importantly, E5 is not expressed in most human papillomavirus (HPV) positive cancers. This suggests that E5 primarily functions to perpetuate the benign papillomatous state rather than participate in more severe oncogenesis (Howley and Lowy, 2001).

The E7 protein exerts its transforming effect primarily through deregulation of the cell cycle (Munger et al., 2001; Munger et al., 2004). The E7 and E6 proteins are expressed in a bicistronic mRNA from the early viral promoter p97 (Stacey et al., 2000). The product of the retinoblastoma tumor suppressor gene (pRB) is a primary regulator of the cell cycle. The hypophosphorylated form of pRB, present in the G0/G1 phase of the cell cycle, is bound to the cellular transcription factor E2F-1. The complexed E2F-1 cannot activate transcription and thus the cell is prevented from entering the S phase of the cycle. A cyclin-dependent kinase phosphorylates pRB at multiple serine residues causing the dissolution of the pRB/E2F-1 complex. E2F-1 is then free to initiate transcription and the cell enters the S phase of the cycle. At the M/G0 boundary, pRB is returned to a hypophosphorylated state by a specific phosphatase where it once again binds E2F-1 and arrests the cell cycle at G0. To induce transformation, the E7 protein preferentially binds the hypophosphorylated form of pRB and disrupts the pRB/E2F-1 complex thus releasing the E2F-1 transcriptional activation factor. This action also leads to the ubiquitinization and proteasome degradation of E7 (Wang et al., 2001). The cell

cycle then proceeds to the synthesis phase unimpeded. Importantly, E7 proteins from low risk (mildly oncogenic) HPVs such as HPV 6 and 11, bind pRB at a much lower affinity than those of high risk HPVs such as type 16 and 18 (Howley and Lowy, 2001). High-risk HPV E7 proteins also bind cyclin-dependent kinase inhibitors p21 and p27 presumably to block their action thus enhancing cycling-dependent kinase phosphorylation of pRB and also associates with cyclins A and E acting to increase their concentration (Longworth and Laimins, 2004). However, it appears that the ability of E7 to facilitate the progression of the cell to S-phase is limited to a subset of differentiated cells that either contain low levers of the cyclin-dependent kinase inhibitors p21 and p27 or high levels of E7 production because these inhibitors can form inactive complexes with E7 and the cell cycle is unaffected (Noya et al., 2001). E7 can also bind histone deacetylases (HDACs) whose relevant action here is to deacetylate E2F resulting in a loss of function (Marks et al, 2001). This binding also appears to be essential for maintaining the viral episome and facilitating immortalization in some cells lines (Longworth and Laimins, 2004). The mechanism by which this particular E7-HDAC interaction exerts its affect is unknown. Some high-risk E7 proteins can also induce host genomic instability by interfering with centrosome function resulting in aneuploidy (Duensing et al., 2000).

The E6 protein works in conjunction with the E7 product to efficiently transform cells. E6 protein from low risk HPVs has little to no transforming capacity. However, E6 proteins from high risk HPVs have been shown to efficiently bind the tumor suppressor gene product, p53, but low risk E6 proteins bind no detectable amount. Production of increased concentrations of p53 occurs in response to cell stress or

damage. The p53 protein can transcriptionally activate the p21 gene which inhibits the activity of the cyclin-dependent kinases that regulate the advance of the cell cycle. Therefore, the activity of p53 results in either the arrest of the cell cycle or propels the cell down the apoptotic pathway. High-risk E6 proteins bind p53 in conjunction with a cellular protein E6-associated protein (E6AP). E6AP does not bind p53 without the presence of E6, and the normal substrate of E6AP is unknown (Munger et al, 2004). This complex then functions as an ubiquitin ligase. Ubiquitin tags signal the cell that this product has been designated for destruction by the proteasome. Hence in papillomavirus transformed cells, the p53 levels are decreased. Loss of p53 activity results in deregulation of the cell cycle and no arrest or apoptosis can halt the rapidly proliferating cells (Howley and Lowy, 2001). HPV E6AP also targets some proteins of the src kinases that interact with signaling networks and can associate with other pro-apoptotic proteins including Bak and Bax (Frame, 2002; Li and Dou, 2000). While the significance of this activity are not understood at this time, it may have implications regarding trafficking of cellular proteins and inhibition of the apoptotic pathways. Additionally, degradation of p53 alone is not sufficient to ensure immortalization. E6 also appears to reduce PDZ protein binding domains (Liu et al., 1999). PDZ binding domains are found at areas of cell-cell contact and the PDZ family of proteins function at these domains acts as molecular scaffolding and aid in signal transduction. The binding of E6 to the PDZ domain is sufficient to induce cell proliferation in the superficial basilar layers and may contribute to metastasis by disrupting normal cell adhesion (Nguyen et al., 2003). HPV E6 can also activate the hTERT subunit of telomerase by binding both Myc and its cofactor Max, leading to transcription activation of the hTERT

promoter (Veldman et al., 2003). Telomerase activation shortens the telomeres leading to cellular senescence. Quantitative experiments have shown that hTERT activity is more important that p53 degradation in regard to immortalization (Longworth and Laimins, 2004, Munger et al., 2004). However, this observation tends to be dependent on the cell type and system and certainly other factors such as E7 activity are involved.

The activities of E6 and E7 in the normal life cycle of papillomaviruses are probably necessary to create a suitable environment for efficient viral replication. E1 and E2 proteins are necessary for the initiation of viral DNA replication, but also other cellular factors such as DNA polymerase, PCNA, and TK. Papillomaviruses only undergo productive replication in terminally differentiated cells that have normally deactivated transcription of genes necessary for DNA replication. E6 and E7 normally function to establish a perpetually cycling cell that is actively producing the necessary products for completion of viral replication. However, in high-risk papillomavirus strains, the virus can no longer exert "controlled disorder", because there is more efficient binding of E6 for p53 and E7 for pRB as compared to low risk strains. So, without either cellular or viral restraints on the cell cycle, malignancies result (Howley and Lowy, 2001).

Late gene functions associated with capsid protein synthesis, productive DNA synthesis, and virion assembly occur only in the terminally differentiated cells. Genes included in the late gene category are L1, L2, and interestingly E4. The E4 gene is designated as an early gene because its locus lies within the early gene region, but is considered a late gene because its expression is from the late promoter and its proteins function late in papillomavirus infection. L1 and L2 expression occurs only in the terminally differentiated cells where they form the structural basis of the capsid with L1

being expressed after L2 facilitating assembly of infectious particles in the upper layers of the epithelium (Florin et al., 2002). The L2 protein accumulates near nuclear structures known as PML bodies and recruits L1 to these domains (Doorbar, 2005). It has been suggested that these PML bodies are the sites of papillomavirus DNA replication and that the capsid proteins are drawn to these sites where capsid assembly occurs (Swindle et al., 1999). The papillomavirus is then shed from the epithelial surface in an enveloped form (Bryan and Brown, 2001). It is thought that delaying release of the virions until cells have reached the uppermost epithelial layers may assist in avoiding host immune detection, particularly because the virus limits presentation of viral epitopes to the immune system in the lower epithelial layers (Marchetti et al., 2002; Matthews et al., 2003).

The E4 protein has several splice forms and is found in the suprabasilar layer through the differentiated layer. It is believed that these different E4 proteins serve different functions in the various cell types. Studies have suggested that the E4 protein found in the terminally differentiated cells induces the collapse of the cytokeratin network and this action may assist the virus in its egress from the cell and assembly of the envelope (Lehr et al., 2004; Wang et al., 2004). Late gene expression is regulated from elements within the late region. The transcripts from the late gene are initiated from a single late promoter and polyadenylated from a different site than those of the early genes. What induces the switch from early to late expression is not well understood, but it is presumably cell factors that are found in the different cell types as the virus transits the epithelial layers acting on the late promoter. Control also appears to be exerted at the transcription/post-transcriptional level. Rearrangement of the chromatin as the late

promoter site has been observed to occur prior to late gene transcription in some HPVs (del Mar Pena and Laimins, 2001). While the mechanisms governing the switch from early to later expression are not clear, there are some significant findings that suggest possible pathways. A negative regulatory control element in the 3' untranslated late region acts to decrease steady state levels of late mRNAs during the steady-state phase of infection. It is thought that this element may alter the definition of the 3' exon region of the late genes, and terminates or pauses transcription. This termination prevents the use of the late poly (A) site. Therefore, transcripts encoding only early proteins using the early poly (A) site are preferred. One model suggests that cellular factors produced only in undifferentiated cells, may contribute to transcriptional pausing perhaps in association with the negative control element. These undifferentiated cell factors would be titrated out as the cell evolves to a terminally differentiated state and thus permit use of the late poly (A) site and complete transcription of late genes proceeds (Howley and Lowy, 2001).

The process of capsid assembly and virion release is not well understood. In the terminally differentiated cell, host DNA transcription must be reactivated so that cellular factors essential for papillomavirus replication are available. This reactivation is presumably the normal function of the E6 and E7 proteins. The late genes are transcribed and capsid assembly and packaging of the genome follow. E4 appears to disrupt the cellular cytoskeleton leading to the release of infectious virions.

Psittacid herpesvirus

Psittacid herpesviruses (PsHV) are enveloped linear double-stranded DNA viruses of the subfamily *Alphaherpesvirinae* and are in the genus Iltovirus (α4) (Davison, 2002; McGeoch et al., 2000; McGeoch et al., 2005; VanDevanter et al., 1996; Tomaszewski et al., 2003). The genome has been sequenced, GenBank accession no. AY372243, and is 163 kbp in length and is organized into unique long (UL) and unique short (US) regions with corresponding genes mapping to homologs of other alphaherpesvirues (Thureen and Keeler, 2003).

The phylogenetically closest alphaherpesvirus relatives to PsHV are PaHV 1 and GaHV 1 based on sequence from the UL30 gene. However, the lack of sequence data for PaHV 1 makes this placement questionable. However, GaHV 1 has been completely sequenced and is closely related based on partial sequence data from the UL30 gene of PsHV 1 (Davison, 2002, Tomaszewski et al., 2003).

Previous investigations suggested that PsHV demonstrated a significant degree of genetic polymorphism by restriction endonuclease analysis (Aini et al., 1993; Gravendyck et al, 1996; Günther et al., 1997). Further investigation showed PsHVs be classified into four genotypes; number 1, 2, 3, and 4 based on the partial sequence data from the UL16 gene (host range protein) (Tomaszewski et al., 2003). These four genotypes generally correspond to known PsHV serotypes where genotype 1 and 4 viruses mapped to serotype 1, genotype 2 viruses mapped to serotype 2, genotype 3 viruses mapped to serotype 3, and two specimens, designated serotype 2/3, mapped to a unique branch of genotype 3 viruses. (Gravendyck et al., 1996; Tomaszewski et al. 2003).

These genotypes are thought to have co-evolved with several species of Neotropical (Central and South American) parrots (Phalen et al., 2004). They are not believed to cause disease in their host species, however an acute fatal disease (Pacheco's disease) may result when parrots are infected with a PsHV genotype to which they have not been evolutionarily adapted (Phalen et al., 2004; Tomaszewski et al., 2003). Because extensive testing has not been performed on parrots in the wild, it is difficult to establish which species serve as carriers for a specific genotype. However, testing for PsHV in captive birds may reflect trends in the wild. PsHV 1 genotype 4 is found in Patagonian conures (*Cyanoliseus patagonus*), PsHV 1 genotype 3 is a common finding in hyacinth macaws (*Andorynchus hyacinthinus*), PsHV 1 genotype 1 has been identified in wild-caught *Aratinga* conures, and PsHV 1 genotype 2 was found in two wild-caught redh-shouldered macaws (*Diopsittaca nobilis*) (Phalen et al., 2004).

Data also suggests that some PsHV genotypes are more pathogenic to some species of parrots that others (Phalen et al., 2004; Tomaszewski et al., 2003). For example, genotype 4 is usually lethal to macaws and conures but genotype 3 rarely causes death in these species, and genotypes 1 and 2 may not cause disease in these species. Genotype 4 has not been reported to cause disease in Amazon parrots in the United States, but genotype 3 may be lethal to this species. Species of Pacific distribution appear to be susceptible to all genotypes equally (Tomaszewski et al., 2003).

Pacheco's disease

Pacheco's disease (PD) was first described as an infectious disease of psittacine birds in 1929 (Pacheco and Bier, 1929). PD is an acute and generally fatal disease of parrots caused by one or more genotypes of psittacid herpesvirus characterized by acute onset of illness with few premonitory signs (Tomaszewski *et al*., 2001). The disease is almost exclusively confined to psittacine birds, but has been described in a starling and is suspected in other passerines including a Pacheco's-like herpesvirus infection in a toucan (Charlton et al., 1990; Phalen et al., 2004; Tomaszewski et al., 2004). While many parrot species are susceptible, the disease has been predominately reported in Amazon parrots (*Amazona* spp.), African grey parrots (*Psittacus erithacus*), macaws (*Ara* spp.), cockatoos (*Cacatua* spp.), and conures (*Aratinga* spp. and *Pyrrhura* spp.) (Cho and McDonald, 1980; Dharma and Sudana, 1983; Randall et al., 1979; Tomaszewski et al., 2001; Tomaszewski et al., 2003). Parrots manifesting clinical signs seldom survive but those that do may exhibit depression, anorexia, and biliverdinuria, which may be accompanied by elevations of serum enzymes (Godwin et al, 1982). Gross necropsy results have been described as variable and non-specific, but an enlarged liver with mottled-tan coloration is a common finding (Graham, 1980; Panigrahy and Grumbles, 1984; Tsai et al., 1993). PD has been generally characterized by acute hepatic necrosis with minimal associated inflammation accompanied by the presence of viral intranuclear inclusion bodies (Panigrahy and Grumbles, 1984). Multiple organ systems are involved and hepatitis is a consistent finding, but other microscopic characteristics may include splenic necrosis, enteritis, pancreatitis, tracheitis, and ingluvitis (Graham, 1980; Gravendyck et al., 1998; Panigrahy and Grumbles, 1984;

Tomaszewski *et al*., 2001). Diagnosis of PD in the past relied on serology or viral isolation but has been replaced by PCR (Gravendyck et al., 1998; Tomaszewski et al., 2001; Tomaszewski et al., 2003).

PD became a disease of concern when collections of captive psittacine birds were assembled by aviculturists worldwide in the latter half of the 20th century (Durham et al., 1977; Gough and Alexander, 1993; Mutlu et al., 1991; Persin and Bratrsovsky, 1986; Simpson et al., 1975; Simpson and Hanley, 1977; Tsai et al., 1993; Westerhof et al., 1988). The evolution of PD as a disease of significance was linked to the growth of the avicultural industry from the late 1960s to the 1990s. The industry's growth was facilitated by the large-scale importation of parrots that had become practical and more affordable with the advent of commercial airfreight and transportation development within the nations of origin (Nilsson, 1981; Styles, 2002). Prior to this time, parrots were expensive and difficult to obtain. They were imported directly from the nation of origin to the pet dealer or collector and kept by small groups of aviculturists (Styles, 2002). Trends in the pet industry coupled with modern transportation methods contributed to popularity and availability of parrots (Styles, 2002, Nilsson, 1981).

In the early days of the industry, little attention was given to the infectious diseases of exotic birds. They could be easily replaced by another wild-caught specimen because few to no restrictions were placed on their importation. However, a worldwide pandemic of viscerotropic velogenic Newcastle disease (VVND) in the early 1970s caused devastating losses in the poultry industry, and the disease was shown to have been introduced into this country in a shipment of parrots (Pierson and Pfow, 1975). The outbreak led to the destruction of millions of chickens and many parrot collections at

great cost in attempts to contain it. In response to this crisis, the United States Department of Agriculture established in 1973 a quarantine system through which all birds imported into the US were channeled and tested for VVND (Pierson and Pfow, 1975; Pilchard, 1991). The quarantine stations were privately owned and operated, but were managed by USDA agents. Station owners took measures to reduce costs: the population density of birds within the station was high and hygiene was not ideal due to understaffing (Nilsson, 1981). This caused birds from multiple continents to be forced to cohabitate in poor conditions in close proximity to each other. These quarantine stations typically held the birds for 30-45 days until the VVND testing results were available (Pilchard, 1991). If no VVND was detected, then the birds were released into the care of the importer. While these measures prevented the entry of VVND into the country, they did nothing to prevent the dissemination of diseases that were infectious to parrots and other birds. Birds that would not normally come into contact with each other were now housed closely together under stressful conditions, making the quarantine stations incubators of disease.

At about the same time, conservationists became concerned about the population status of the parrots in the wild (Nilsson, 1981). This eventually led to the creation of national and international laws regulating their commerce. In 1992, the Wild Bird Conservation Act closed import to all psittacine birds unless by special permit (Styles, 2002; Wright et al., 2001). The economic impact of the quarantine measures in concert with restricted importation served to increase the cost of the imported birds. Therefore, more attention was given to the infectious diseases affecting psittacine birds and

Pacheco's disease emerged as one of the most serious diseases challenging by the aviculture industry (Simpson and Hanley, 1977).

Classical PD was only one manifestation of the disease. Evidence began to accumulate relating a constellation of pathological conditions that developed in the exposed birds and survivors of PD outbreaks. The first of these conditions to be clinically recognized was mucosal papillomatosis.

Mucosal papillomatosis

Mucosal papillomas are benign or pre-malignant neoplasms of mucosal epithelial surfaces. Mucosal papillomas have been described in many species of captive Neotropical parrots, with the highest incidence of these lesions occurring in Amazon parrots (*Amazona*) and macaws (*Ara* spp.) (Cribb, 1984; Graham, 1988; Graham, 1991; Johne et al., 2002; McDonald, 1988; Van der Heyden, 1988).

Clinically, the disease appears to be spread to in-contact birds like an infectious agent (McDonald, 1988). The lesions wax and wane and may not be grossly evident but may be present at a microscopic level (Phalen et al., 1998; Taylor and Murray, 2002). Histologically they appear as papillary structures having a highly vascularized connective tissue core with overlying proliferative stratified cuboidal to pseudostratified columnar mucosal epithelium depending on the location (Schmidt et al., 2003). Lymphoplasmacytic infiltrate is a variable feature of mucosal papillomas, but may correspond to their waxing and waning behavior (Schmidt et al., 2003).

Mucosal and cutaneous papillomas in mammals are known to be caused by papillomaviruses (Brentjens et al., 2002). It was also known that cutaneous papillomas

in wild finches (Lina et al., 1973; Osterhaus et al., 1977; Moreno-Lopez et al., 1984) and African grey parrots (*Psittacus erithacus*) (Jacobson et al., 1983; O'Banion et al., 1992) were caused by species-specific avian papillomaviruses. Therefore, it was assumed that mucosal papillomas in psittacine birds had a similar etiology. However, investigations could find little evidence supporting the existence of papillomaviruses in mucosal papillomas, and efforts to experimentally transmit the disease using homogenates from mucosal papillomas have failed (Sundberg et al., 1986). Papillomavirus virions could not be detected in any mucosal papillomas by electron microscopy (Sundberg et al., 1986). Papillomavirus proteins could not be found in mucosal papillomas using antibodies directed against papillomavirus group-specific antigens, including the conserved L1 major capsid protein, except one study reporting positive immunoreactivity in one macaw and one Amazon parrot (Bonda et al., 1998; Sundberg et al., 1986). Finally, papillomavirus DNA could not be detected in mucosal papillomas using a *Psittacus erithacus* papillomavirus (African grey parrot papillomavirus or PePV) virion DNA probe by Southern blotting (Sundberg et al., 1986); by in-situ hybridization, in-situ PCR, or nested PCR using primers derived from PePV conserved sequence except one African grey parrot (Latimer et al., 1997); or by direct PCR using both degenerate primers that amplify a wide range of different mammalian papillomavirus L1 sequence and primers derived from PePV L1 sequence except one Amazon parrot (Johne et al., 2002). Because no convincing evidence for an association between papillomaviruses and mucosal papillomas could be found, efforts to identify other potential causes were initiated.

Observations were made that mucosal papillomas occurred in survivors and exposed birds subsequent to outbreaks of PD (Van der Heyden, 1988). It was generally held that birds surviving the acute manifestations of the disease become latently infected with PsHV following a sub-clinical or mild infection (Gaskin, 1989; Grund and Schlippenbach, 2002; Phalen et al., 2001). Evidence began to accumulate supporting PsHV as a potential cause for mucosal papillomas. One report described herpesvirus virions in a mucosal papilloma from an orange-fronted conure (*Aratinga canicularis*) (Goodwin and McGee, 1993). Investigations in other species showed an association between herpesviruses and cutaneous papillomas in rainbow smelt (*Osmerus mordax*) (Herman et al., 1997), koi (*Cyprinus carpio*) (Calle et al., 1999), and marine sea turtles (Quackenbush et al., 1998; Quackenbush et al., 2001).

There were also reports describing herpesvirus-like particles in the cutaneous papillomas from the feet and legs of macaws (*Ara* spp.) (Lowenstine, 1982; Phalen, 1997) and cockatoos (*Cacatua* spp.) (Lowenstine, 1982; Lowenstine et al., 1983; Phalen, 1997). Further support for the association between PsHV and mucosal papillomas was presented in a study that found that parrots with mucosal papillomas had circulating anti-PsHV antibodies, whereas parrots without papillomas did not (Phalen et al., 1997). Two other investigations detected PsHV DNA in mucosal papillomas by PCR from four of four birds in one study (Phalen et al., 1998), and 9 of 12 birds in another (Johne et al., 2002). These findings were supported by the observations that the prevalence of PsHV infection in parrots with mucosal papillomas was higher than that seen in the general population of captive parrots (Grund and Schlippenbach, 2002) and parrots without mucosal papillomas (Tomaszewski et al., 2002).

Therefore, to test the hypothesis that mucosal papillomas of Neotropical parrots are caused by one or more genotypes of PsHV, mucosal papillomas from 30 Neotropical psittacine birds were examined by PCR with PePV-specific, FPV-specific, and PsHV-specific primers; and a subset of five mucosal papillomas were also examined by immunohistochemistry for L1 major capsid protein immunoreactivity. The findings of this study are discussed in chapter II of this work.

During the investigation of the putative association between mucosal papillomas and PsHV, a unique PsHV sequence was discovered in the periophthalmic papilloma of a non-Neotropical psittacine bird, an African grey parrot (*Psittacus erithacus*), that differed significantly from the PsHVs previously described by Tomaszewski et al. (2003). This finding led to further investigation of other African grey parrots to determine the prevalence of this unique PsHV.

A novel psittacid herpesvirus in African grey parrots

The cause of papillomas in African grey parrots had been attributed to a species-specific *Psittacus erithacus* papillomavirus (PePV) (Jacobson et al., 1983; O'Banion et al., 1992). However during the investigation of the putative association between PsHVs and mucosal papillomas, a phylogenetically unique PsHV sequence was detected in the biopsy of a periophthalmic papilloma from an African grey parrot. This same sequence was detected in two other living African grey parrots including a bird exhibiting a cloacal papilloma, and a bird with a histologically normal cloaca. The sequence was also found in the histologically normal cloaca of a fourth African grey parrot that died of

causes unrelated to PsHV. This was the first report of a truly mucosal papilloma in an African grey parrot.

PsHV was known to kill African grey parrots (*Psittacus erithacus*) (Tomaszewski et al., 2003), but it had also been shown that PsHV existed latently in other species of parrots either asymptomatically or in parrots exhibiting mucosal papillomas (Grund and Schlippenbach, 2002; Johne et al., 2002; Phalen et al., 1998; Phalen et al., 2001). PsHV associated with PD had not been reported to exist latently in African grey parrots. Therefore, this novel PsHV sequence demonstrated both phylogenetic as well as clinical variations compared to previously described PsHVs.

The results of this investigation are reported in Chapter III where this novel α4 herpesvirus is phylogenetically characterized in relation to both known PsHVs and the broader family of alphaherpesviruses based on sequence data derived from the UL16 (host range protein) and UL30 (DNA polymerase protein) genes. This new psittacid herpesvirus is related but phylogenetically distinct from previously described psittacid herpesviruses (Tomaszewski et al., 2003).

Internal papillomatous disease and other associated tumors

Observations were made about seemingly related neoplastic diseases that were often coincidental with the presence or a history of IPD (Graham, 1991; Hillyer et al., 1991; Phalen et al., 1997). These tumors associated with IPD were adenomas and carcinomas of the bile ducts and pancreatic ducts (Graham, 1991; Elangbam and Panciera, 1988; Gibbons et al., 2002; Hillyer et al., 1991; Phalen et al., 1997; Potter et al., 1983). These tumors had the same Neotropical species distribution as had been described for mucosal

papillomas (Graham, 1991; Hillyer et al., 1991; Phalen et al., 1997). Clinically, these tumors were either locally invasive or space occupying lesions, but infrequently metastasized (Schmidt et al., 2003). However, these neoplasms differ from mucosal papillomas in that once they are established, they do not wane but appear to progress along a spectrum from relatively benign adenomatous change to a malignant carcinomatous state. Other tumors that have been speculated to have an association with IPD were carcinomas of the proventriculus, ventriculus, and cloacal mucosa.

The cause of IPD and related diseases was unknown, but studies by Phalen et al. (1997 and 1998) suggested that these neoplasms might be associated with infection by one or more genotypes of PsHV. Based on these preliminary findings, an investigation into the cause of IPD was performed building on the knowledge acquired from the work with mucosal papillomas.

Methods used to investigate theses associated neoplasms associated with IPD included conventional PCR of DNA extracted from the fresh and formalin-fixed paraffin-embedded (FFPE) tissues of birds with IPD and an associated tumor using probes that detected all known PsHVs. Techniques were also performed in an attempt to define the exact cellular location of any PsHV found in the neoplastic tissues to establish a clear link between the presence of PsHV and the neoplastic tissue. For example, in a liver with bile duct carcinoma, there are affected neoplastic sections and histologically normal sections. The liver was determined to contain PsHV DNA by extracting DNA from digests of the whole tissue, including carcinoma and unaffected tissue, and examining it with PsHV specific probes by conventional PCR. However, the question remained whether the neoplastic bile duct cells had significantly higher concentrations of

PsHV compared to the adjacent unaffected hepatocytes. The first technique used in an attempt to localize the PsHV was laser capture microdissection (LCM). The laser was used as a microsurgical instrument to collect samples within the affected and unaffected regions of the tissue. The DNA was then extracted from these samples and examined by conventional PCR using PsHV specific probes to determine if any relative difference in concentration of PsHV existed between the neoplastic and unaffected tissue. In situ hybridization was similarly employed to examine FFPE tissues of birds with IPD associated tumors using both random and specific biotinylated probes made from known PsHVs or amplicons of PsHV genes. Lastly, in situ PCR was also used to examine the same tissues using PsHV specific primers that amplified all known PsHV genotypes with biotinylated nucleotides as the detection agents. The results of this work are detailed in Chapter IV.

CHAPTER II

PSITTACID HERPESVIRUSES ASSOCIATED WITH MUCOSAL PAPILLOMAS IN NEOTROPICAL PARROTS*

Introduction

Papillomas are benign or premalignant neoplasms of cutaneous, mucocutaneous, and mucosal epithelial surfaces. All papillomas in mammals appear to be caused by papillomaviruses (Brentjens et al., 2002). In contrast, cutaneous papillomas in rainbow smelt (*Osmerus mordax*) (Herman et al., 1997), koi (*Cyprinus carpio*) (Calle et al., 1999), and marine sea turtles (Quackenbush et al., 1998; Quackenbush et al., 2001) are associated with herpesvirus infections.

Both cutaneous and mucosal papillomas have been described in birds. Cutaneous papillomas have been reported in wild finches (*Fringilldae*) (Jennings, 1968; McDonald, 1965; Sironi et al., 1992), African gray parrots (*Psittacus erithacus*) (Jacobson et al., 1983), a Cuban Amazon parrot (*Amazona leucocephala*) (Johne et al., 2002), macaws (*Ara* spp.) (Phalen, 1997) and cockatoos (*Cacatua* spp.) (Lowenstine et al., 1983; Schmidt et al., 2003). The etiologic agents responsible for cutaneous papillomas in the finch, African gray parrot, and Cuban Amazon parrot are papillomaviruses. Lesions caused by these viruses contain intra-nuclear inclusion bodies consisting of crystalline arrays of virus particles (Jacobson et al., 1983; Lina et al., 1973; Osterhaus et al., 1977).

Papillomaviruses from a finch (*Fringilla coelebs* papillomavirus [FPV]) and African gray parrot (*Psittacus erithacus* papillomavirus [PePV]) have been purified, cloned, and sequenced. The genomes of FPV and PePV are structurally similar to mammalian papillomaviruses but share little genetic homology to them or each other (Moreno-Lopez et al., 1984; O'Banion et al., 1992; Tachezy et al., 2002; Terai et al., 2002). Nevertheless, the L1 major capsid proteins of both FPV (Dillner et al., 1991) and PePV (Jacobson et al.; 1983, Lim et al.; 1990, O'Banion et al., 1992) are immunologically cross-reactive with the L1 protein of mammalian papillomaviruses.

In contrast to the cutaneous papillomas of finches and African gray parrots, the cutaneous lesions found in cockatoos and macaws appear to be caused by herpesviruses. These lesions of the unfeathered skin of the foot are characterized by intranuclear eosinophilic inclusion bodies that contain herpesviruses virions (Lowenstine et al., 1983, Schmidt et al., 2003). To date, the viruses associated with these lesions have not been characterized.

Mucosal papillomas have been described in many species of captive Neotropical psittacine birds, with the highest incidence of these lesions occurring in Amazon parrots (*Amazona*) and macaws (*Ara* spp.) (Graham, 1991; Johne et al., 2002; McDonald, 1988; Van der Heyden, 1988). There is a single report of an oral papilloma in an African gray parrot (Latimer et al., 1997). However, this lesion was of squamous epithelium and not of the mucous membranes and therefore cutaneous in nature. Mucosal papillomas occur primarily in the oral cavity and cloaca (vent). Less frequently, mucosal papillomas are found in the crop, esophagus, proventriculus, ventriculus, and conjunctiva (Graham, 1991). These lesions cause considerable discomfort to affected birds, may interfere with

reproduction, and in severe cases result in the bird's death. Bile duct and pancreatic duct adenocarcinomas are also reported to occur with a relatively high prevalence in birds with mucosal papillomas (Graham, 1991; Hillyer et al., 1991).

PePV is suspected to be the etiological agent of the one oral papilloma described in an African gray parrot because it contained PePV DNA (Latimer et al., 1997). However, the etiology of mucosal papillomas in Neotropical parrots remains unknown. Clinical observations suggest that mucosal papillomas are caused by an infectious agent as they appear to spread from affected birds to in-contact birds (McDonald, 1988; Van der Heyden, 1988). However, four parrots inoculated with a mucosal papilloma homogenate did not develop disease (Sundberg et al., 1986).

Efforts to detect papillomaviruses in mucosal papillomas have been largely unsuccessful. Virus particles have not been found in these lesions. PCR with degenerate primers capable of detecting most mammalian papillomavirus sequences and with primers derived from the PePV sequence have failed to detect papillomavirus DNA in the mucosal papillomas of parrots (Johne et al., 2002; Latimer et al., 1997; Sundberg et al., 1986). Additionally, a previous investigation failed to detect L1-like proteins in mucosal papillomas using anti-L1 serum made against conserved epitopes of mammalian papillomaviruses (Sundberg et al., 1986). However, there is a report describing L1-like immunoreactivity present in epithelial cells of a cloacal papilloma from a hyacinth macaw (*Anodorhynchus hyacinthinus*) and a lilac-crowned Amazon parrot (*Amazona finschi*) (Bonda et al., 1998).

Psittacid herpesviruses are a heterogeneous group of viruses divided into four genotypes and four serotypes that are typically associated with an acute fatal infection in

parrots (Pacheco's disease) (Tomaszewski et al., 2003). However, some birds survive the acute manifestations of the disease and may become latently infected while others appear to become latently infected following an inapparent infection (Grund and Schlippenbach, 2002; Phalen et al., 2001). Mucosal papillomas have been observed to develop in parrots that survived PsHV infections (Van der Heyden, 1988) and herpesvirus virions were observed in a mucosal papilloma from an orange-fronted conure (*Aratinga canicularis*) (Goodwin and McGee, 1993). It is therefore likely that one or more of these PsHV genotypes are involved in the development of mucosal papillomas.

Epidemiologic evidence suggests that the prevalence of PsHV infection in parrots with mucosal papillomas is higher than that seen in the general population of captive parrots (Grund and Schlippenbach, 2002) and parrots without mucosal papillomas (Tomaszewski et al., 2002). An initial study found that parrots with mucosal papillomas had circulating anti-PsHV antibodies, whereas parrots without papillomas did not (Phalen et al., 1997). Two studies have used PCR to detect PsHV DNA in these lesions. In a preliminary study, PsHV DNA was detected in mucosal papillomas from four of four birds (Phalen et al., 1998), and in a second study performed by other investigators, PsHV DNA was found in 9 of 12 (75%) mucosal papillomas (Johne et al., 2002). However, if PsHVs are indeed associated with the development of mucosal papillomas, then one would expect to find PsHV DNA in 100% of the samples and yet Johne et al. (2002) reported only a 75% detection rate.

To test the hypothesis that mucosal papillomas of Neotropical parrots are caused by PsHV and not a papillomavirus, mucosal papillomas from 30 Neotropical psittacine

birds were examined by PCR with PePV-specific, FPV-specific, and PsHV-specific primers. Five mucosal papillomas were also examined by immunohistochemistry for L1 major capsid protein immunoreactivity. The genotypes of PsHVs detected in mucosal papillomas were determined by comparing their sequence to the sequences of previously reported psittacid herpesviruses (Tomaszewski et al., 2003).

Results

Immunohistochemistry of mucosal papillomas using anti-HPV6b L1 serum

A single investigation reports L1-like immunoreactivity in two mucosal papillomas from Neotropical parrots that were stained with an antibody made to a fusion protein containing a portion of the HPV 6b L1 protein (Bonda et al., 1998; Strike et al., 1989). To verify this finding, we used this same anti-L1serum to stain the papilloma from a hyacinth macaw (SC98-0018) that had been reported by Bonda et al. (1998) to contain L1 immunoreactivity, three mucosal papillomas from macaws and one mucosal papilloma from an Amazon parrot. Two human cervical papillomas were used as positive controls. A sagittal section taken through a Neotropical psittacine embryo was used as a negative control.

The human cervical papillomas showed distinct nuclear and cytoplasmic staining in the basalar epithelial layer and again at the non-keratinized superficial (lumenal) cell layer (Fig. 2.1a). Most tissues in the negative control psittacine embryo showed diffuse mild to moderate cytoplasmic staining and some showed a distinct nuclear staining pattern. Importantly, the cloacal mucosa showed intense cytoplasmic and moderate

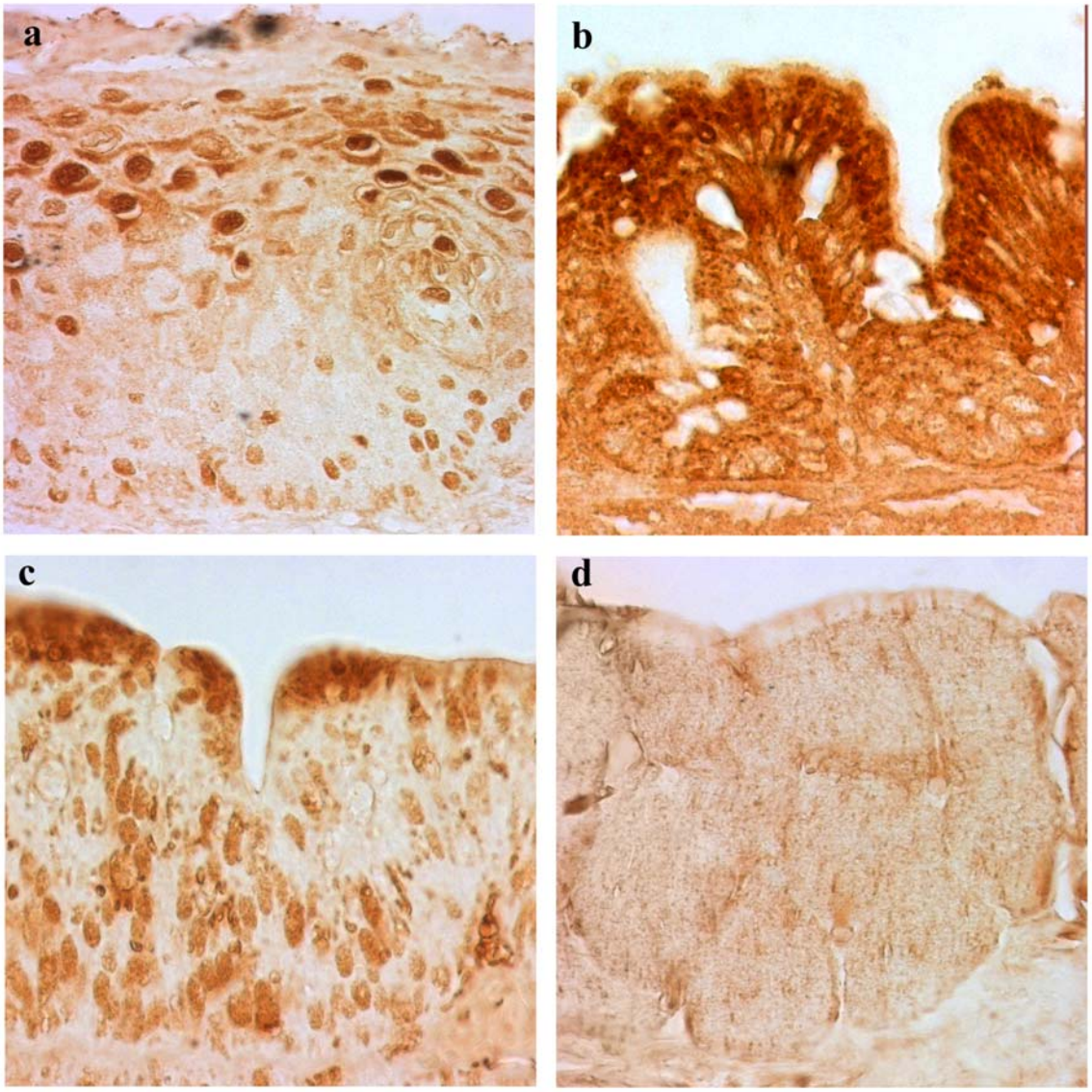

Fig. 2.1. Immunohistochemical results of staining with the anti-L1 fusion protein antiserum. (2.1a) human cervical papilloma showing distinct nuclear and cytoplasmic staining; (2.1b) prominent cytoplasmic and moderate nuclear staining seen in the normal cloacal mucosa of developing psittacine embryo SC01-0019; (2.1c) nuclear and cytoplasmic staining observed in a cloacal papilloma of hyacinth macaw SC98-0018; (2.1d) mucosal papilloma from the cloaca of a macaw DP01-0044 showing only background staining with the anti-L1 antiserum.

nuclear staining (2.1b). This staining pattern was consistent in multiple trials and the intensity observed was not due to artifact. The cloacal papilloma from the hyacinth macaw (SC98-0018) previously reported by Bonda et al. (1998) again stained strongly with the anti-L1 fusion protein antiserum (Fig. 2.1c). Both cytoplasmic and nuclear staining was observed. Importantly, this pattern closely resembled that seen in some tissues of the developing embryo. Three other mucosal papillomas from other macaws and one from an Amazon parrot showed only faint background staining. (Fig. 2.1d).

Electron microscopy

It was assumed that if the cloacal papilloma from the hyacinth macaw (SC98-0018) actually contained L1 proteins, then it would also contain papillomavirus virions. Exhaustive examination of thin sections of the papilloma failed to demonstrate any such virions.

PCR with PePV- and FPV-specific primers

A single oral papilloma from an African gray parrot was previously shown to contain PePV DNA (Latimer et al., 1997). DNA extracted from all the mucosal papillomas in this study were screened for papillomavirus using PePV and FPV specific primers by PCR. None of the 41 papillomas contained PePV or FPV DNA.

Table 2.1

Primer sequences for PePV, FPV, and PsHV

Virus	Primers	Sequence (5'-3')	Amplicon[1]	Reference
PePV:	ppv6621fmod (forward)	GGGCGGATATGACTTTCTGG	204	(Johne, 2002 and
	ppv6810r (reverse)	GCAGTGCGCACGCCTG		Tachezy, 2002)
FPV:	FPV L1f (forward)	AGGTGCTCAGGAGAATAATGC	351	(Terai, 2002)
	FPV L1r (reverse)	AGAAGGTCGTCCGAAAGCCGC		
PsHV:	Amplifying: PsHV-Johne			
	PsHV-s	CATGAACGGCATGCTGCCGT	178	(Johne, 2002)
	PsHV-as	GACTGCCACGGAGTATTG		
	Amplifying 23F			
	23Ff5a (forward)	TGCGTGGGGTTAAACTCGGAACTAGAAG	667	(Tomaszewski, 2003)
	23Fr3(reverse)	CGACTACACGAGCCTAACATC		
	Sequencing 23F			
	23Ff5b (forward)	GGGGTTAAACTCGGAACTAGAAG	662	(Tomaszewski, 2003)
	23Fr3 (reverse)	CGACTACACGAGCCTAACATC		

[1]amplicon length in nucleotides

Amplification of DNA from known PsHVs using primers from the DNA polymerase gene

Previously, Johne et al. reported that they were able to amplify PsHV from nine of twelve papillomas with PCR using primer set PsHV-Johne that was derived from the DNA polymerase gene (UL30) (Table 2.1). In order to determine if the primers used by these investigators would consistently and efficiently amplify DNA from all known PsHV genotypes, an optimized PCR reaction was performed with DNA from two representative samples from each of the four PsHV genotypes. These same samples were also amplified using the 23F-amplifying primer set (derived from the UL16/UL17 region) that detects all known PsHV genotypes (Table 2.1). Amplification products

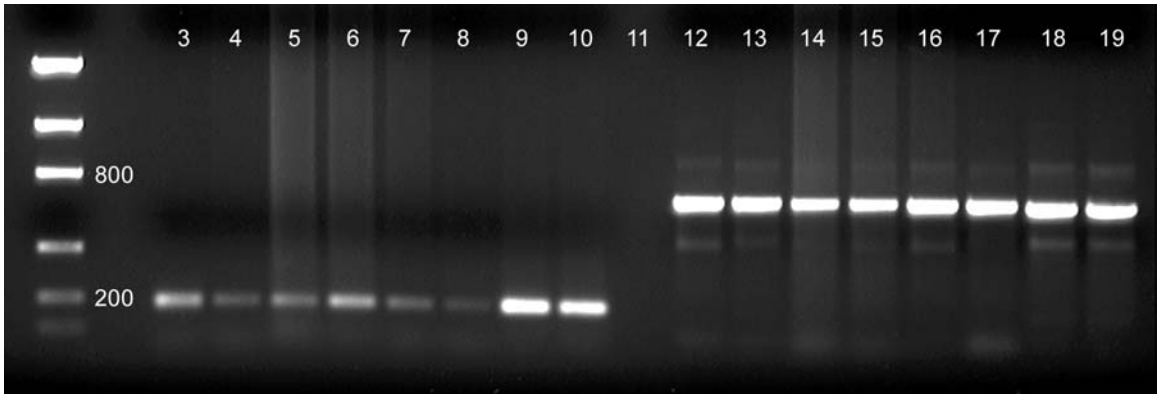

Fig. 2.2. Comparison of the amplification efficiency of primer sets PsHV-Johne and 23F-amplifying using paired DNA samples representative of each of the four PsHV genotypes. Lanes 3-10 contain a 178 bp amplicon generated by primer set PsHV-Johne (Table 2.1). Lanes 12-19 contain a 667 bp amplicon was generated by primer set 23F amplifying (Table 2.1). DNA from genotypes 1, 2, and 3 was extracted from mucosal papillomas (Table 2.2). Genotype 4 DNA was extracted from tissues of birds dying with Pacheco's disease (Table 2.2). Specific samples amplified were: lanes 3 and 12, SC02-022 (genotype 1); lanes 4 and 13, SC02-038 (genotype 1); lanes 5 and 14, SC93-790 (genotype 2); lanes 6 and 15, SC94-056 (genotype 2); lanes 7 and 16, SC94-1038 (genotype 3); lanes 8 and 17, SC02-047 (genotype 3); lanes 9 and 18, 88-97 (genotype 4); lanes 10 and 19, 91-864 (genotype 4). Amplicons were electrophoresed on a 1.5% agarose gel.

were detected in all of the reactions (Fig. 2.2). Both primer sets amplified DNA from all four PsHV genotypes. However, the primer set used by Johne et al had a markedly reduced amplification efficiency compared to the 23F primer set with PsHV genotypes 1, 2, and 3 (Fig. 2.2).

PCR with PsHV specific primers

There are two previous studies using a limited number of tissues demonstrating PsHV DNA in all (Phalen et al., 1998) or many of the papillomas that they examined (Johne et al., 2002). In order to determine if PsHV DNA is in fact consistently present in mucosal papillomas, primer sets known to amplify all known PsHV genotypes (Table 2.1; 23F-amplifying) were used with PCR to screen 41 papillomas from 30 Neotropical parrots; a biopsy from the cloacal mucosa of a macaw without papillomas that was in contact with another macaw with a papilloma; and 12 histologically normal mucosa samples from birds that had papillomas. PsHV DNA was detected in all of the mucosal papillomas examined. The estimated number of copies of the target gene per 100 pg of DNA ranged from approximately 1,000 to over 1,000,000 and serial 10-fold dilutions of a plasmid containing the UL 16/17 region showed that the sensitivity of this assay was 1,000 copies of target DNA (Fig. 2.3).

PsHV DNA was not amplified from three mucosal samples that were collected from the mucosa adjacent to papillomas. Nine histologically normal digestive mucosal samples were collected from birds with papillomas. Seven were negative, but one proventricular sample was weakly positive (approximately 1,000-10,000 copies of target DNA) and another was moderately positive (approximately 10,000-1,000,000 copies of target DNA). PsHV DNA was also detected in the cloacal biopsy from an in contact macaw (SC02-0043) not exhibiting mucosal papillomas. There was no PsHV DNA amplified from DNA extracted from the tissues of a near hatch conure embryo (SC01-0019) or the blood from an incubator-hatched African gray nestling (SC03-0005). Also, PsHV DNA was not found in the brachial plexus, lumbosacral plexus, or brain of 3 birds

with papillomas. Herpesvirus DNA was consistently amplified from DNA purified from control tissue known to contain PsHV DNA.

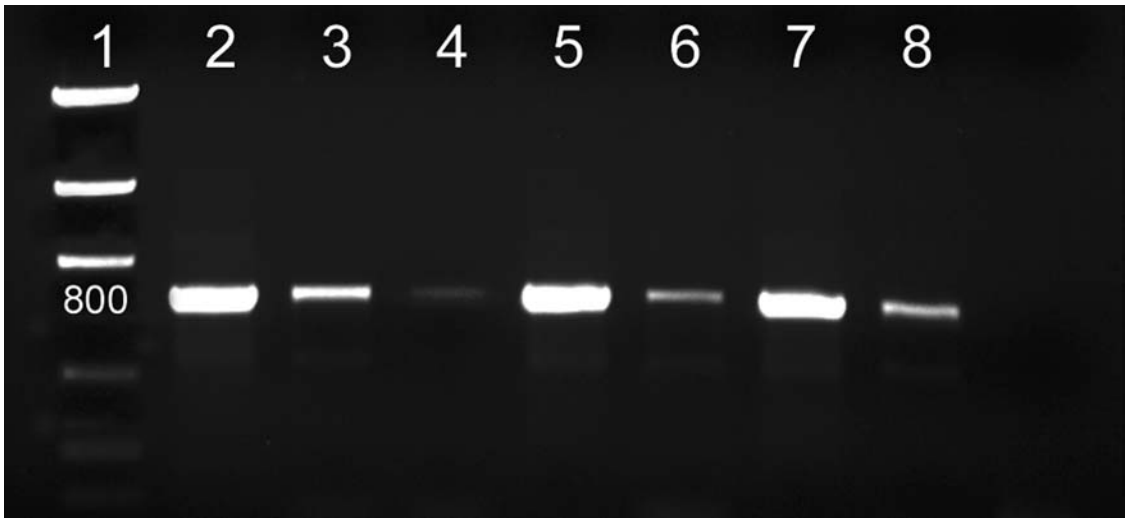

Fig. 2.3. Evaluation of the sensitivity of the 23F primer set to detect PsHV in serial dilutions of a PsHV clone of known concentration (clone 23F-from which the 23F primer sets were derived), and from 100 ng of genomic DNA extracted from mucosal papillomas. Lanes 2-4 contain a 667 bp amplicons from the serial dilutions of the PsHV clone 23F in order of decreasing concentration (plasmids/µl): lane 2 – 10^6; lane 3 – 10^4; lane 4 –10^3. Lanes 5, 6, and 7 contain the 667 bp amplicons generated from DNA derived from mucosal papillomas and lane 8 contains DNA derived from the cloaca of an asymptomatic macaw (Table 2). Specific samples are: lane 5 - SC02-022 (genotype 1); lane 6 – SC00-020 (genotype 2); lane 7 – SC02-047 (genotype 3); lane 8 – SC02-0043 (genotype 4).

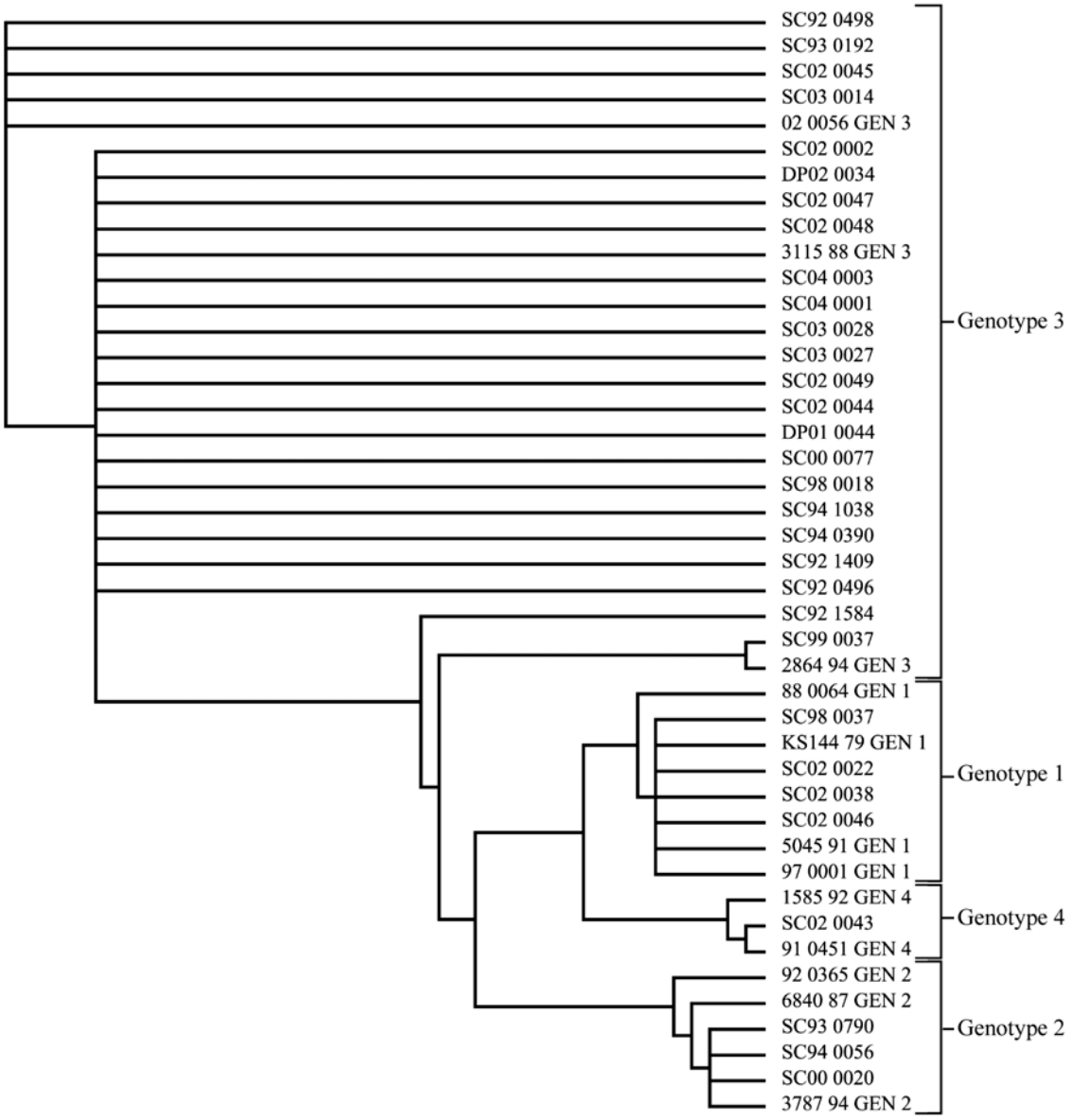

Fig. 2.4. Consensus phylogenetic tree (cladogram) of a set of 419 bp PsHV sequences generated by bootstrap re-sampling (numbers of repetitions=1000), distance optimality, and neighbor-joining search using PAUP 4.0. Sequences preceded by "SC" or "DP" were derived from mucosal papillomas except for sequence SC02-0043 that was derived from the cloaca of an asymptomatic macaw (Table 2.2). Sequences followed by the suffix "GEN X" were reference sequences of known genotype where X=genotype.

Phylogenetic analysis

The sequence of a 419 bp fragment of each amplicon was compared to the sequences of PsHVs of known genotype (Tomaszewski et al., 2000). A cladogram of all the sequences is shown (Fig. 2.4). A phylogram with representatives of each genotype showing branch-lengths and bootstrap values was also generated (Fig. 2.5). Of the 30 sequences from the mucosal papillomas, four were genotype 1 (13.3%); three were genotype 2 (10.0%); and 23 were genotype 3 (76.7%). The herpesvirus found in the cloaca of a macaw without papillomas (SC02-0043) was genotype 4. In six birds, PsHV amplicons were sequenced from an upper gastrointestinal papilloma and a cloacal papilloma from the same bird. The sequences from both lesions from each bird were identical.

Estimation of the distribution of genotypes in the population of birds with mucosal papillomas

If PsHV sequence is amplified from a mucosal papilloma selected at random, then the probabilities of mapping into each PsHV genotype are: p (genotype 1, n= 4) = 0.13 ± 0.12; p (genotype 2, n=3) = 0.10 ± 0.11; and p (genotype 3, n=23) = 0.77 ± 0.15.

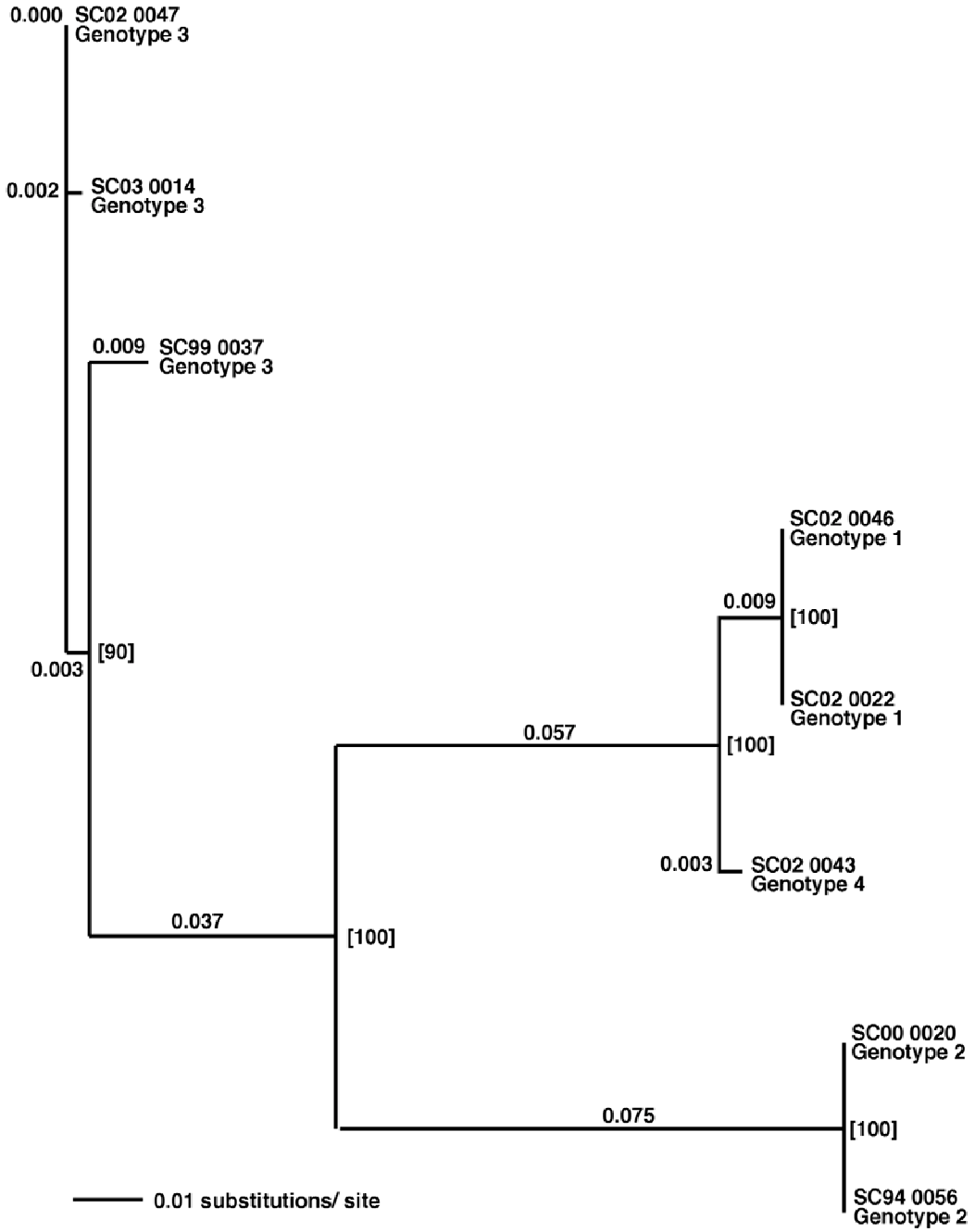

Fig. 2.5. Phylogram displaying branch lengths and bootstrap values [bracketed at nodes] for one representative of each genotype of the 419 bp PsHV sequence. This tree was generated by bootstrap re-sampling (numbers of repetitions=1000), distance optimality, and neighbor-joining search.

Significance of the observed PsHV prevalence in papillomas as compared to the estimated prevalence rates in the general psittacine population

The prevalence of PsHV infection in macaws and Amazon parrots submitted to the Texas Veterinary Medical Diagnostic Laboratory was 0% (0/25) (Tomaszewski et al., 2002). An overall seroprevalence of PsHV infection in a recent study of aviary parrots in Europe was 37% (200/750) (Grund et al., 2002). The rate of detection of PsHV in the mucosal papilloma sample was 100% (30/30). The prevalence of the PsHV infection in birds with mucosal papillomas was significantly higher ($p \leq 0.05$) than the prevalence of infection in previously sampled USA and European populations.

Discussion

Investigations into the etiologic agent of mucosal papillomas of Neotropical parrots have focused on two possible etiologies, papillomaviruses and PsHVs. Our work, coupled with the work of other investigators, provides increasingly strong evidence that PsHVs play a direct role in the development of mucosal papillomas. Prior to this investigation, it had been noted that mucosal papillomas were seen in birds that survived acute systemic PsHV infections (Van der Heyden, 1988). Additionally, birds with mucosal papillomas had been shown to have circulating antibody to PsHVs (Phalen et al., 1997); PsHV DNA had been demonstrated in the epithelial layer of a mucosal papilloma by DNA-DNA in situ hybridization (Johne et al., 2002); and PsHV DNA had been found in four of four mucosal papillomas (Phalen et al., 1998) and nine of twelve mucosal papillomas by Johne et al. (2002). In our current study, we found that 100% of the 41 mucosal papillomas collected from 30 birds were positive for PsHV DNA.

Importantly, PsHV DNA was not found in three histologically normal mucosal samples that were adjacent to papillomas, and was only found in two of seven normal mucosal samples taken from other parts of the digestive system. The 100% prevalence of PsHV infection in the birds with papillomas was found to be significantly higher than any reported infection prevalence in parrots and therefore it is extremely unlikely that presence of PsHVs in papillomas was coincidental.

If PsHVs were the causative agent of mucosal papillomas in Neotropical parrots, then it would be expected that they would all contain PsHV. Yet, Johne et al. (2002) only detected PsHV DNA in 9 of 12 papillomas that they examined. PsHV occurs in variable concentrations in the mucosal papillomas. Our data shows that the 23F primer set is highly sensitive and capable of detecting PsHV at minimal concentrations of at 10^3 copies or potentially less (Fig. 2.3). However, the primers used by Johne et al. are not as efficient at amplifying PsHV genotypes 1, 2, or 3 and therefore would not have detected PsHV at the lower concentrations found in some of the papillomas described by our study (Fig. 2.3). Thus, we speculate that their negative samples may have actually been positive and contained concentrations of PsHV lower than the limit of detection by their primer set.

If the hypothesis that PsHV infection is necessary for the development of mucosal papillomas in Neotropical parrots is correct, then the phylogenetic data presented here provides evidence as to the epizootiology of this disease. Only PsHV genotypes 1, 2, and 3 were found in the mucosal papilloma sample population, and the majority (77%) of the viruses were genotype 3. The most common genotypes to cause Pacheco's disease in the United States are genotypes 3 and 4, while genotypes 1 and 2 are less

commonly implicated (Tomaszewski et al., 2003). The work by Tomaszewski et al. (2003) suggests that specific PsHV genotypes may be more likely to cause Pacheco's disease in some species and less likely to cause Pacheco's disease in others. Both genotypes 3 and 4 demonstrate some general species preference. Genotype 4 is the most common genotype to be found in macaws that die with Pacheco's disease. In contrast, genotype 3 is rarely found to cause Pacheco's disease in macaws and genotypes 1 and 2 were never found in macaws with Pacheco's disease. These data potentially explain why genotypes 1, 2 and 3 but not 4 are found in macaws with mucosal papillomas. Macaws infected with genotype 4 would be less likely to survive infection and would succumb before developing papillomas. However, infections with the other genotypes may result in asymptomatic persistent infection and the development of papillomas. The unexpected discovery of a PsHV genotype 4 in from the cloacal biopsy of an asymptomatic macaw (SC03-0043) suggests that not all genotype 4 infections in macaws result in death from Pacheco's disease and other factors such as individual host immune response may determine the ultimate outcome resulting from infection.

It has also been observed that infection with PsHV genotype 3 usually results in a fatal infection in Amazon parrots but rarely macaws. However, our study found that 60% of Amazon parrots with papillomas were infected with genotype 3. This data again suggests that there may be factors other than genotype that determine what disease process may be observed.

These variations of individual species sensitivity to PsHV infection could result in the inapparent spread of genotypes 1, 2, and 3 from bird to bird, and would explain why mucosal papillomas can disseminate throughout a collection without an ensuing

outbreak of Pacheco's disease. These data also suggest that under some conditions, birds with mucosal papillomas may be potential sources of outbreaks of Pacheco's disease.

Epizootiological evidence suggests that the PsHVs present in the papillomas go through periods where they produce virions. However, it also appears these viruses are predominantly present in the mucosal papillomas in a latent state, or at the very least are slowly replicating. Inclusion bodies and virions have only been detected in a single papilloma and transmission trials with a mucosal papilloma homogenate failed to induce mucosal papillomas or Pacheco's disease (Goodwin and McGee, 1993; Sundburg et al., 1986). If PsHVs do maintain a latent state in the mucous membranes of parrots, this would be a novel strategy for an alpha herpesvirus, as they typically infect nerve cells and establish latency. Whether or not PsHVs also latently infect other tissues will require further investigation. Dorsal root nerve ganglia are common location for persistent alphaherpesvirus infection. We were not able to detect PsHV DNA in nervous tissue from 3 birds, but we did not examine dorsal root ganglia or employ intensive amplification techniques such as nested PCR.

Mucosal papillomas in Amazon parrots are most often confined to the mucosa of the cloaca. Macaws also typically have cloacal papillomas as well as papillomas of the upper digestive tract. This difference in distribution of the lesions appears to be the result of host factors because the same genotypes found in macaw mucosal papillomas were also found in Amazon parrot papillomas. This is further supported by the observations that the genotypes found in the upper digestive tract papillomas were the

same as the genotypes found in the cloaca papillomas in all of the birds examined with lesions in both locations.

In a previous study, a variant of the genotype 3 viruses was identified that could be neutralized by serum raised to either serotype 2 or serotype 3 viruses (Gravendyck et al., 1996). In that study, both viruses were European isolates and it was not known if a similar virus was present in the USA. A virus with an identical sequence to this variant was detected in one cloacal papilloma from a macaw (sample SC99-0037, Fig. 2.4) in this study, suggesting that this variant of the genotype 3 with its unusual serotype is also present in the USA.

The only evidence that a papillomavirus may play a role in the development of mucosal papillomas are the findings of PePV DNA in an oral papilloma in an African gray parrot (Latimer et al., 1997) and L1 immunoreactivity in papillomas from a hyacinth macaw and an Amazon parrot (Bonda et al., 1998). The oral papilloma from the African gray parrot should not be considered as a mucosal papilloma, because the lesion was characterized by keratinized squamous epithelium and was not of mucocutaneous epithelium. Therefore, we feel that this lesion is more representative of the cutaneous papillomas seen in African gray parrots that are known to be caused by PePV. Previous studies failed to document PePV DNA in mucosal papillomas (Sundburg et al., 1986; Latimer et al., 1997). Similarly, neither PePV nor FPV DNA was amplified from the mucosal papilloma population in our study. However, we cannot exclude the existence of a novel avian papillomavirus that was not detected by our PePV or FPV primer sets in the lesions. We chose to use these specific primers because they are derived from the conserved L1 major capsid protein sequence of the only known

avian papillomaviruses, and the nucleotide homology between PePV and FPV is too diverse to make degenerate primers capable of detecting both viruses and the and any intervening variants.

In order to determine whether the L1 immunoreactivity previously reported in the hyacinth macaw was the result of papillomavirus antigen, we repeated this experiment with the same antibody and papilloma from the hyacinth macaw (SC98-0018) reported by Bonda et al. (1998), papillomas from 3 other macaws and an Amazon parrot, a human cervical papilloma as a positive control, and a conure embryo as a negative control. We were able to demonstrate L1 immunoreactivity in the hyacinth macaw papilloma. However, we also showed a similar degree and pattern of immunoreactivity in the psittacine embryonic control tissue. By contrast, the four other mucosal papillomas examined did not contain this immunoreactive protein. Therefore, given that virions were not observed in this papilloma and FPV or PePV DNA was not found, we conclude that the immunoreactivity is either nonspecific or is due to a protein that is cross-reactive to the L1 anti-serum but is not in fact papillomavirus L1 major capsid protein. Importantly, we did amplify PsHV genotype 3 DNA from this mucosal papilloma (Fig. 2.4).

In conclusion, all the evidence to date strongly suggests that PsHVs play an essential role in the development of mucosal papillomas in Neotropical parrots and explain their apparent infectious nature. In contrast, there is no evidence to suggest that a papillomavirus is present in these lesions.

Materials and Methods

Source of specimens

Tissues from thirty-one Neotropical parrots, one old world parrot, and one finch were used in the mucosal papilloma study (Table 2.2). The papillomas examined in this study were harvested from Amazon parrots (30%), macaws (67%), and a single conure (3%). All birds were from aviaries in the United States of America. Tissues were diagnostic specimens (biopsies or whole birds) that were submitted to the Schubot Exotic Bird Health Center (Texas A&M University, College Station, TX). An additional sample, a cloacal biopsy from an unaffected blue and gold macaw (SC02-0043) that was in contact with another bird with mucosal papillomas was also examined. When tissues were available from more than one bird from the same source, only tissues from one of the birds were examined.

Tissues were collected using cleaned, autoclaved instruments that had been bleached and formalin-soaked. A different set of instruments was used to collect each tissue to prevent DNA contamination. Tissues were either processed immediately or stored at -80°C until use. Papillomas were taken from more than one site in 7 birds. The total number of papillomas examined was 44: choana=2, cloaca=23, crop=4, esophagus=3, glottis=5, oropharynx=6, proventriculus=1. Histologically normal digestive tract mucosal samples were available from 6 birds with mucosal papillomas: choana=1, crop=4, esophagus=1, proventriculus=3. In two of these birds, three additional samples

Table 2.2

Tissues and sources

Isolate Number	Genebank Access #/Use[1]	Species Examined	Geno[2]	Tissues Sequenced
SC88-0097	immunohist.[3]	*Ara macao*		
SC91-0864	immunohist.	*Amazona aestiva*		
SC92-0496	AY421976	*Ara macao*	3	cloacal papilloma
SC92-0498	AY421977	*Ara macao*	3	cloacal papilloma
SC92-1409	AY421978	*Amazona auropalliata*	3	glottal papilloma
SC92-1584	AY421979	*Ara ararauna*	3	cloacal papilloma
SC93-0192	AY421980	*Ara ararauna*	3	choanal papilloma
SC93-0790	AY421981	*Ara chloroptera*	2	esophageal papilloma
SC94-0056	AY421982	*Ara chloroptera*	2	cloacal papilloma
SC94-0188	immunohist.	*Ara macao*		
SC94-0390	AY421983	*Aratinga* spp.	3	glottal papilloma
SC94-1038	AY421984	*Ara chloroptera*	3	cloacal papilloma
SC98-0018	AY421985	*Anodorhynchus hyacinthinus*	3	cloacal papilloma
SC98-0037	AY421986	*Amazona aestiva*	3	cloacal papilloma
SC99-0037	AY421987	*Ara macao*	3	cloacal papilloma
SC00-0020	AY421988	*Amazona oratrix*	2	cloacal, crop papillomas
SC00-0077	AY421989	*Amazona oratrix*	3	cloacal papilloma
DP01-0044	AY421990	*Ara ararauna*	3	cloacal, glottal papillomas
SC01-0019	(-) control DNA	*Pyrrhura rupicola*		
DP02-0034	AY421991	*Ara chloroptera*	3	cloacal, glottal papillomas
SC02-0002	AY421992	*Amazona autumnalis*	3	cloacal papilloma
SC02-0022	AY421993	*Ara ararauna*	1	cloacal papilloma
SC02-0038	AY421994	*Amazona barbadensis*	1	cloacal papilloma
SC02-0043	AY421995	*Ara ararauna*	4	cloaca (no papilloma)
SC02-0044	AY421996	*Ara ararauna*	3	cloacal papilloma
SC02-0045	AY421997	*Amazona aestiva*	3	cloacal papilloma
SC02-0046	AY421998	*Amazona aestiva*	1	cloacal papilloma
SC02-0047	AY421999	*Amazona aestiva*	3	cloacal papilloma
SC02-0048	AY422000	*Amazona oratrix*	3	cloacal papilloma
SC02-0049	AY422001	*Ara macao*	3	cloacal papilloma
SC03-0005	(-) control DNA	*Psittacus erithacus*		
SC03-0014	AY563422	*Ara chloroptera*	3	cloacal papilloma
SC03-0027	AY563423	*Ara ararauna*	3	oropharyngeal papilloma
SC03-0028	AY563424	*Ara ararauna*	3	oropharyngeal papilloma
DP04-0009	(-) control DNA	*Lonchura striata*		
SC04-0001	AY563425	*Ara chloroptera*	3	glottal papilloma
SC04-0003	AY563426	*Ara chloroptera*	3	cloacal papilloma

[1]GeneBank accession number or tissue use
[2]PsHV genotype
[3]"immunohist."=tissues used for immunohistochemistry

were taken from mucosa that was the immediately adjacent to papillomas but was histologically normal. When only a single papilloma was available, the amplicon from that papilloma was sequenced. There were six birds that had papillomas in the upper digestive tract and cloaca. In these birds, amplicons from both the cloacal lesion and an upper digestive tract lesion were sequenced. One bird had multiple papillomas of the upper digestive tract; only one of the amplicons from these papillomas was sequenced. Neural tissue was available from 3 birds: brachial plexus=1, lumbosacral plexus=2, and brain=1.

Histological evaluation of the mucosal papillomas and unaffected tissue

Sections of the tissues were fixed in formalin, embedded in paraffin, and thin sections were routinely stained with hematoxylin and eosin. The tissue was considered to contain papillomas if the normal mucosa was replaced with papillary growths consisting of a narrow to broad base with the mucosal fronds containing a fine fibrovascular core covered by a cuboidal to tall columnar epithelium of variable thickness.

Immunohistochemistry of mucosal papillomas using papillomavirus anti-L1 major capsid protein antibody

Formalin-fixed paraffin-embedded mucosal papillomas from one Amazon parrot (SC91-0864) and four macaws (SC88-0097, SC94-0188, SC98-0018, DP01-0044) were affixed to 3-aminopropyl-trimethoxysilane coated glass slides (Sigma, St. Louis, MO.). Tissues were incubated with rabbit polyclonal antiserum made against an HPV-6b L1 ß-galactosidase fusion protein (Bonda et al., 1998). The antibody has been previously

shown to detect a highly conserved epitope within the L1 protein of mammalian papillomaviruses (Strike et al., 1989). Positive controls were two human cervical papilloma biopsies. The negative control was a near-hatch conure embryo (SC01-0019) (Table 2.2). The embryo was sagittally sectioned to include cloaca and other viscera.

Microwave antigen retrieval was performed in 10 mM citrate buffer using methods similar to those described by Shi et al (Shi et al., 1991). Tissues were permeabilized with 0.5% Triton X 100 in PBS, and endogenous peroxidase was quenched with 0.3% H_2O_2 in water. Tissues were blocked with 3% BSA / 0.1% glycine /10% normal goat serum in 0.2% Tween 20 in PBS. The tissues were then incubated with L1 fusion protein antiserum, diluted 1:500 in a solution of 10% normal goat serum / 0.2% Tween 20 in PBS, for 12 h at 4ºC. Immunoreactions were visualized using HRP-conjugated goat anti-rabbit antibody (Bethyl Labs, Montgomery, Tex.), with diaminobenzidine tetrahydrochloride (Sigma) and imidazole (EM Science, Gibbstown, NJ.) as chromogenic substrates (protocol from CHEMICON International, Inc., www.chemicon.com). Reactions were stopped at 15 minutes, and sections were examined by light microscopy (Fig. 2.1a-d).

Electron microscopy

Formalin-fixed, paraffin-embedded papillomatous tissue from the hyacinth macaw (SC98-0018) that had stained positively with the anti-L1 antibody was deparafffinized and fixed in 3% uranyl acetate in 100% ethanol. The tissue was then embedded in Spur's firm epoxy resin and 60-90 nm sections were placed on a copper grid. The

sections were post stained with 3% uranyl acetate and Reynold's lead citrate. Thin sections were examined for papillomavirus virions at 10,000 X for two hours.

DNA purification

DNA was extracted from the tissues using the Purgene® Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN.) following the instructions for fresh or frozen solid tissue.

PCR of papilloma tissues with PePV- and FPV-specific primers

All PCR reactions were performed on an Eppendorf Mastercycler® Personal Thermocycler (Eppendorf, Hamburg, Germany). Primer set were derived from the conserved papillomavirus L1 major capsid protein region and DNA extracted from the tissues was examined for the presence of PePV DNA using the PePV-specific primers ppv6621fmod and ppv6810r and for FPV DNA using FPV L1f and FPV L1r by PCR (Table 2.1). The primer set for PePV amplification was slightly modified from that published by Johne (Johne et al., 2002) to agree with the PePV sequence reported by Tachezy, et al (2002). The negative control for the PePV PCR was DNA extracted from a pre-hatch conure embryo (SC01-0019). DNA from the liver of a society finch free of papillomas (DP04-0009) was used as the negative control for the FPV PCR. The positive controls were a clone of *Psittacus erithacus* papillomavirus cloned into the *Sal* I site of pBR322 and a clone of finch papillomavirus cloned into the *Eco* RI site of pBR328 (Terai et al., 2002).

PCR was performed as described by Johne et al. (Johne et al., 2002). Briefly, a 25 µl reaction mix containing, 100 ng genomic DNA, 25 pmol of each primer, 0.1 mM of each of the 4 deoxynucleotide triphosphates, 2.5 mM magnesium chloride, 0.75 units Taq polymerase and 1X buffer A (Promega, Madison, WI.) was used in the reaction.

The thermocycler parameters were: initial denaturation at 95°C for 5 minutes; followed by 35 cycles of denaturing at 94°C for 30 seconds, annealing at 60°C for 30seconds, extension at 72°C for 30 seconds, and then a period of final extension at 72°C for 10 minutes. Amplification products were imaged on an ethidium bromide agarose gel.

Amplification of DNA of known PsHVs using primers from the DNA polymerase gene

Johne et al. (2002) detected PsHV DNA in eight of twelve papillomas they examined. To determine if the primers (Table 2.1) used in their study would efficiently amplify DNA from all 4 genotypes of the PsHV, we first optimized the magnesium and dNTP concentrations in the PCR reaction mix for these primers using PsHV genotype 1 viral DNA. The optimum reaction conditions using 100 ng of DNA (avian genomic and viral) were determined to be: 25 pmol of each primer, 0.05 mM of each of the 4 deoxynucleotide triphosphates, 2.5 mM magnesium chloride, 1X buffer A (Promega) and 0.75 units Taq polymerase per 25 µl reaction. The thermocycler parameters were the same as those reported by Johne et al (1998, 2002): initial denaturation at 95°C for 5 minutes; followed by 35 cycles of annealing at 56°C for 30 seconds, extension at 72°C for 30 s, denaturing at 94°C for 30 s, and then a period of final extension at 72°C for 10

min. Using the optimized reaction conditions, viral DNA was amplified from four viruses of each genotype. The intensity of the amplification products was compared to those produced by PCR of the same viruses using primer set 23F-amplifying (Fig. 2.2).

PCR of DNA from papilloma tissues with PsHV-specific primers

DNA was examined for the presence of PsHV DNA by PCR. The primer set 23F-amplifying (Table 2.1) was used to amplify PsHV DNA (Tomaszewski et al., 2000). The amplicon produced was 667 bps in length and spanned a section of UL16/UL17 (host-range protein / DNA-cleaving and packaging protein) including the UL16 initiation codon. Negative controls were DNA extracted from the blood of an incubator-hatched African gray parrot (SC03-0005) held in isolation and a pre-hatch conure embryo (SC01-0019). The positive control was DNA containing PsHV extracted from tissues of an Amazon parrot that died of Pacheco's disease (Tomaszewski et al., 2000).

PCR was performed as previously described (Tomaszewski et al., 2000). Briefly, a 25 µl reaction mix containing, 100 ng genomic DNA, 25 pmol of each primer, 0.1 mM of each of the 4 deoxynucleotide triphosphates, 2.5 mM magnesium chloride, 0.75 units Taq polymerase and 1X buffer A was used in the reaction.

The thermocycler parameters were: initial denaturation at 94°C for 5 minutes; followed by 40 cycles of annealing at 60°C for 45 seconds, extension at 72°C for 90 seconds, denaturing at 94°C for 30 seconds, and then a period of final extension at 72°C for 5 minutes. PCR amplification products were imaged on ethidium bromide agarose gels.

The sensitivity of the assay was determined by amplifying viral DNA from 10-fold dilutions of a plasmid containing the UL16/17 ORF (Fig. 2.3). The approximate copy number of PsHVs in each 500 ng of genomic DNA was estimated by comparing the intensity of the amplification products from the papilloma samples to those of the amplification products from the known concentrations of plasmid DNA (Fig. 2.3).

DNA sequencing of PsHV amplicons

PCR products were purified using QIAquick® PCR Purification Kit (Qiagen Inc., Valencia, Cal.). The sequencing reaction was performed using an ABI Prism® Big Dye™ Terminators v3.0 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA) using sequencing primers 23Ff5b (forward) and 23Fr3 (reverse) (Table 2.1). Products were sequenced using an ABI 377 DNA sequencer or ABI 3100 DNA sequencer (Applied Biosystems).

Phylogenetic analysis

A 419 bp fragment beginning at the UL16 start codon was used for the phylogenetic analysis (Tomaszewski et al., 2000). All sequences were aligned using Clustal X 1.81 (Thompson et al., 1997). Aligned sequences were imported into PAUP for analysis (Swofford, 1998). Phylogenetic trees were generated by performing a neighbor-joining search using bootstrap re-sampling (1,000 replications) employing distance optimality. A cladogram including all sequences was included in this analysis to define the specific branches of the phylogenetic tree (Fig. 2.4) (Tomaszewski et al., 2000). There is also a

phylogram showing branch-lengths and bootstrap values employing representative sequences of each genotype (Fig. 2.5).

Statistical analysis

A test of the normal approximation of the binomial was performed to estimate the distribution of the genotypes in the population of mucosal papillomas (Johnson, 1984). This test was performed using only those birds that exhibited mucosal papillomas and did not include the in contact macaw (SC02-0043). A chi-square test of independence (Johnson, 1984) was performed to demonstrate that the prevalence of PsHV in the mucosal papilloma population was significantly greater than the prevalence rate in one population of parrots from the USA (Johne et al., 2002) and the prevalence rate of PsHV infection in European aviaries (Grund and Schlippenbach, 2002).

CHAPTER III

A NOVEL PSITTACID HERPESVIRUS FOUND IN AFRICAN GREY PARROTS (*PSITTACUS ERITHACUS ERITHACUS*)*

Introduction

Herpesviruses have been identified in a broad range of birds (Sui et al., 1995; VanDevanter et al., 1996; Ehlers et al., 1999; Alfonso et al., 2001; Wellehan et al., 2003). Molecular characterization of several of these viruses shows that they all are alphaherpesviruses. However, avian herpesviruses are genetically diverse and are found in at least three genera of the *Alphaherpesvirinae* (Davison, 2002). The α4 genus exclusively contains the avian herpesviruses, Gallid herpesvirus 1 (GaHV-1) (VanDevanter et al., 1996; Davison, 2002), Passerid herpesvirus 1 (PaHV-1) (Wellehan et al., 2003), and Psittacid herpesvirus (PsHV) (Davison, 2002; Tomaszewski et al., 2003). PsHV is the only herpesvirus identified to date in psittacine birds (parrots). PsHV consists of 4 genotypes that have distinct biological characteristics (Tomaszewski et al., 2003). These genotypes appear to have co-evolved with several species of Neotropical (Central and South American) parrots. In their host species, they are not thought to cause disease (Tomaszewski et al., 2003). However, an acute fatal disease (Pacheco's disease) may result when parrots are infected with a PsHV genotype to which they are not evolutionarily adapted.

Additionally, PsHV genotypes 1, 2, and 3 are believed to cause mucosal papillomas in subclinically infected Neotropical parrots and survivors of Pacheco's disease (Styles et al., 2004). Cutaneous papillomas have been described in macaws and cockatoos and are thought to be caused by an uncharacterized herpesvirus (Lowenstine et al., 1983; Phalen, 1997). However, cutaneous papillomas in African grey parrots appear to be caused by *Psittacus erithacus* papillomavirus (PePV) (O'Banion et al., 1992).

In this study, we report the discovery of a novel α4 herpesvirus in the tissues of four African grey parrots (*Psittacus erithacus erithacus*). This new psittacid herpesvirus is related but phylogenetically distinct from previously described psittacid herpesviruses (Tomaszewski et al., 2003).

Results

Histological examination of tissues

Tissues were examined by light microscopy to determine if papillomatous changes were present. Specimens SC00-0020, SC02-0022, SC02-0025, and SC02-0047 manifested mucosal papillomas characterized by normal mucosa replaced with papillary growths consisting of a narrow to broad base with the mucosal fronds containing a fine fibrovascular core covered by a cuboidal to tall columnar epithelium of variable thicknesses (Table 3.1). Specimen SC01-0030 had a cutaneous periophthalmic papilloma characterized by undulating folds of hyperkeratotic proliferating epidermis. Specimens DP00-0078, SC02-0043, and SC03-0001 had no histologically detectable lesions.

Table 3.1

Tissues and sequences

Specimen # (PsHV/Genotype)	Species	Tissue Sequenced	Gene (partial) Sequenced	Genebank Accession #
DP00-0078 (PsHV-2)	*Psittacus erithacus*	Cloaca	UL 16 UL 30	AY623129 AY623121
SC00-0020 (PsHV-1/2)	*Amazona oratrix*	Cloacal Papilloma	UL 16 UL 30	AY421988 AY623125
SC01-0019 (Neg. Control)	*Pyrrhura rupicola*	Embryo		
SC01-0030 (PsHV-2)	*Psittacus erithacus*	Periophthalmic Papilloma	UL 16 UL 30	AY623131 AY623122
SC02-0022 (PsHV-1/1)	*Ara ararauna*	Cloacal Papilloma	UL 16 UL 30	AY421993 AY623126
SC02-0025 (PsHV-2)	*Psittacus erithacus*	Cloacal Papilloma	UL 16 UL 30	AY623132 AY623123
SC02-0043 (PsHV-1/4)	*Ara ararauna*	Cloaca	UL 16 UL 30	AY421995 AY623128
SC02-0047 (PsHV-1/3)	*Amazona aestiva*	Cloacal Papilloma	UL 16 UL 30	AY421988 AY623127
SC03-0001 (PsHV-2)	*Psittacus erithacus*	Cloaca	UL 16 UL 30	AY623130 AY623124
SC03-0005 (Neg. Control)	*Psittacus erithacus*	Blood		

PCR with PsHV specific primers amplifying regions of UL9, UL16/17, and UL30

Amplicons were produced from the four African grey parrot samples using PsHV primer sets 23F and ILK/IYG, but not with primer sets 11R or 39F (Table 3.2 and Table 3.3). PsHV DNA was amplified from the positive PsHV reference controls representing

Table 3.2

Primer sequences for PsHV and PePV

Gene	Primers	Sequence (5'→ 3')	AP[a]	Reference
PsHV:				
UL9[b]	11Rf8	AGTTGCTGAATCGGTACTGGTTCTC	537	Tomaszewski 2001
	11Rr4	GGATGGGAATTTGCACCTCCT		
UL16[c]	23Ff5a	TGCGTGGGGTTAAACTCGGAACTAGAAG	667	Tomaszewski 2003
	23Fr3	CGACTACACGAGCCTAACATC		
	23Ff5b	GGGGTTAAACTCGGAACTAGAAG	662	Tomaszewski 2003
	23Fr3	CGACTACACGAGCCTAACATC		
UL30[d]	ILK	TCCTGGACAAGCAGCARNYSGCNMTNAA	266	VanDevanter 1996
	IYG	CACAGAGTCCGTRTCNCCRTADAT		
	39Ff	AGCCTCTACCCGAGCATAATCCAG	634	Tomaszewski[e]
	39Fr	GTGAAGCTAGAGTGCGAGAAAGTGT		
PePV:				
L1[f]	ppv6621fmod	GGGCGGATATGACTTTCTGG	205	Styles 2004
	ppv6810r	GCAGTGCGCACGCCTG		

[a] Amplification product length in nucleotides
[b] Origin of binding protein
[c] Host-range protein
[d] DNA polymerase protein
[e] Tomaszewski unpublished work
[f] Major capsid protein

all four genotypes using 11R, 23F, 39F, and ILK/IYG primer sets (Table 3.3). Amplification products were not detected in the negative control samples from the incubator-hatched African grey parrot chick or *Pyrrhura* conure embryo. PePV DNA was not detected in any samples, but was amplified from the positive PePV clone control.

Table 3.3

PsHV amplification results for primer sets 11R, 23F, 39F, and ILK/IYG

Specimen # (PsHV/Genotype)	Species	Tissue Examined	Primer Set[a] 11R	23F	39F	ILK/IYG
DP00-0078 (PsHV-2)	*Psittacus erithacus*	Cloaca	-	+	-	+
SC00-0020 (PsHV-1/2)	*Amazona oratrix*	Cloacal Papilloma	+	+	+	+
SC01-0019 (Neg. control)	*Pyrrhura rupicola*	Embryo	-	-	-	-
SC01-0030 (PsHV-2)	*Psittacus erithacus*	Periopthalmic Papilloma	-	+	-	+
SC02-0022 (PsHV-1/1)	*Ara ararauna*	Cloacal Papilloma	+	+	+	+
SC02-0025 (PsHV-2)	*Psittacus erithacus*	Cloacal Papilloma	-	+	-	+
SC02-0043 (PsHV-1/4)	*Ara ararauna*	Cloaca	+	+	+	+
SC02-0047 (PsHV-1/3)	*Amazona aestiva*	Cloacal Papilloma	+	+	+	+
SC03-0001 (PsHV-2)	*Psittacus erithacus*	Cloaca	-	+	-	+
SC03-0005 (Neg. control)	*Psittacus erithacus*	Blood	-	-	-	-

[a] The results of primer sets were designated as "+" if an amplicon resulted and a "-" if no amplification.

Sequencing of PsHV amplicons from UL16/17 and UL30

Sequence data was generated for all samples from the UL16/17 amplicon using primer set 23Ff5b/23Fr3. UL30 sequence data for the PsHV reference samples was produced using primer set 39F. UL30 sequence data for the African grey parrot samples was generated using primer set ILK/IYG.

Phylogenetic analysis

An 88 amino acid sequence was translated from a 266 bp fragment amplified from UL30 of all samples and was compared to homologous sequence from four representative mammalian and five non-psittacine avian alphaherpesviruses (Figure 3.1). Only 57 amino acids (177 bp) of PaHV-1 UL 30 sequence were available, so the phylogram distance and location is approximate for this virus. A 139 amino acid sequence translated from a 419 bp fragment amplified from the UL16 gene was used to compare PsHV genotypes to the herpesvirus sequences obtained from the African grey parrots (Figure 3.2). Comparison of the UL16 ORF of other non-psittacid avian α-herpesviruses was not possible because GaHV-1 does not contain an UL16 ORF and this sequence has not been determined for PaHV-1.

Phylogenetic trees were generated using both nucleotides and amino acid sequences by performing a neighbor-joining search using bootstrap re-sampling (1,000 replications) employing distance optimality as previously described (Styles et al., 2004). Both nucleotide and amino acid sequences yielded similar phylograms, but only the amino acid sequence data is illustrated.

Discussion

This is the first report of a mucosal papilloma in an African grey parrot, bird SC02-0025. Papillomas from African grey parrots in previous reports have been cutaneous in character (Jacobson et al.; 1983; Latimer et al., 1997). The significance of this finding is that mucosal papillomas are found in psittacine species other than Neotropical parrots.

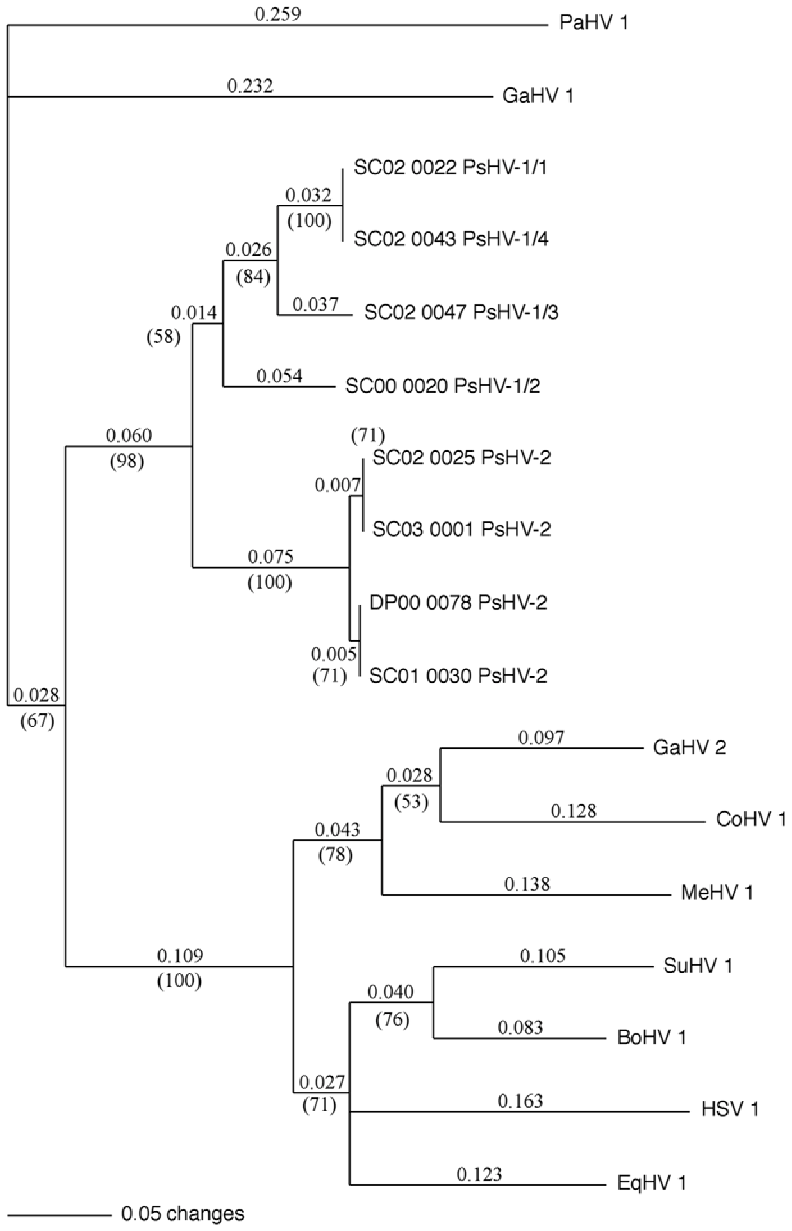

Fig. 3.1. Unrooted consensus phylogram of an 88 amino acid fragment (except PaHV-1 where only 57 amino acids were available) from the DNA polymerase gene (UL30) generated by bootstrap re-sampling (repetitions=1000), distance optimality, and neighbor-joining search using PAUP 4.0. Distance values are displayed on the branches and bootstrap values are in parentheses. All sequences beginning with "SC" or "DP" are psittacine birds. PsHV-1 samples are followed by a slash and the corresponding genotype. The African grey parrots are designated by PsHV-2. The remaining herpesvirus sequences are BoHV 1 = bovine; CoHV 1 = columbiform (pigeon); EqHV 1 = equine; GaHV 1 = gallid (chicken) 1 or infectious laryngotracheitis; GaHV 2 = gallid 1 or Marek's disease; HSV 1 = human herpes simplex 1; MeHV 1 = meleagrid (turkey); PaHV 1 = passerid (finch); and SuHV 1 = suid.

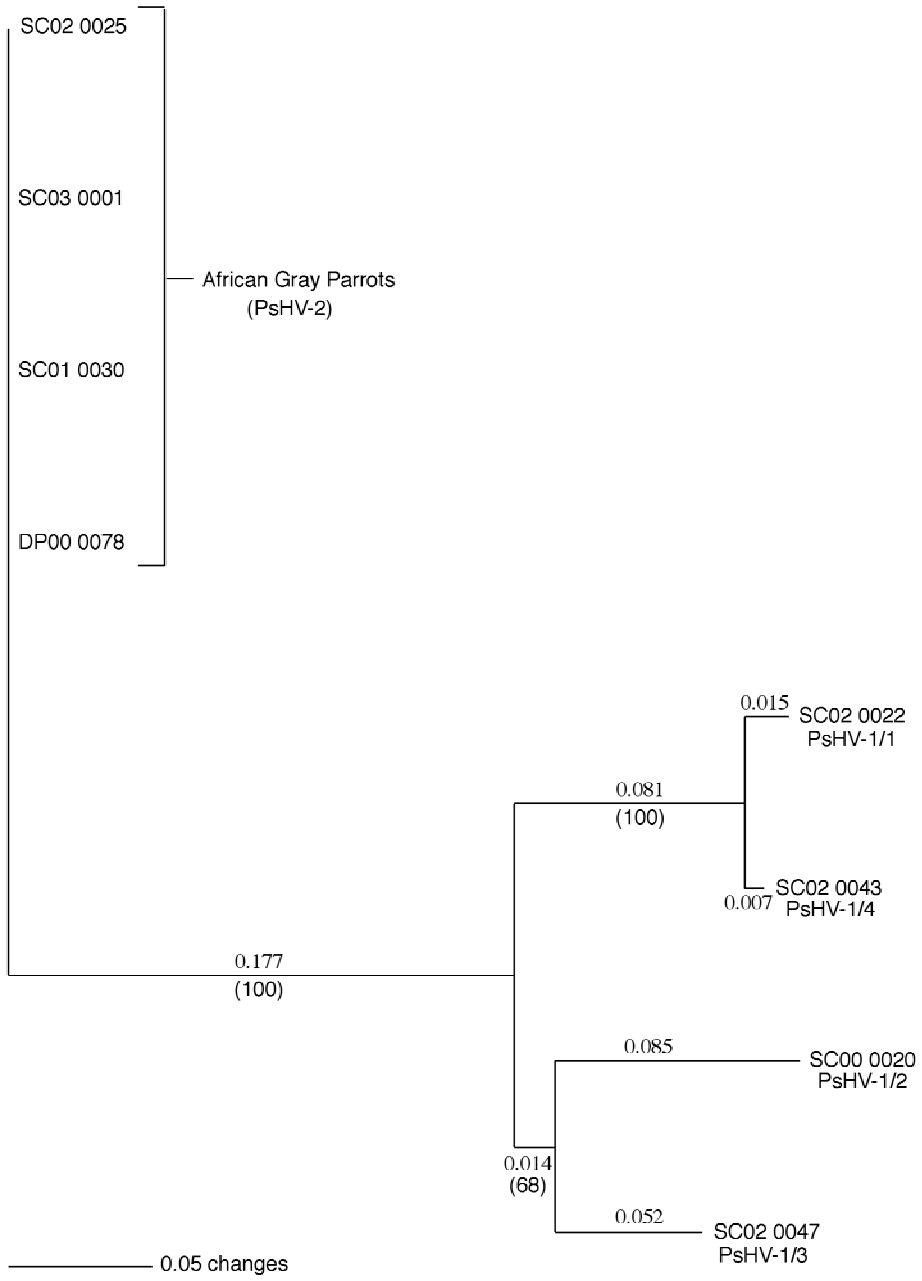

Fig. 3.2. Unrooted consensus phylogram of a 139 amino acid fragment (except SC02-0043 PsHV-1/4 which is 137 amino acids due to a two residue deletion) from the host range protein gene (UL16) of African grey parrots and four PsHV-1 reference sequences from Neotropical parrots generated by bootstrap re-sampling (repetitions=1000), distance optimality, and neighbor-joining search using PAUP 4.0. Distance values are displayed on the branches and bootstrap values are in parentheses. PsHV-1 samples are followed by a slash and the corresponding genotype. The African grey parrots are designated by PsHV-2.

Amplicons were produced from the African grey parrot samples using primer sets 23F and ILK/IYG, but not with primer sets 11R and 39F. Experiments by Tomaszewski et al. (2001) described an atypical amplification product using primer set 11R found in the DNA extracted from tissues of African grey parrots that died of Pacheco's disease. However, no such product was amplified from the African grey parrot samples in this study.

The UL16 products for the African grey parrot samples and the PsHV positive reference controls were sequenced with primer set 23Ff5b/23Fr3. However, obtaining the UL30 products for sequencing was more complicated. Primer set 39Ff/39Fr amplified the PsHV reference controls but not the African grey samples. Primer set ILK/IYG amplified both the PsHV positive reference controls and the African grey samples. However, while primer set ILK/IYG was successfully employed in sequencing the African grey parrot samples, it failed to produce suitable products for sequencing the PsHV reference samples despite the fact that appropriately sized products were visualized on the agarose gel. This failure may have been due to the fact that primer set ILK/IYG is degenerate and did not work equally well as a sequencing primer set with all PsHV reference genotypes, but was sufficient for amplification. Fortunately, the 266 bp (base pair) ILK/IYG sequence was nested within the 634bp product created by primer set 39F that did permit amplification and sequencing of the PsHV reference samples.

The phylogram generated using UL30 sequences showed that the herpesviruses from the four African grey parrots were virtually identical α4 herpesviruses and were most closely related to PsHV (Figure 3.1). This relationship was supported by analysis of the UL16 gene data (Figure 3.2). While only limited sequence data was available from

UL16 and UL30 genes, the phylogenetic analysis of both genes shows identical placement of the previously described PsHVs and the African grey sequences. This independent analysis supports the hypothesis that these two viruses are related but distinct. The sequences of the African grey viruses showed nucleotide diversity from the closest PsHV genotype (genotype 2) of 20% in UL30 to 23% in UL16. Therefore, in accordance with the guidelines of the International Committee on the Taxonomy of Viruses, it is proposed that these alphaherpesviruses found in the African grey parrots be designated as PsHV-2 and the PsHVs found primarily in Neotropical parrots as PsHV-1. It is understood that further study of the biological properties of PsHV-2 will be necessary in order to adequately characterize it as a unique psittacid herpesvirus. However, the phylogenetic analysis of the sequence data from the four African grey parrots in this study is sufficient to suggest the existence of this novel psittacid herpesvirus and its placement within the herpesvirus family tree.

The discovery of PsHV-2 in wild caught African grey parrots from three unique origins suggests that PsHV-2 may be adapted to this species and predictably could be the first indigenous herpesvirus described in psittacines from the African continent. However, all of the African grey parrots examined in this study were exposed to other psittacine species from multiple geographic locations around the world during the quarantine process and afterwards in the aviary. It is possible that they could have become infected with PsHV-2 some time after importation rather than being infected in the wild, but the birds came from different sources and were presumably imported at different times. Therefore, it is likely that the birds were infected in the wild rather than in captivity. Given the diversity of PsHV-1, it is possible that multiple genotypes of

PsHV-2 exist in other African parrot species, but verification will require screening of parrots in Africa.

It appears that PsHV-2 is a relatively nonpathogenic virus since it has not been detected in birds with Pacheco's disease. However, we detected PsHV-2 DNA in a mucosal and a cutaneous papilloma, suggesting that like PsHV-1 genotypes 1, 2, and 3; PsHV-2 may have the potential to induce papillomas. This is also the first report of a purely mucosal papilloma in an African grey parrot. Cutaneous papillomas in this species are rare and reported to be caused by PePV (O'Banion et al., 1992); but no PePV DNA was detected in this lesion. Therefore, if PsHV-2 has oncogenic potential, then infections are either uncommon or the virus is minimally oncogenic.

Infection by PsHV-2 may complicate the identification of PsHV-1 infected birds. This is because primer set 23F, which is used in PCR diagnostic testing, detects all known PsHV-1 genotypes and PsHV-2. Because PsHV-1 can cause Pacheco's disease in many species of psittacine birds including African grey parrots, infected parrots should be segregated from susceptible birds. However, infection with PsHV-2 may have less clinical significance, and differentiating these infections by the use of virus-specific PCR primers is indicated.

Psittaciforms probably evolved on the Gondwanaland super-continent and radiated outward with continental drift (Hedges et al., 1996; Cracraft, 2001). If indeed PsHV-2 is found in wild African parrots, then the close relationship between PsHV-1 and PsHV-2 could suggest that the ancestral predecessor of the PsHVs probably originated in Gondwanaland before the radiation of the parrots occurred rather than PsHV-2 arising *de novo* or migrating into African parrots from another avian species. Therefore, it is

possible that additional undiscovered herpesviruses exist in other psittacine species originating in Gondwanaland and Australian parrots are the likely candidates for screening.

Materials and Methods

Sample history and collection

Tissues from four African grey parrots (*Psittacus erithacus erithacus*) kept as pets or aviary specimens in the USA were examined in this study (DP00-0078, SC01-0030, SC02-0025, SC03-0001) (Table 3.1). Three of these birds were wild-caught (DP00-0078, SC02-0025, SC03-0001); the origin of the fourth bird was unknown (SC01-0030). Tissues examined included a biopsy from a parrot with a cloacal papilloma (SC02-0025); the histologically normal cloacal mucosa of this bird's cage-mate that died of peritonitis unrelated to PsHV (SC03-0001); a biopsy of a periophthalmic cutaneous papilloma (SC01-0030); and the histologically normal cloacal mucosa of a parrot that died of atherosclerosis (DP00-0078) (Table 3.1). Tissues were collected using cleaned, autoclaved instruments that had been bleached and formalin-soaked. A different set of instruments was used to collect each tissue to prevent DNA contamination. Tissues were either processed immediately or stored at -80°C until use.

Negative control DNA was extracted from the blood of an incubator-hatched African grey parrot chick (SC03-0005) and a near hatch psittacine embryo (SC01-0019). Four DNA samples representing all known PsHV genotypes were used as positive reference controls (SC02-0022 PsHV-1 genotype 1; SC00-0020 PsHV-1 genotype 2; SC02-0047 PsHV-1 genotype 3; SC02-0043 PsHV-1 genotype 4) (Tomaszewski et al., 2003) (Table

3.1). The positive control for PePV was a clone of *Psittacus erithacus* papillomavirus inserted into the *Sal I* site of pBR322 (Terai et al., 2002; Styles et al., 2004).

Histological evaluation of the tissue

Sections of the tissues were fixed in formalin, embedded in paraffin, and thin sections were routinely stained with hematoxylin and eosin. These sections were then microscopically examined for the presence of any lesions.

DNA extraction and polymerase chain reaction

DNA was extracted from the tissues using the Purgene® Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN.) following the instructions for fresh or frozen solid tissue. Samples were either processed immediately or stored at -80°C until use.

All PCR reactions were performed on an Eppendorf Mastercycler® Personal Thermocycler (Eppendorf, Hamburg, Germany). PCR screening for PsHV DNA was performed as previously described (Tomaszewski et al., 2001). Briefly, a 25 µl reaction mix containing, 100 ng genomic DNA, 25 pmol of each primer, 0.1 mM of each of the 4 deoxynucleotide triphosphates, 2.5 mM magnesium chloride, 0.75 units Taq polymerase and 1X buffer A was used in the reaction. The thermocycler parameters were: initial denaturation at 94°C for 5 minutes; followed by 40 cycles of annealing at 60°C for 45 seconds, extension at 72°C for 90 seconds, denaturing at 94°C for 30 seconds, and then a period of final extension at 72°C for 5 minutes. PCR amplification products were imaged on ethidium bromide agarose gels. The samples were examined for PsHV DNA

using primer sets 11R (11Rf8/11Rr4); 23F (23Ff5a, 23Ff5b/23Fr3); 39F (39Ff/39Fr) (Tomaszewski et al., 2001; Tomaszewski et al., 2003), and degenerate primer set ILK/IYG (VanDevanter et al., 1996) (Table 3.2).

PCR screening for PePV DNA was performed as previously described (Styles et al., 2004). Briefly, a 25 µl reaction mix containing, 100 ng genomic DNA, 25 pmol of each primer, 0.1 mM of each of the 4 deoxynucleotide triphosphates, 2.5 mM magnesium chloride, 0.75 units Taq polymerase and 1X buffer A (Promega, Madison, WI.) was used in the reaction. The thermocycler parameters were: initial denaturation at 95°C for 5 minutes; followed by 35 cycles of denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and then a period of final extension at 72°C for 10 minutes. Amplification products were imaged on an ethidium bromide agarose gel. Samples were examined for PePV DNA using primer set ppv6621fmod/ppv6810R (Terai et al., 2002; Styles et al., 2004) (Table 3.2).

PCR product purification and sequencing

Amplicons generated by the 23F and 39F primer sets were column purified using a QIAquick® PCR Purification Kit (Qiagen Inc., Valencia, Cal.). ILK/IYG products were extracted from a 1% agarose gel using a QIAquick® Gel Extaction Kit (Qiagen Inc., Valencia, CA.).

All sequencing reactions were performed using an ABI Prism® Big Dye™ Terminators v3.0 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA) employing sequencing primers 23Ff5b/23Fr3 for the UL16 product of all bird samples;

39Ff/39Fr (UL30) for the four PsHV reference control samples; and ILK/IYG (UL30) for the four African grey parrot samples (Table 3.2). Products were sequenced using an ABI 3100 DNA sequencer (Applied Biosystems).

Phylogenetic analysis

Both nucleotide and amino acid sequences were aligned using Clustal X 1.81 (Thompson et al., 1997). Aligned sequences were imported into PAUP for analysis (Swofford, 1998). Phylogenetic trees were generated by performing a neighbor-joining search using bootstrap re-sampling (1,000 replications) employing distance optimality. Phylograms showing branch-lengths and bootstrap values for UL30 and UL16 are illustrated in Figures 3.1 and 3.2 respectively.

CHAPTER IV

INTERNAL PAPILLOMATOUS DISEASE AND OTHER TUMORS ASSOCIATED WITH PSITTACID HERPESVIRUS INFECTIONS IN PARROTS

Introduction

Internal papillomatous disease (IPD) affects predominately captive Neotropical parrots. The greatest prevalence has been reported in macaws (*Ara* spp.), Amazon parrots (*Amazona* spp.), conures (*Aratinga* spp.), and hawk-headed parrots (*Deroptyus accipitrinus*) (Graham, 1991; Gibbons et al., 2002; Hillyer et al., 1991; Phalen et al., 1997; Van der Heyden, 1988). IPD is characterized by the presence of mucosal papillomas primarily affecting the epithelium of the oral cavity and cloaca, but also observed in the esophagus, crop, proventriculus, ventriculus, conjunctiva, nasolacrimal duct, and bursa (Graham, 1991). Grossly, mucosal papillomas are pink to grey-tan verrucous lesions (Graham, 1991; Phalen et al., 1997). Histologically, they appear as papillary structures having a highly vascularized connective tissue core with overlying proliferative stratified cuboidal to pseudostratified columnar mucosal epithelium depending on the location, with lymphoplasmacytic infiltrate as a variable feature of the lesions (Phalen et al., 1997; Schmidt et al., 2003). These papillomas will wax and wane (Phalen et al., 1997) and the presence of the lymphoplasmacytic infiltrates may be related to this behavior.

The clinical manifestations of IPD depend on the species of parrot, stage of the disease and the lesions. Mucosal papillomas (MP) are found most frequently in the

cloaca of Amazon parrots and the oral cavity, upper digestive tract, and cloaca of macaws and conures (Phalen et al., 1997; Phalen et al., 1998). The lesions in macaws and conures are more likely to be progressive and less likely to display the waxing and waning behavior (Phalen et al., 1997). While mucosal papillomas are benign, they can result in clinical complications where they ulcerate and hemorrhage, obstruct the respiratory tract (choana and glottis), upper digestive tract (esophagus and crop), or lower digestive tract (proventriculus, ventriculus, and cloaca) (Graham, 1991; McDonald, 1988; Phalen et al., 1997). Birds with chronic mucosal papillomatosis were observed to develop other tumors including adenomas and carcinomas of the bile and pancreatic ducts (Graham, 1991; Elangbam and Panciera, 1988; Gibbons et al., 2002; Hillyer et al., 1991; Phalen et al., 1997; Potter et al., 1983). Clinically, these bile and pancreatic neoplasms result in parenchymal destruction or space occupying lesions, but metastasize infrequently (Schmidt et al., 2003).

IPD and associated tumors can be found in a wide age-range of parrots. They are normally found in adult birds but one macaw was observed to develop a cloacal papilloma within 6 months of its hatch-date, and within 1.5 years this bird had developed bile duct carcinoma and died at the age of two from the disease (Styles, 2004).

The prominent feature of IPD is the development of mucosal papillomas. Because papillomas in other animals were known to be caused by papillomaviruses, it seemed likely that a papillomavirus might be associated with the development of IPD. Cutaneous and mucosal papillomas in mammals (Brentjens et al., 2002) and cutaneous papillomas in finches (Moreno-Lopez et al., 1984) and African grey parrots (O'Banion et al., 1992) were shown to be caused by species-specific papillomaviruses. However,

there was little evidence supporting papillomaviruses as the cause of IPD in parrots. Efforts to detect papillomavirus group-specific antigens were largely unsuccessful (Sundberg et al., 1986) and papillomavirus DNA could not be found in mucosal papillomas (Johne et al., 2002; Latimer et al., 1997; Sundberg et al., 1986). However, there is one report of papillomavirus L1 protein-like immunoreactivity in the epithelial cells of the cloacal papillomas from two parrots, but further investigation provided evidence that the observed staining was non-specific (Bonda et al., 1998; Styles et al., 2004). While no papillomaviruses could be found in the IPD lesions, mucosal papillomas and associated tumors appeared to have an infectious etiology because unaffected birds developed mucosal papillomas after being exposed to birds with IPD (Graham, 1991; McDonald, 1988; Phalen et al., 1997; Van der Heyden, 1988). Therefore, in an effort to differentiate those papillomas known to be caused by papillomaviruses from the mucosal papillomas and associated tumors of Neotropical parrots, Graham (1991) introduced the term internal papillomatous disease to make this distinction. Due to the paucity of evidence associating papillomaviruses with IPD, investigations were conducted to identify other potential causes.

Studies suggested that these IPD might be associated with infection by one or more genotypes of the alphaherpesvirus, psittacid herpesvirus 1 (PsHV 1). In one investigation, 18/18 (100%) parrots with IPD were seropositive for anti-PsHV antibody while 11 clinically normal parrots were seronegative (Phalen et al., 1997). Two studies reported finding PsHV DNA in the mucosal papillomas from a small group of parrots (Phalen et al., 1998; Phalen et al., 1999), and from the papillomas of 9/12 (75%) parrots in another study (Johne et al., 2002).

Herpesviruses are known to cause neoplasia in some animal species including the development of papillomas. Unclassified herpesviruses have been associated with the development of cutaneous fibropapillomas in rainbow smelt (*Osmerus mordax*) (Herman et al., 1997), koi (*Cyprinus carpio*) (Calle et al., 1999), marine sea turtles (Quackenbush et al., 1998; Quackenbush et al., 2001), macaws (*Ara* spp.) (Lowenstine, 1982; Phalen, 1997), and cockatoos (*Cacatua* spp.) (Lowenstine, 1982; Lowenstine et al., 1983; Phalen, 1997). Herpesviruses have also been associated with the development of other types of tumors. Adenocarcinomas of frogs (*Rana* spp.) were found to be caused by an unclassified herpesvirus (Epstein, 2001). B-cell lymphomas (Burkitt's lymphoma), nasopharyngeal carcinomas, and gastric carcinomas affecting humans were associated with the gammaherpesvirus Epstein-Barr virus (EBV or human herpesvirus 4) (Epstein, 2001; Takada, 2000). Vascular endotheliosarcomas (Kaposi's sarcoma) were caused by the gammaherpesvirus Kaposi's sarcoma-associated herpesvirus (KSHV or human herpesvirus-8) (Boshoff et al., 1995; Damania, 2004). However, only one herpesvirus was known to cause tumors in avian species which is Marek's disease, that causes T-cell lymphomas to develop gallinaceous birds caused by the alphaherpesvirus Marek's disease herpesvirus (MDHV or gallid herpesvirus-2 (GaHV 2)) (Venugopal, 2000; Lupiani et al., 2004).

A recent investigation into the cause of mucosal papillomas detected PsHV 1 DNA in the mucosal papillomas of 30/30 (100%) Neotropical parrots, but was not detected in the histologically normal tissue from the same bird (Styles et al., 2004). Given this evidence and the previous work of Phalen et al. (1997, 1998, 1999), it seemed likely that the bile

and pancreatic duct carcinomas associated with IPD were similarly associated with PsHV 1.

Other tumors involving the mucosal epithelium of Neotropical parrots have also been described including proventricular and ventricular carcinomas, and cloacal carcinomas (Schmidt et al., 2003). During the investigation of mucosal papillomas, PsHV DNA was detected in a cloacal carcinoma from a bird that no other lesions (Styles, 2004). Given the association of PsHV with mucosal papillomas, it was also possible that these mucosal tumors could be similarly associated with the virus. Therefore, the hypothesis tested in this study was that the bile and pancreatic tumors associated with IPD, and the endoventricular and cloacal carcinomas found in Neotropical parrots are caused by one or more genotypes of PsHV 1.

Several methods were employed to test this hypothesis. Conventional PCR using PsHV 1 specific primers was used to show that PsHV DNA was found in the tumors from affected birds using both fresh and formalin-fixed paraffin-embedded (FFPE) tissue. To determine if any PsHV DNA detected in the tissue was localized to the tumors, experiments were performed to show that PsHV DNA was in significantly higher concentration in the tumors compared to the adjacent histologically normal tissue. These efforts included examining the DNA from tumors and adjacent histologically normal tissue collected by laser capture microdissection (LCM) with conventional PCR and PsHV 1 specific probes, and using in situ hybridization with PsHV 1 biotinylated probes or direct in situ PCR using PsHV 1 specific primers.

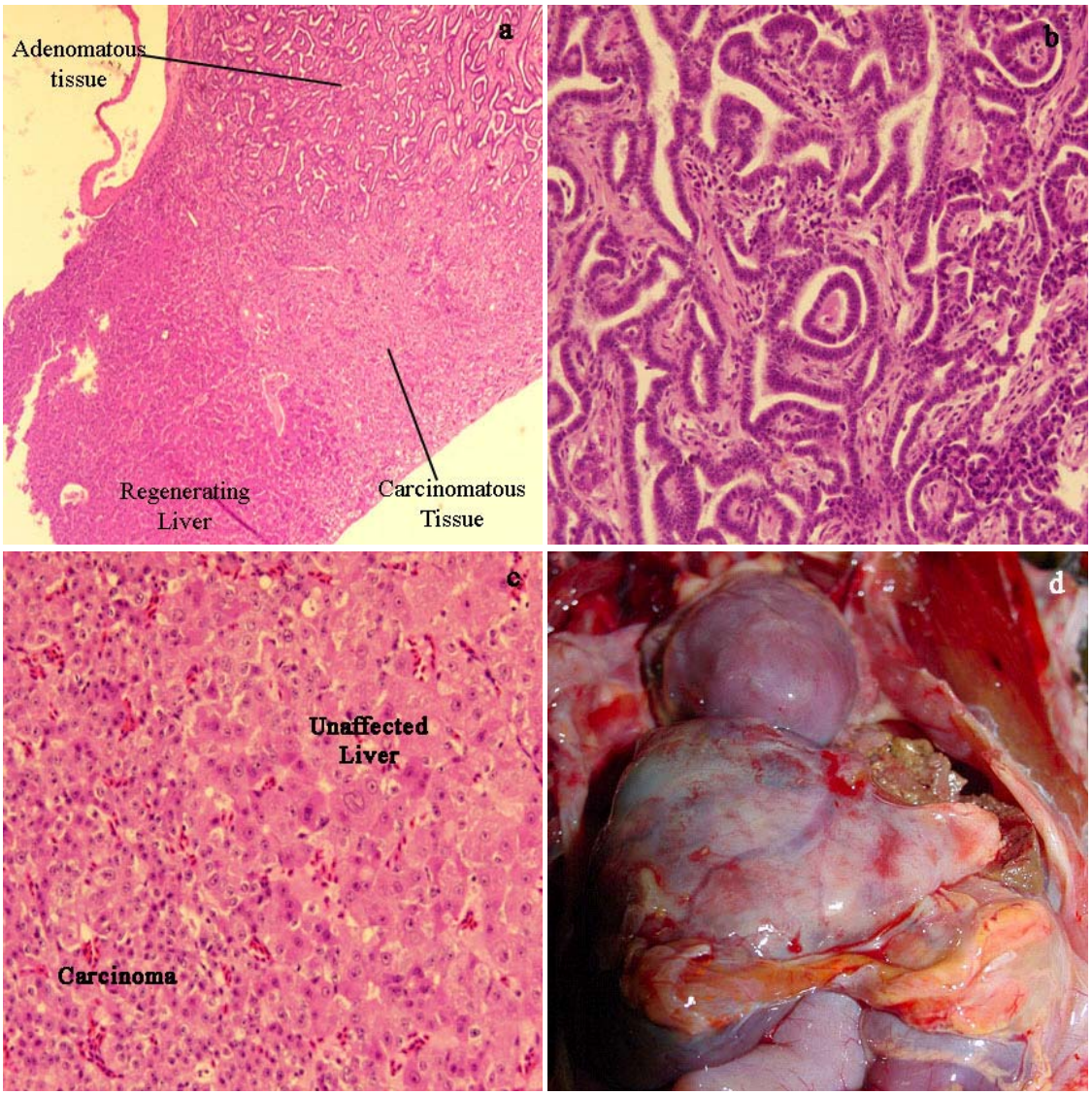

Fig. 4.1. Gross and microscopic views of the spectrum of change in two parrots with bile duct carcinoma. (a) 10x magnification of the liver showing adenomatous ducts blending into the carcinomatous tissue accompanied by a nodule of liver regeneration (SC96-0604), (b) 40 x magnification of the adenomatous ducts (SC96-0604), (c) 40 x magnification of the carcinomatous tissue showing highly undifferentiated cells with some attempts to form a glandular architecture (SC96-0604), and (d) gross view of a carcinomatous liver from bird (SC04-0012).

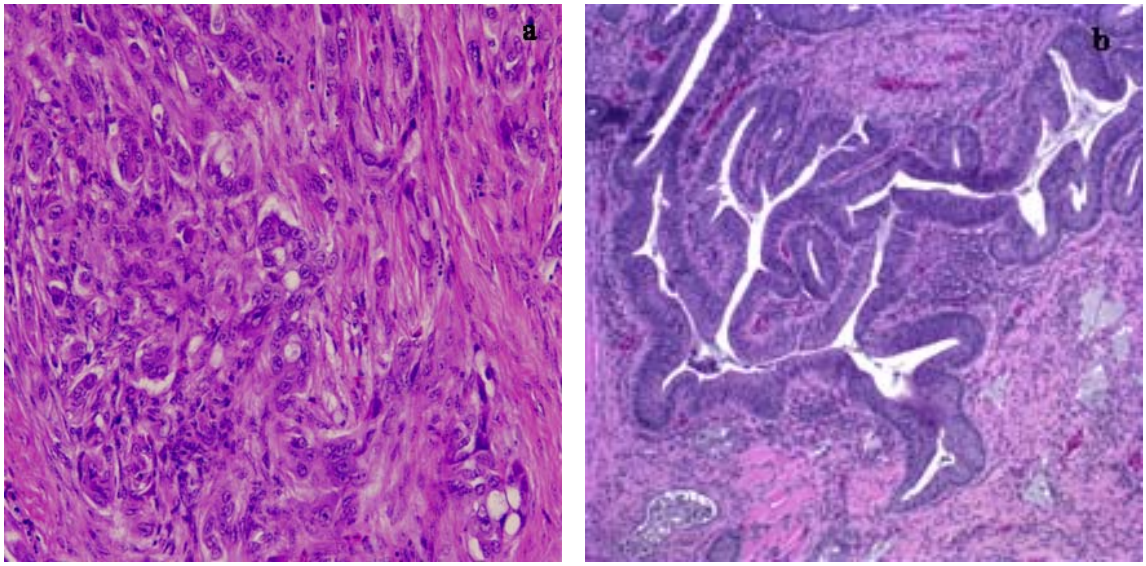

Fig. 4.2. Microscopic views of an endoventricular and cloacal carcinoma. (a) Endoventriculuar carcinoma from sample SC96-0604 showing highly undifferentiated cells with some attempts to form a ductular architecture. Goblet cells producing mucin can also be seen. (b) Cloacal carcinoma from sample SC04-0010 showing aberrant ducts, fibroplasia, and mucin lakes.

In this investigation, a single PsHV 1 genotype was found in all the affected tissue examined except one bird. Efforts to localize the virus to the affected tissue were inconclusive.

Results

Histological evaluation of tissues

Adenomatous and/or carcinomatous neoplasms were observed in the liver (Fig 4.1 a-d) and pancreas. Fourteen of the birds had bile duct neoplasms and three of these birds also had pancreatic tumors (Tables 4.1 and 4.2). All birds with bile or pancreatic duct

Table 4.1

Fresh IPD tissues, samples and sequence results

Isolate Number	GenBank Accession#	Species Examined	Tissues	PCR[1] Result
SC92-0496	AY421976	*Ara macao*	cloacal papilloma	+
			bile duct carcinoma	+
SC92-0498	AY421977	*Ara macao*	cloacal papilloma	+
			crop (normal)	-
			bile duct carcinoma	+
SC92-1409	AY421978	*Amazona auropalliata*	glottal papilloma	+
			bile duct carcinoma	+
SC93-0192	AY421980	*Ara ararauna*	choanal papilloma	+
			bile duct carcinoma	+
SC94-0390	AY421983	*Aratinga spp.*	glottal papilloma	+
			bile duct carcinoma	+
SC00-0020	AY421988 (+) Control	*Amazona oratrix*	Genotype 2	+
SC00-0077	AY421989	*Amazona oratrix*	cloacal papilloma	+
			crop (normal)	-
			bile duct adenoma	+
SC01-0019	(-) Control	*Pyrrhura rupicola*	embryo	-
DP02-0034	AY421991	*Ara chloroptera*	cloacal papilloma	+
			crop (normal)	-
			bile duct carcinoma	+
SC02-0022	AY421993 (+) Control	*Ara ararauna*	Genotype 1	+
SC02-0024	Sample	*Ara ararauna*	cloacal carcinoma	+
SC02-0043	AY421995 (+) Control	*Ara ararauna*	Genotype 4	+
SC02-0047	AY421999 (+) Control	*Amazona aestiva*	Genotype 3	+
SC03-0005	(-) Control	*Psittacus erithacus*	blood	-
DP04-0006	Sample	*Eclectus roratus*	cloacal papilloma	+
			crop (normal)	-
			bile duct adenoma	+
			proventriculus (normal)	-
SC04-0010	Sample	*Cyanoliseus patagonus*	glottal papilloma	+
			cloacal carcinoma	+
			proventriculus (normal)	-
SC04-0012	Sample	*Ara severa*	bile duct carcinoma	+
			choanal papilloma	+
			cloacal papilloma	+
			crop (normal)	-
			pancreatic adenoma	+
			proventriculus (normal)	-

[1] PCR Result: amplicon produced = (+), no amplicon produced = (-). Samples accompanied by a GenBank submission number were also used in Chapter II (Styles et. al., 2004).

Table 4.2

PCR results of formalin-fixed paraffin-embedded tissues

Sample Number[1]	GenBank Access#/Use[2]	PsHV Geno[3]	Species Examined	Tissue Examined	PCR[4] +/-
SC91-1299	Sample	3	*Cacatua moluccanus*	bile duct adenoma	+
				brain	+/-
				cloacal papilloma	+
				pancreatic adenoma	-
SC92-0058	(-) Control		*Cacatua alba*	liver	-
SC92-0573	(+) Control Pachecos DZ	Unk		liver	+
SC93-0780	(+) Control Pachecos DZ	Unk	*Psittaucs erithacus*	liver	+
SC95-0028	Sample	3	*Amazona oratrix*	bile duct adenoma	+
				brain	+/-
				crop papilloma	+
				lung	+/-
				pancreatic adenoma	+
SC96-0604	Sample	3	*Amazona ochrocephla (history of papillomas)*	bile duct adenoma	+
				brain	+
				lung	+
				proventricular carcinoma	+
				skeletal muscle metastasis	+
SC96-0622	Sample	3	*Amazona ochrocephla*	bile duct adenoma	+
				lung	-
				ventricular carcinoma	+
SC97-0365	Sample	3	*Amazona oratrix*	bile duct adenoma	+
				brain	-
				cloacal papilloma	+
				endoventric. carcinoma	+
				lung	-
SC00-0020	AY421988	2	*Amazona oratrix*	cloacal papilloma	+
SC01-0019	(-) Control		*Pyrrhura rupicola*	embryo	-
SC02-0022	AY421993	1	*Ara ararauna*	cloacal papilloma	+
SC02-0043	AY421995	4	*Ara ararauna*	cloaca	+
SC02-0047	AY421999	3	*Amazona aestiva*	cloacal papilloma	+

[1] Samples were fixed in formalin for less than 72 hours and then paraffin-embedded except samples SC01-0030, SC00-0020, SC02-0022, SC02-0043 and SC02-0047 that were collected as fresh tissues and used as representatives of each PsHV genotype to develop the internal primer sets for the UL16/17 region.

[2] Tissues with a GeneBank Accession # were sequenced as fresh tissues using primer set 23Ff5b/23Fr3 and FFPE tissues with primer set 23Ff5a/23Fr+114D. "Unk" are unknown PsHV genotypes.

[3] All samples were PsHV 1 geontypes. "Unk" designates an unknown PsHV 1 genotype (not sequenced).

[4] PCR result given as (+) for successful amplification and (-) if no amplification

neoplasia had mucosal papillomas present with the exception of one sample that had a history of papillomas. Two birds had cloacal carcinomas and three birds had carcinomas of the proventriculus or ventriculus (Fig 4.2 a-b; Table 4.2). Of the two birds with cloacal tumors, one bird was accompanied by a mucosal papilloma and the other had not papillomas. Of the three bird with proventricular or ventricular carcinomas, all three birds were accompanied by bile duct tumors, one bird exhibited a papilloma, one had a history of papillomas, and one had no overt or history of papillomas (Table 4.2).

PCR of fresh IPD tissues with PsHV-specific primers

The purpose of this experiment was to determine if PsHV 1 DNA could be detected in the tumors and not in histologically normal mucosal tissue by conventional PCR using PsHV 1 specific probes. Fresh tissues were available from 11 parrots with mucosal tumors and DNA was extracted from neoplastic and histologically normal tissue (Table 4.1). Primer sets used to amplify any PsHV 1 DNA found in the tissues produced a 667 bp amplicon from the UL16/UL17 region of the genome (Table 4.3).

PsHV 1 DNA was consistently amplified from DNA purified from the positive reference controls that were infected with PsHV of known genotype. There was no PsHV DNA amplified from the negative control tissues of a near hatch conure embryo or the blood from an incubator-hatched African grey parrot (Table 4.1).

PsHV 1 DNA was amplified from all tumors. PsHV 1 DNA was not detected in any of the adjacent unaffected tissue or histologically normal mucosal tissue available in six of the birds.

Table 4.3

Primer sequences for PsHV and cytochrome oxidase I

Gene	Primers	Sequence (5'→ 3')	AP[1]	Reference
Psittacine ctyochrome oxidase I protein				
COI	H7523	ACTGTGAATATGTGGTGGGC	207	Eberhard 2004
	COIf	CCTGCAGGAGGAGGAGAYCC		
UL9: Origin of binding protein				
	11Rf8	AGTTGCTGAATCGGTACTGGTTCTC	537	Tomaszewski 2001
	11Rr4	GGATGGGAATTTGCACCTCCT		
PsHV 1 UL16/17: Host range protein/DNA cleaving-packaging protein				
	23Ff5a	TGCGTGGGGTTAAACTCGGAACTAGAAG	667	Tomaszewski 2003
	23Fr3	CGACTACACGAGCCTAACATC		
	23Ff5b	GGGGTTAAACTCGGAACTAGAAG	662	Tomaszewski 2003
	23Fr3	CGACTACACGAGCCTAACATC		
	23Ff-131D	GAGACCCTYGACGAAGTGTGG	194	Styles[2]
	23Fr+63	GTTGACGTCGTTGAGAAGTCG		
	23Ff5a	TGCGTGGGGTTAAACTCGGAACTAGAAG	320	Tomaszewski 2003
	23 Fr+114D	CTTGGCBGTMAGGATGGTC		Styles[2]
	23Ff+43	GACTTCTCAACGACGTCAACG	223	Styles[2]
	23Fr+266D	GCMGTTATGTGAACGTTGC		
PsHV 1 UL30: DNA polymerase protein				
	39Ff	AGCCTCTACCCGAGCATAATCCAG	634	Tomaszewski[2]
	39Fr	GTGAAGCTAGAGTGCGAGAAAGTGT		
	39FfRad	ATGAACGGCATGCTGC	197	Tomaszewski[2]
	39Fr2	GAGTCGGTGTCCCCGTAGAT		

[1] Amplification product length in nucleotides

[2] Unpublished work

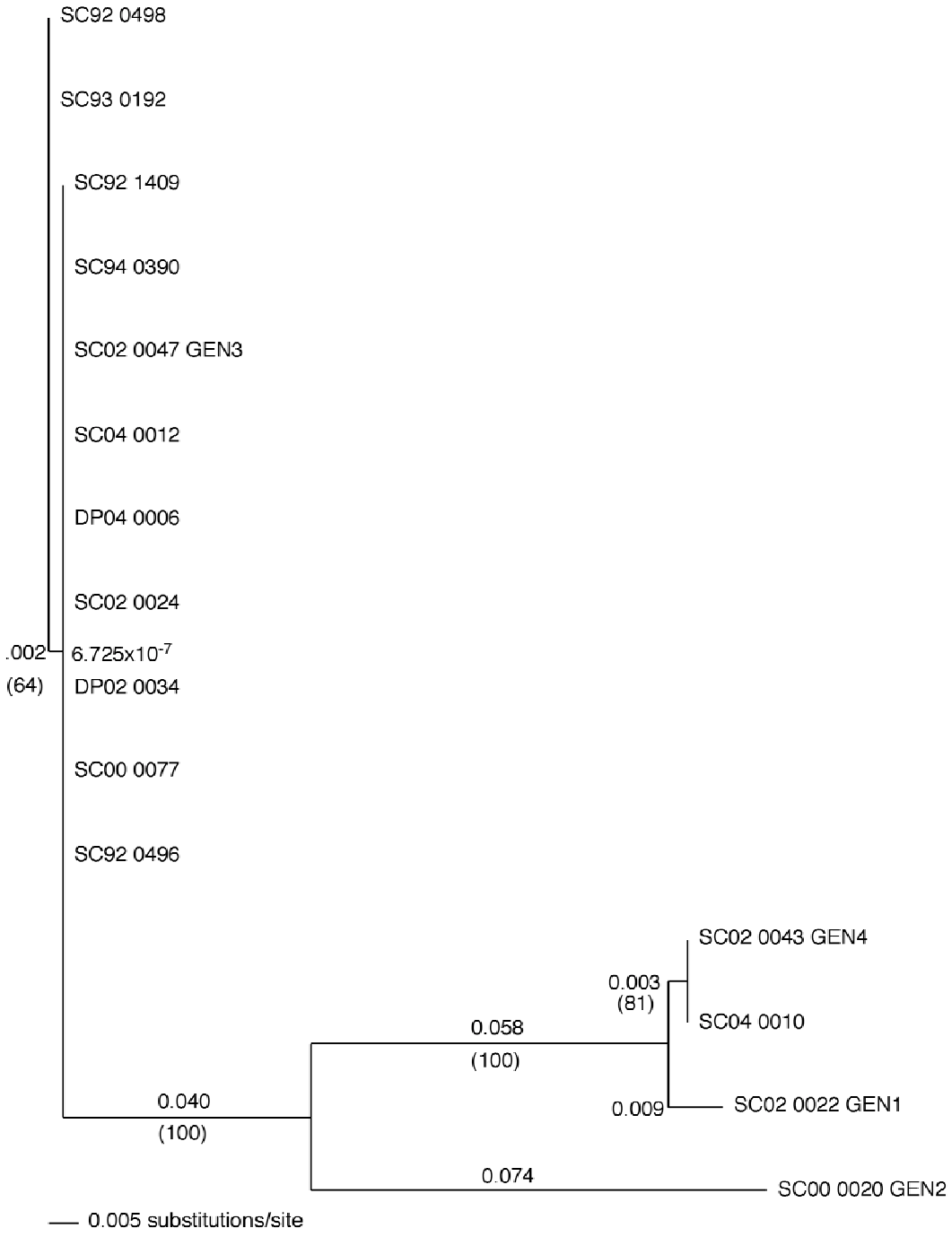

Fig. 4.3. Consensus phylogenetic tree of a set of 419 bp PsHV sequences generated by bootstrap re-sampling (1000 repetitions), distance optimality, and neighbor-joining search using PAUP 4.0. Branch lengths are provided and bootstrap values are displayed in parentheses. Sequences preceded by "SC" or "DP" denote samples exhibiting lesions of IPD (Table 1). Sequences followed by the suffix "GEN X" were reference sequences of known genotype where X=genotype.

Phylogenetic analysis of PsHV DNA sequences from the fresh IPD tissues

A 419 bp fragment was parsed from the 667 bp PsHV amplicon produced by the 23F primers as described by Tomaszewski et al. (2003) for phylogenetic comparison. This fragment began at the start codon of the PsHV 1 UL16 (host range protein) gene and was compared to analogous sequences from PsHVs of known genotype. A phylogram with representatives of each genotype showing branch-lengths and bootstrap values was generated as described by Tomaszewski et al. (2003) (Fig. 4.3). All tumor sample sequences were determined to be PsHV 1 genotype 3 except one sample that was PsHV 1 genotype 4 (SC04-0010).

PCR of formalin-fixed paraffin-embedded IPD tissues with PsHV specific primers

Because fresh samples of birds with IPD and associated tumors are difficult to obtain in a timely fashion, an attempt was made to expand the data set using archived FFPE histopathology samples of five IPD cases from the Schubot Exotic Bird Health Center (Texas A&M University, College Station, TX). DNA was extracted from the tissues of five parrots with bile duct tumors and examined by conventional PCR using primers derived from a 662 bp sequence spanning a section of the PsHV 1 UL16/UL17 genes that produced amplicons ranging from 194-320 bp in length (Table 4.3). Primers were designed to produce smaller amplicons to overcome any DNA degradation due to the fixation process. The UL16/UL17 region was chosen so that sequence comparison could be made to homologous PsHV 1 sequences of known genotype as described by Tomaszewski et al. (2003).

Three sets of primers were tested to determine which sets consistently amplified the greatest number of formalin-fixed samples. These primer sets were tested to determine which was superior at detecting PsHV in the tissue (Table 4.3). Some of the individual FFPE tissues had no amplification results with all of the three primer sets presumably because of damage to the DNA. Most notably the pancreatic tumors from the FFPE tissues gave no amplification results with two of the primer set but did with primer set 23Ff5a/23Fr+114D. DNA damage in the pancreas may be compounded due to release of autolytic enzymes at the time of death followed by damage due to fixation. While some samples were amplified by all primer sets, most produced detectable products from primer set 23Ff5a/23Fr+114D. Therefore, primer set 23Ff5a/23Fr+114D was used to probe the samples and primer set 23Ff5b/23Fr+114D was used to sequence any amplicons (Fig. 4.4).

PsHV 1 DNA was detected in the positive control tissues, but not from the negative controls. PsHV 1 DNA was amplified from all tumors with the exception of one pancreatic adenoma (SC91-1299) (Table 4.2). This negative result was most likely attributed to pancreatic enzymes degrading the DNA post-mortem. The intensity of the PCR signal acquired from each sample was used as an indirect measure of whether the sample was positive or possibly contaminated. Strong signals were interpreted as true positives (i.e. the signal was gave similar intensity to the known positive control). Results from samples giving weak signals were less clear and possible interpretations of this result are described below.

One concern with using archived specimens is that they are usually not collected using precautions to prevent DNA contamination from tissues containing high

Fig 4.4. A 1% agarose gel showing PCR results using primer set 23Ff5a/23Fr+114D with tissues of three different samples (Table 4.2 and Table 4.3). The product is 320bp in size.

concentrations of virus to other tissues. For example, mucosal papillomas were shown to have detectable quantities of PsHV DNA while adjacent histologically normal mucosa from the same organ was not (Styles et al., 2004). Contamination could be a problem when using conventional PCR to detect PsHV DNA with FFPE tissues, but if contamination is present it may only be superficial and possibly below the limit of detection of the primer set used in this experiment which was determined to be about 10^3 copies of virus/µl (Styles et al., 2004). In an attempt to determine if DNA contamination had occurred with the FFPE tissues, the brain and lung were used as negative internal controls because they might be expected to contain a concentration of PsHV DNA below

the level of detection of the primer sets (Styles, 2004). PsHV DNA was not detected in the brain or lung of sample SC97-0365 or the lung of SC96-0622, but was consistently detected in the brain and lung of sample SC96-0604 (Table 4.2). The brain of sample SC91-1299 and the brain and lung of sample SC95-0028 varied between positive and negative PCR results depending on the specific DNA extraction (multiple extractions were performed in an attempt to eliminate the possibility of experimental contamination) and the individual primer sets used to amplify the DNA. This result suggests several possibilities: that contamination may have been a factor in some samples, variable concentrations of PsHV DNA found in these samples were at or below the limit of detection of the primers and the result depended on the concentration of virus in a particular extraction, or these tissues may have been positive. However, analogous tissues from fresh samples of birds with IPD were consistently negative (Styles, 2004; Styles, unpublished data).

Phylogenetic analysis of PsHV DNA sequences from the FFPE-IPD tissues

The sequence of a 238 bp fragment spanning the PsHV 1 genes UL16/UL17 was derived from the amplicon produced by primer set 23Ff5a/23Fr+114D and subjected to phylogenetic analysis using analogous PsHV 1 sequences of known genotype (data not shown) (Styles et al., 2004). All samples were determined to be PsHV 1 genotype 3.

PCR of tissue collected by laser capture microdissection

One important aspect of this investigation was to show that the PsHV viral concentrations found in the neoplastic tissue were significantly higher than that found in

histologically normal adjacent or distant mucosal tissue. The FFPE tissues from four parrots were affixed to glass slides, visualized microscopically, and the samples of neoplastic and adjacent histologically normal tissue were collected using LCM (Table 4.4). LCM is a microsurgical technique using a precision cutting laser that can excise minute sections of tissue and collect it on a plastic cap.

DNA was extracted from the three samples and controls collected by LCM using the Pico Pure ™ DNA Extraction Kit and then either used directly as recommended by the kit or further purified in an attempt to improve the quality of the sample with a Purgene® Genomic DNA Purification Kit. The signal integrity of the psittacine cytochrome oxidase I housekeeping gene was diminished by further purification with the Purgene® kit so only the Pico Pure ™ kit was used. The DNA was then examined by PCR using the same three primer sets described above in the section of PCR with FFPE tissues (Table 4.2 and Table 4.3).

The primer sets failed to produce amplicons from positive control (SC93-0780) or tumor samples, even though these tissues had been shown to contain PsHV DNA by PCR of their FFPE sections (Table 4.4). However, amplicons were produced with the Neotropical psittacine cytochrome oxidase I internal control primers (Eberhard and Bermingham, 2004) from the unaffected pancreas, pancreatic duct adenoma, and choanal papilloma of parrot SC04-0012, but not from the unaffected liver, bile duct carcinoma, or cloacal papilloma. This result suggests that multiple factors such as length of fixation prior to embedding, formalin permeability differences in tissues, and the thicknesses to which tissues are cut may affect the DNA integrity and ability to detect DNA in FFPE tissues.

Table 4.4

Tissues examined using LCM

Sample Number[1]	Genebank Accession# or Use	PsHV Geno[2]	Species Examined	Tissue Examined
SC93-0780	(+) Control	Unk	*Psittacus erithacus*	liver (Pacheco's disease)
SC96-0622	Sample	3	*Amazona ochrcephala*	bile duct adenoma liver (unaffected) ventricular carcinoma
SC00-0077	AY421989/Sample	3	*Amazona oratrix*	bile duct adenoma
SC01-0019	(-) Control	N/A	*Pyrrhura rupicola*	embryo
DP04-0006	Sample	3	*Eclectus roratus*	bile duct adenoma liver (unaffected)
SC04-0012	Sample	3	*Ara severa*	bile duct carcinoma choanal papilloma cloacal papilloma liver (unaffected) pancreas (unaffected) pancreatic adenoma

[1] Samples were collected as fresh tissues and then formalin fixed for less than 72 hours and then paraffin embedded except SC93-0780 and SC96-0622 that were submitted as FFPE tissues. Samples SC93-0780 and SC96-0622 were positive for PsHV DNA by PCR from digestion of tissue taken from the paraffin block. All other samples were positive on PCR using DNA extracts of fresh tissue.

[2] All samples were PsHV 1 types. Samples SC98-0019, SC00-0077, DP04-0006, and SC04-0012 were sequenced and genotyped using primer set 23Ff5b/23Fr3; sample SC96-0622 was sequenced and genotyped using primer set 23Ff5a/23Fr+114D. "Unk" designates an unknown PsHV 1 genotype (not sequenced)

N/A = not appilicable

Tissues from bird SC04-0012 were also fixed with paraformaldehyde. Paraformaldehyde has been reported to demonstrate a higher detection rate of DNA from fixed samples in some applications, but not necessarily for PCR (Schmid et al., 1991; Greer et al., 1991). However, paraformaldehyde-fixed tissues were deemed unsuitable for LCM or any subsequent use of microscopically sectioned tissue due to poor fixation and loss of tissue architecture. Therefore, due to the inability to amplify PsHV DNA from FFPE tissues, LCM was determined not to be an effective method for showing

differences in concentration of PsHV DNA in neoplastic and histologically normal FFPE tissues.

In situ hybridization

In situ hybridization (ISH) was also used to determine relative differences in PsHV concentration in neoplastic and normal tissue. Biotinylated DNA probes were generated with either random primers or specific primers. Biotinylated DNA probes 50-300 bp in length were generated using random primers with whole PsHV 1 genotype 1 and 4 virus, and using both random primers and specific primers with sections of four different PsHV 1 genotype 3 genes, UL9, UL16/17, and UL30 gene (Tomaszewski et al., 2001; Tomaszewski et al., 2003). The PsHV positive control sample SC92-0142 was the crop of a bird that died of Pacheco's disease exhibiting PsHV intranuclear inclusion bodies in the crop mucosal epithelium (Table 4.5). This positive control sample was used to screen the efficiency of the different biotinylated probes to detect the PsHV. A signal amplification system was used to enhance the sensitivity of the probes and crimson stain was produced.

PsHV 1 DNA was detected in the inclusion bodies of the positive control tissue and the strongest signal was observed using those random biotinylated DNA probes generated from the whole PsHV 1 genotype 1 and genotype 4 viruses (Fig. 4.5a).

The negative control samples exhibited non-specific staining of the cytoplasm of the hepatocytes in the liver of samples SC92-0111 and the glandular mucosa of samples SC01-0019 (Fig. 4.5b). However, non-specific staining was not observed in the crop or cloacal mucosa of negative control SC01-0019 suggesting that ISH may still have utility

Table 4.5

Tissues examined by in situ hybridization and in situ PCR

Sample Number	Genbank Accession# or Use[1]	PsHV Gen[2]	Species Examined	Tissue Examined
SC92-0111	(-) Control		*Eclectus roratus*	liver
SC92-0142	(+) Control (Pacheco's disease)	Unk	*Amazona farinosa guatemalae*	crop, pancreas
SC97-0365	Sample	3	*Amazona oratrix*	endoventric. carcinoma
SC98-0018	AY421985/Sample	3	*Anodorhynchus hyacinthinus*	cloacal papilloma
SC00-0077	AY421989/Sample	3	*Amazona oratrix*	bile duct adenoma
SC01-0019	(-) Control		*Pyrrhura rupicola*	embryo
SC02-0002	AY421992/Sample	3	*Amazona autumnalis*	cloacal papilloma
SC04-0012	Sample	3	*Ara severa*	bile duct carcinoma spleen

[1] All samples were formalin-fixed and paraffin-embedded for less than 72 hours. Samples SC98-0018, SC00-0077, SC02-0002, and SC04-0012 were collected as fresh tissues and then fixed while the remainder of the tissues were submitted as routine diagnostic histopathology submissions.
[2] Freshly collected tissues were sequenced and genotyped using primer set 23Ff5b/23Fr3. Histopathology submissions were sequenced and genotyped using primer set 23Ff5a/23Fr+114D. "Unk" are unknown PsHV genotypes.

in a some mucosal tissues (Fig. 4.5c). It was assumed that this non-specific staining was due to an endogenous protein, probably biotin that reacted with the streptavidin, but was in such abundance that it could not be blocked.

Staining was not observed in any of the neoplastic tissues, but non-specific cytoplasmic staining was observed in the cytoplasm of the hepatocytes (Fig. 4.5d). Therefore, ISH with biotinylated DNA probes was not a suitable method for demonstrating PsHV 1 DNA in ISD tissues. The problems arising from the non-specific staining of histologically normal tissue coupled with the non-staining of neoplastic tissues eliminated this method. The concentration of intact PsHV 1 DNA may have been

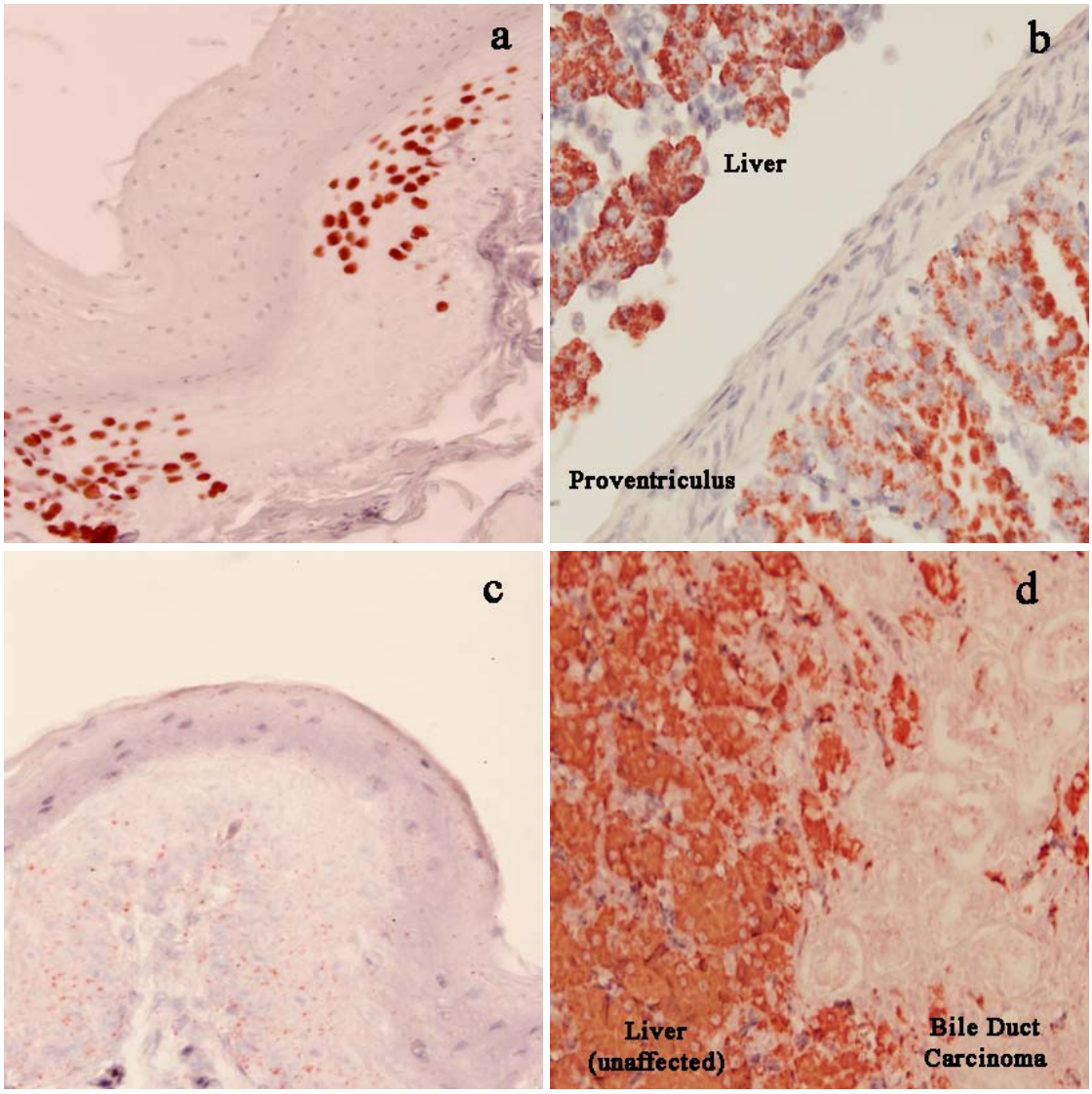

Fig. 4.5. In situ hybridization results of three control tissues and a liver with bile duct carcinoma. (a) Using random probes generated from a PsHV genotype 4 whole virus staining PsHV intranuclear inclusion bodies in the crop of sample SC92-0142; (b) Non-specific staining observed in the cytoplasm of hepatocytes and proventricular glands of negative control sample SC01-0019 using probes random probes generated from the UL16/17 amplicon; (c) Lack of non-specific staining in the crop of negative control sample SC01-0019 using the same probes as in (b); (d) Unaffected liver exhibiting non-specific staining of hepatocyte cytoplasm with adjacent bile duct carcinoma showing no staining of sample SC04-0012 using the same probes as in (b).

below the limit of detection of this technique because its presence had been established by PCR with DNA extracted from the whole tissue in earlier experiments.

Direct in situ PCR

The negative results in the experiments using ISH suggested that the concentration of PsHV 1 might be below the level of detection for that method. Most ISH methods have sensitivities in the 10^4 range while conventional PCR techniques are in the range of 10^3 (Morel and Raccurt, 2003). Therefore, in a final attempt to show a difference in concentration of PsHV 1 DNA in neoplastic versus histologically normal tissue, in situ PCR (ISPCR) was employed. Perhaps the level of detection could be improved by this method since the process would amplify the target DNA. The same sample tissues and controls used in the ISH experiments were examined by ISPCR using the three primer sets used to amplify PsHV 1 DNA from the FFPE tissues (Table 4.2 and Table 4.3). The primers were used individually and in combination in case the genome had sustained more damage from fixation at one locus compared to another. Biotinylated dCTP was added to the amplification mix so that the label could be incorporated into the amplicons and the sample signal amplification/detection system was used.

PsHV 1 DNA was amplified from the inclusion bodies of the positive control tissue most reliably using primer sets 23Ff-131D/23Fr+63 and 39RADFf/39Fr3 (Fig. 4.6 a-f). These two primer sets produced the greatest signal intensity in the control tissues and infected cells could be easily discerned. However, there was staining due to non-specific amplification of host DNA that contributed to the background. Attempts to reduce this background are described below. Also, the negative control tissue showed the same

Panel 1 Panel 2

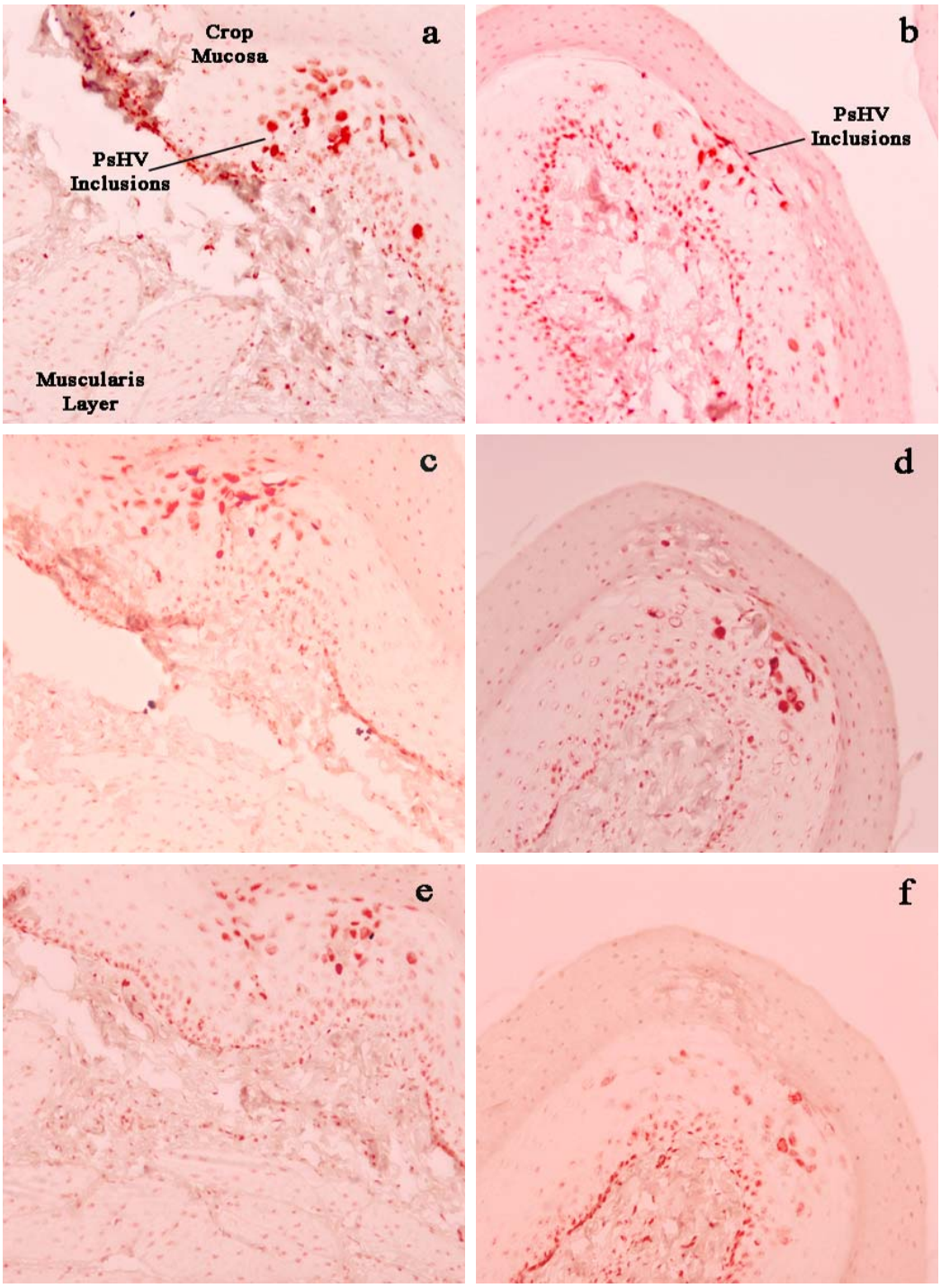

Fig. 4.6. In situ PCR results from two sections of crop. Panel 1 (first section) is (a, c, e) and Panel 2 (second section) is (b, d, f) from sample SC92-0142 using primer sets 23Ff-131D/23Fr+63 and 39RADf/39Fr2. (a) No S1 or ddTTP treatment; (b) No S1 or ddTTP treatment; (c) S1 treatment; (d) S1 treatment; (e) ddTTP treatment; (f) ddTTP treatment.

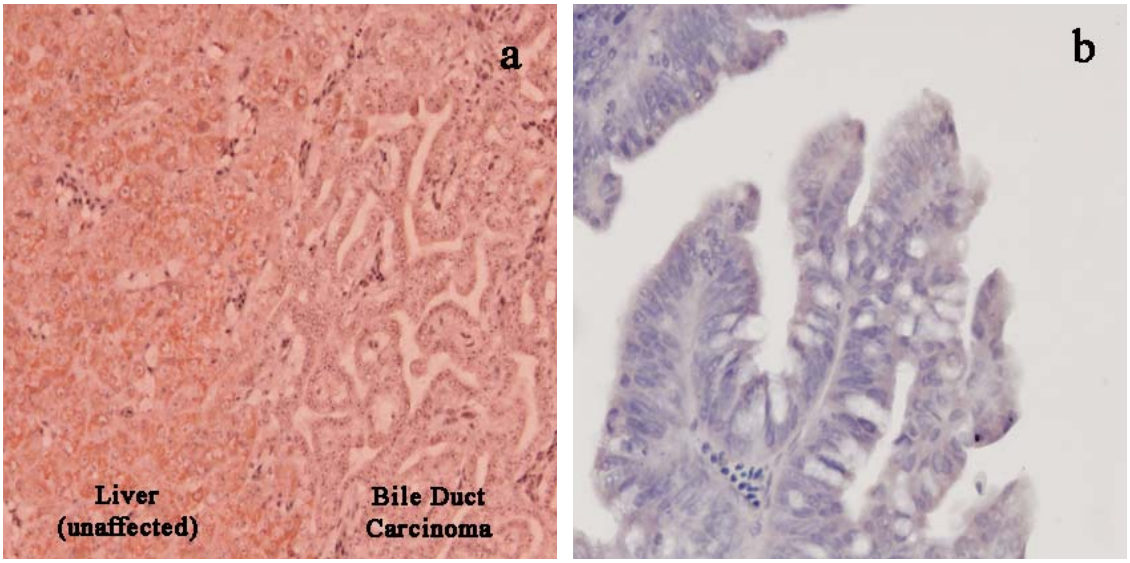

Fig. 4.7. In situ PCR results using a combination of PsHV primers 23Ff-131D/23Fr+63 (UL16/17 genes) and 39RADf/39Fr2 (UL30 gene) with S1 nuclease treatment. (a) a bile duct adenoma from sample SC00-0077 showing non-specific staining of the hepatocytes and no staining of the neoplastic cells; (b) a cloacal papilloma from sample SC98-0018 showing no positive staining.

intense non-specific staining in the cytoplasm of the hepatocytes and the mucosal epithelium of the proventriculus, but not the mucosal surfaces of the crop or cloaca that was observed in the ISH experiments. Again this was due to presumably and endogenous protein that could not be blocked in the samples.

Non-specific amplification of fragmented host DNA resulted because the *taq* polymerase initiated a nascent DNA chain at points of DNA fragmentation that were created by the fixation process (Morel and Raccurt, 2003; Nuovo, 1997). Two methods were tested to try and reduce this non- specific amplification artifact. Tissues were treated with S1 nuclease that cleaved any overhanging nucleotides from fragmented host DNA creating a blunt end, and ddTTP that acted as a chain terminator. While both

methods were effective at reducing non-specific amplification, they did not completely eliminate it. One method did not appear to be superior to the other and both treatments appeared to diminish the intensity of the signal (Fig. 4.6a-f). For most applications, the S1 nuclease was employed due to the cost difference between the enzyme and ddTTP.

The results of the ISPCR procedure were that neoplastic tissues did not show any amplification (Fig. 4.7a and 4.7 b) but non-specific staining was again observed in the cytoplasm of the hepatocytes (Fig. 4.7a).

The results of this experiment suggest that as with the ISH results, the concentration of intact PsHV 1 in the IPD tissues was below the level of detection for this method. Therefore, it does not appear that in situ PCR can be used to detect PsHV 1 DNA in IPD tissues.

Discussion

Work done by our laboratory demonstrated the association between PsHV 1 genotypes 1, 2, or 3 and the development of mucosal papillomas in Neotropical parrots (Styles et al., 2004). Tissues of 17 parrots with IPD related tumors and carcinomas of the digestive tract were probed using PCR for PsHV 1 DNA. With the exception of one sample (SC04-0010), PsHV 1 genotype 3 DNA was amplified from all bile tumors, two out of three pancreatic duct tumors, all proventricular/ventricular carcinomas, all cloacal carcinomas, and all mucosal papillomas, but not in histologically normal tissue. These findings suggest that PsHV 1 genotype 3 is associated with IPD-related and other mucosal tumors and is not a systemic infection.

Phylogenetic analysis was performed on a 219 bp fragment of UL16 amplified from the fresh tissues (Fig. 4.1) and a 238 bp fragment spanning a segment of UL16/UL17 of the FFPE tissues (data not shown). PsHV 1 genotype 3 was identified in all birds except a Patagonian conure (SC04-0010), where a PsHV 1 genotype 4 was amplified.

It had been previously shown that PsHV 1 DNA could not be amplified with conventional PCR from the histologically normal tissue adjacent to mucosal papillomas (Styles et al., 2004). To show that PsHV in the neoplastic tissue was in significantly higher concentration than adjacent histologically normal tissue, LCM was used to selectively collect neoplastic and histologically normal FFPE tissue from birds with tumors associated with IPD and other mucosal tumors. However, PsHV 1 DNA was not amplified from any of the neoplastic tissue (Table 4.4), although these tissues were known to contain PsHV DNA by PCR.

The psittacine cytochrome oxidase I gene housekeeping gene was not consistently amplified from all samples suggesting that the integrity of the DNA was varied with the sample and tissue. Given these results, it is possible that an insufficient number of cells containing PsHV DNA were collected, or that the concentration of intact DNA was inadequate to permit amplification.

Optimal PCR amplification of viral DNA from FFPE tissues depends on several factors, the particular virus, whether the virus exists as an episome or in an integrated state, the particular tissue, the type and length of fixation, and length of storage of the fixed tissues (Biedermann et al., 2004; Chen et al., 1991; Cohen et al., 2002; Greer et al., 1991; Evans et al., 2003; Yazdi et al., 2004). Amplification from LCM collected tissues has greater success with tissues that are either frozen or formalin-fixed for less than 24

hours. The archived FFPE tissues used in this experiment were fixed for an average of about 48-72 hours. Also, the target DNA such as a host gene or viral DNA integrated into the host genome demonstrates greater amplification efficiency compared to episomal viral DNA because the integrated virus is protected from fixation degradation along with the host DNA (Evans et al., 2003). PsHV DNA exists in a latent state probably as an episome in these mucosal cells, and therefore would be exposed to a greater degree of damage from the fixation process (Evans et al., 2003).

Viral concentration plays an important role in the success of these types of detection methods. The viral copy number for latent herpes simplex viruses has been estimated to be greater than 20 copies per neuron (Simmons et al., 1992), but a recent report by Cai et al. (2002) places the estimate between 2-20 copies per neuron. At best, there would have been 200-2000 copies of PsHV DNA/µl in the LCM samples. The limit of detection of the primers used in this experiment was about 10^3 virus/µl and the concentration of the PsHV DNA was at that limit (Styles et al., 2004). However, the integrity of this episomal DNA was poor as evidenced by the intermittent amplification of the cytochrome oxidase I gene, further reducing the amount of useful DNA for detection. Low viral copy numbers coupled with insufficient numbers cells containing intact PsHV DNA was likely to be responsible for the failure to amplify DNA from the LCM samples.

Further attempts to localize PsHV in these tumors included in situ hybridization and in situ PCR using biotin-labeled probes or nucleotides for detection. The sensitivity of both of these techniques is similar to that for conventional PCR with the limit of detection for in situ hybridization for cell preparations and 10^4 copies per cell for fixed

tissues with no signal amplification techniques (Mabruk, 2004; Kim, 2004; Speel et al., 1999). The range is similar with in situ PCR at about 10^3 copies per cell being only slightly more sensitive without signal amplification techniques. However in this experiment, a chromagen-tyramide signal amplification system was used that was reported to detect 1-2 copies of integrated HPV DNA in FFPE tissues (Evans et al., 2003). The results for both the in situ techniques were that no PsHV DNA could be detected in the tumors, but PsHV DNA was detected in the positive control tissue from a bird that died from Pacheco's disease where presumably high copy numbers existed in the tissues. The failure of PsHV DNA to be detected by in situ hybridization or in situ PCR could be explained for some of the same reasons discussed with the LCM results including the likelihood that the concentration of virus was below the limit of detection for the method used. One other important factor regarding the inability to amplify PsHV DNA using these in situ techniques is that PsHV is assumed to exist as an episome, and as previously discussed, would be at higher risk for degradation due to the fixation process.

The problems with non-specific staining with the streptavidin/biotin system could not be overcome. It is likely that an endogenous protein existed in the tissues (probably biotin) in concentrations sufficient to overcome blocking that caused this artifact. It was observed that tissues of the embryonic control stained more intensely than did the analogous adult tissues. Biotin is found in high concentrations in avian embryos and an overabundance of this protein was most likely responsible for the artifact (Vanio and Imhof, 1996). This may be a shortcoming of using the chromagen-tyramide

amplification system in avian tissues, except that the non-specific staining in mucosal epithelium could be greatly reduced by manipulating experimental factors.

While attempts to localize PsHV 1 DNA in the tumors failed, it is important to note that only PsHV 1 genotype 3 DNA was amplified from the lesions with the exception of one bird that will be discussed below. Previous work also found that 76% of the mucosal papillomas contained PsHV 1 genotype 3 DNA (Styles et al., 2004). These results suggest that genotype 3 viruses may possess a higher oncogenic potential than other genotypes, or that other genotypes are equally oncogenic but genotype 3 may exert a tropism for mucosal cells of the bile and pancreatic ducts, and glandular mucosa of the proventriculus and cloaca. Further studies will need to be done to determine what factors are involved with the oncogenic potential of genotype 3 viruses, but it must first be definitively shown that PsHV is localized to the neoplastic tissue.

There were two non-Neotropical species reported in this study that had IPD and associated tumors. These were an eclectus parrot (DP04-0006) and a Moluccan cockatoo (SC91-1299). This is the first report of IPD in these species. PsHV-1 genotype 3 DNA was amplified from the lesions of both birds. The history of the Moluccan cockatoo is largely unknown except that it was a wild-caught bird living in an aviary until it died from tumors associated with IPD. However, the eclectus parrot originated from a collection that had suffered a Pacheco's disease outbreak. This bird showed clinical signs consistent with the disease and was treated with the anti-herpesviral drug acyclovir. This bird survived and subsequently developed IPD and bile duct adenoma. All PsHV 1 genotypes appear to be equally lethal to parrots of Pacific distribution (Tomaszewski et al., 2003). It is likely that this bird would have also

succumbed to the disease had it not been rescued with treatment, so this example may not be representative of natural disease.

The finding of PsHV 1 genotype 4 in Patagonian conure (SC04-0010) is the first instance of amplifying a genotype other than genotype 3 from a bird with a mucosal papillloma. This bird had a glottal papilloma and a cloacal carcinoma. Several possibilities may help to explain this finding. It is possible that genotype 4 could have been associated with the lesions or that the bird was co-infected with a genotype 3 virus, but further investigation will need to be done although precedence for such co-infection does exist (Tomaszewski, personal communication). A more likely explanation is that the mechanisms giving rise to the mucosal papilloma and cloacal carcinoma were different. This idea will be elucidated below.

Bile and pancreatic duct neoplasms and perhaps proventricular/ventricular carcinomas are associated with IPD but cloacal carcinomas are not. The sample size for proventricular/ventricular carcinomas is small (n=3), but each is coincidental with bile duct tumors. The data set only has two examples of cloacal carcinomas, one from a macaw that had no evidence of IPD, and one from the conure with genotype 4 that had a glottal papilloma and more tumors need to be examined. Bile duct, pancreatic duct, and endoventricular carcinomas do not readily metastasize in contrast to cloacal carcinomas that do metastasize. Also, these former tumors can develop in a broad age-range of birds, but cloacal carcinomas tend to develop only in older parrots. The difference in age predilection and metastatic behavior separates cloacal carcinomas from the other tumors associated with IPD. While cloacal carcinomas may be coincidental with IPD, they may not be a manifestation of IPD.

The results of this investigation suggest that bile and pancreatic duct tumors are manifestations of IPD, and that proventricular/ventricular tumors may also be related but the numbers are too small to draw clear conclusions. Cloacal carcinomas appear to develop by a different pathway that may be unrelated to IPD. The development of all of these tumors is associated with the presence of PsHV 1 genotype 3. Further studies will need to be performed to elucidate the oncogenic mechanisms of this genotype.

Materials and Methods

Samples: sources, history, and processing

Tissues used in this study were acquired as diagnostic submissions to the Schubot Exotic Bird Health Center (Texas A&M University, College Station, TX) and originated from aviaries within the United States. Tissues were processed as either routine histopathology submissions and available as FFPE specimens or for study as cases of IPD available as fresh tissues (Tables 4.1, 4.3, 4.4, and 4.5). Fresh specimens submitted for study as cases of IPD were collected using cleaned, autoclaved instruments that had been bleached and then soaked in formalin. A different set of instruments was used to collect each tissue to prevent DNA contamination. However, no precautions guarding against PsHV DNA contamination were taken with specimens processed for routine diagnostic submissions for histopathology.

The tissue was considered to contain papillomas if the normal mucosa was replaced with papillary growths consisting of a narrow to broad base with the mucosal fronds containing a fine fibrovascular core covered by a cuboidal to tall columnar epithelium of variable thickness. Adenomatous and/or carcinomatous neoplasms were observed in the

liver and pancreas (Fig. 4.1a). Lesions were classified as bile or pancreatic duct adenomas if they displayed moderately well differentiated ductal architecture accompanied by a variable amount of stroma (Fig. 4.1b) (Schmidt et al., 2003). Bile duct carcinomas were less well differentiated and tended to form nests and cords of poorly formed ducts or individual cells accompanied by a moderate amount of stroma (Fig. 4.1c). Proventricular/ventricular carcinomas were observed in three birds and were more malignant in nature. They consisted of poorly differentiated cells that appeared to be derived from the glandular mucosa of the proventriculus arranged as infiltrative nests and cords displaying extensive stromal proliferation and extended through the muscularis through to the serosa (Fig. 4.2a). Goblet cells were also observed but the mitotic index was very low. Cloacal carcinomas were seen as infiltrative lesions characterized by moderately to poorly differentiated epithelial cells that formed cords and nests supported by a scirrhous stroma (4.2b).

The fresh tissues from 11 parrots and the FFPE tissues of 5 other birds were examined (Tables 4.1 and 4.2). Six of the birds were macaws (38%), 6 were Amazon parrots (38%), 2 were conures (12%), one was an eclectus parrot (6%), and one bird was a cockatoo (6%). Fifteen of these birds (94%) either exhibited mucosal papillomas and a related tumor or had a history of mucosal papillomas, however one macaw (6%) with a cloacal carcinoma had no gross evidence or history of mucosal papillomas. Eight birds had bile duct carcinoma accompanied by at least one mucosal papilloma, one of which also had pancreatic duct adenoma; six birds had bile duct adenomas (two of which also had pancreatic duct adenomas) and five which were accompanied by at least one mucosal papilloma and one that had no evidence or history of papillomas; there were

two birds with proventricular carcinomas one of which had mucosal papillomas, and there were two birds with cloacal carcinomas one of which had mucosal papillomas. Histologically normal digestive tract mucosal samples were available from 6 birds: crop=5, proventriculus=3 (Table 4.1).

Fresh tissues were either processed immediately or stored at -80°C until use. Formalin-fixed paraffin-embedded (FFPE) tissues that had been fixed in formalin for 72 hours or less were selected for the study (Table 4.2).

Tissues used for the LCM experiment (Table 4.4) were FFPE sections that had been shown to be positive by PCR examination of the DNA extracted from either fresh (Table 4.1) or FFPE (Table 4.2) tissues. All tissues were fixed in formalin for 72 hours or less. Tissues included an Amazon parrot with bile duct adenoma, another Amazon parrot with bile duct adenoma and ventricular carcinoma, a macaw with bile duct carcinoma and pancreatic adenoma, and an eclectus parrot with bile duct adenoma (Table 4.4). Both the formalin-fixed and paraformaldehyde-fixed tissues of sample SC04-0012 were examined.

IPD tissues examined by in situ hybridization and direct in situ PCR (Table 4.5) were processed as those described above for LCM. The tissues used in these experiments were FFPE tissues fixed for 72 hours or less and had been shown to contain PsHV DNA by conventional PCR except the spleen of sample SC04-0012 where the concentration of PsHV 1 DNA was below the limit of detection for PCR. Tissues examined were an endoventricular carcinoma from an Amazon parrot, a bile duct adenoma from another Amazon parrot, a bile duct carcinoma and histologically normal spleen from a macaw,

and a cloacal papilloma from a second macaw (Table 4.5). The spleen from SC04-0012 was used as a negative internal control tissue for this bird.

Histological evaluation of the tissues

Sections of the tissues were fixed in formalin, embedded in paraffin, and thin sections were routinely stained with hematoxylin and eosin. These sections were then microscopically examined for the presence of lesions.

Extraction of DNA from fresh IPD tissues

DNA was extracted from the tissues using the Purgene® Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN.) following the instructions for fresh or frozen solid tissue. The extracted DNA was then re-hydrated in nuclease-free water (Promega, cat# P1193, Madison, WI).

Extraction of DNA from formalin-fixed paraffin-embedded tissues

Many of the paraffin blocks contained multiple tissues. Tissues of interest were isolated from those not examined by melting the paraffin block and re-embedding the tissue of interest in another paraffin block separately, so that there was only one tissue present in each block. Blocks were then cut into 5 µm sections using a Leitz 1512 rotary microtome. A new microtome blade was used to cut each tissue and the microtome was cleaned with DNA AWAY™ (Molecular BioProducts, cat# 7010, San Diego, CA), absolute ethanol, and distilled de-ionized water before and between each tissue to prevent contamination. Fifteen to twenty 5 µm paraffin sections containing the

tissue of interest were collected and placed into sterile microcentrifuge tubes. Tissues were deparaffinized using the following protocol of Shi et al. (2002). Briefly, 1 ml of xylene was added to the sections and incubated at room temperature with intermittent mixing for 30 minutes, followed by centrifugation at 14,000 rpm using a Beckman-Coulter Mircofuge™ 18 centrifuge, and then decanting. Then the tissues were similarly treated in 100% and 75% ethanol. Final re-hydration was performed by washing in PBS (pH=7.0) for 15 minutes twice.

DNA was extracted from the tissues using the Purgene® Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN.) following the instructions for fresh or frozen solid tissue except the amount of proteinase K was doubled for the initial digestion for a minimum of 12 hours at 55°C. Following the initial digestion, fresh proteinase K was added to the tissue and digestion was continued for an additional hour or until the tissue had completely dissolved. The extracted DNA was then re-hydrated in nuclease-free water (Promega, cat# P1193, Madison, WI).

PCR of IPD tissues with PsHV 1-specific primers

All PCR reactions were performed on an Eppendorf Mastercycler® Personal Thermocycler (Eppendorf, Hamburg, Germany). PCR was performed as previously described (Tomaszewski et al., 2000). Briefly, a 25 µl reaction mix containing, 100 ng genomic DNA, 25 pmol of each primer, 0.1 mM of each of the 4 deoxynucleotide triphosphates, 2.5 mM magnesium chloride, 0.75 units Taq polymerase and 1X buffer A was used in the reaction (Promega, cat# M1861, Madison, WI).

Primer set 23Ff5a/23Fr3 was used to amplify a 667 bp product that spanned PsHV 1 genes UL16/UL17 from fresh or frozen tissues (Table 4.3). Three other primer sets were used for FFPE tissue including the LCM experiment: 23Ff-131D/23Fr+63, 23Ff5a/23Fr+114D, and 23Ff+43/23F4+266D (Table 4.3). These primer sets amplified different consecutive segments of the UL16/UL17 genes to ensure consistent amplification at different loci in case damage had resulted from the fixation process at any one locus. All primer sets used amplified the four PsHV 1 genotypes.

The positive control for fresh tissues was DNA containing PsHV extracted from the tissues of an Amazon parrot that died of Pacheco's disease. Positive controls for FFPE tissue were DNA extracted from the FFPE liver of two parrots that died of Pacheco's disease (Table 4.2). The negative controls for the fresh tissues were the DNA extracted from a near hatch psittacine embryo and the blood of an incubator hatched African grey parrot held in isolation (Table 4.1). Negative controls for FFPE tissue were DNA extracted from the FFPE liver of a bird that died of causes unrelated to PsHV and from the whole body of a near hatch psittacine embryo (Table 4.4). For the LCM experiment, primer set H7523/COIf amplified a section of Neotropical psittacine cytochrome oxidase I and served an internal control (Eberhard and Bermingham, 2003) (Table 4.3). Reference samples representing each PsHV 1 genotype were used to verify the functionality of the primers used to probe both fresh and FFPE tissues (Styles et al., 2004; Tomaszewski et al., 2003).

The thermocycler parameters were: initial denaturation at 94°C for 5 minutes; followed by 40 cycles of annealing at 60°C for 45 seconds, extension at 72°C for 90

seconds, denaturing at 94°C for 30 seconds, and a final extension at 72°C for 5 minutes. PCR amplification products were imaged on ethidium bromide agarose gels.

DNA sequencing of PsHV amplicons from IPD tissues

PCR products were purified using QIAquick® PCR Purification Kit (Qiagen Inc., Valencia, CA). The sequencing reaction was performed using an ABI Prism® Big Dye™ Terminators v3.0 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA). Amplicons from fresh tissues were sequenced with primer set 23Ff5b/23Fr3 and PCR products from the FFPE tissues were sequenced with primer set 23Ff5b/23Fr+114D (Table 4.3). Products were sequenced using an ABI 377 DNA sequencer or ABI 3100 DNA sequencer (Applied Biosystems).

Phylogenetic analysis of sequences from IPD tissues

Phylogenetic analysis was performed as described by Tomaszewski et al., (2003). A 419 bp product amplified fresh tissues and a 238 bp product amplified from FFPE tissue were subjected to phylogenetic analysis. The 419 bp amplicon began at the UL16 start codon and the 238 bp product started in the UL17 gene, 169 bp in front of the UL16 start codon, and continued for 66 bp beyond the UL16 start codon. All sequences were aligned using Clustal X 1.81 (Thompson et al., 1997). Aligned sequences were imported into PAUP for analysis (Swofford, 1998). Phylogenetic trees were generated by performing a neighbor-joining search using bootstrap re-sampling (1,000 replications) employing distance optimality. A phylogram was generated from the sequence data showing branch-lengths and bootstrap values employing representative sequences of

each genotype for sequences generated from the fresh tissues (Fig. 4.1) and for the FFPE tissues (data not shown).

Preparation and sectioning of tissues for LCM

FFPE tissues were sectioned at 5 μm thickness on a Leitz 1512 rotary microtome and then floated onto 3-aminopropyl-trimethoxysilane coated glass slides (HistoBond +, StatLabs Medical Products Inc., Lewisville, TX). Each individual tissue section was cut with a new microtome blade to prevent contamination. Sections were then permitted to air dry at room temperature after which the sections were fixed to the slides by heating in an oven at 60° C for one hour. The tissues were routinely stained with hematoxylin and eosin (and deparaffinized in the process). Stained tissue sections were placed in a slide box that contained silicone-desiccating packets to maintain low humidity and keep the tissue as dry as possible until use.

Laser capture microdissection (LCM)

Slides containing tissues to be examined were placed onto the sampling stage of an Acrturus PixCell II™ Laser Capture Microdissection instrument (Arcturus, Mountain View, CA). A microscope objective of 10X was selected and the area of interest was identified, that area was centered in the middle of the slide stage, and the slide secured in place with the vacuum slide-holding system. A CapSure™ Macro LCM Cap (Arcturus, cat# 10284-04, Mountain View, CA) was loaded onto the sampling arm of the instrument. The LCM cap was lowered onto the sample surface ensuring that the cap was level, centered, and there was effective contact with the sample. The instrument

settings varied between 7.5-15 microns of cutting diameter; 75-100 mW laser power; and cutting time of 1-3 milliseconds depending on the type and topography of the tissue being sampled. The laser was then activated and tuned for maximum cutting efficiency. As the LCM cap surface became filled with cut sections, the cap was lifted from the surface and manually rotated to expose areas of the cap surface that were empty so that collection could continue. This activity was repeated until between 2000-5000 sections had been acquired.

When sufficient tissue had been collected, then the LCM cap was removed and inserted into a 500 µl microcentrifuge tube. The tissue was held at room temperature in the tube until processed. DNA was extracted from the collected tissue using a Pico Pure ™ DNA Extraction Kit (Arcturus, cat# KIT0103, Mountain View, CA). Once the digestion was completed, the digest was processed in two ways, either used directly for PCR as recommended by the kit or further purified using a Purgene® Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN).

Tissue processing for in situ hybridization and in situ PCR

Sections to be used for in situ hybridization and in situ PCR were processed routinely as described in the section on preparation of tissues for LCM except tissues were not stained and were deparaffinized and re-hydrated by the following protocol: immersion in xylene for 5 minutes; repeat with fresh xylene; immersion in absolute ethanol for 3 minutes; repeat with fresh absolute ethanol; immersion in a 95% ethanol for 3 minutes; repeat with fresh 95% ethanol; immersion in a 70% ethanol for 3 minutes; followed by three washes in distilled de-ionized water for 2 minutes.

DNA retrieval was then performed to enhance the signal integrity. The re-hydrated tissue sections were placed in a glass slide tray and immersed into 1 liter of 10 mM citrate buffer (pH=6.0). Sections were then heated in this solution in a microwave set at maximum power for 5 minutes; the heated solution was then allowed to rest for 5 minutes after initial heating; this was followed by another period of heating at maximum power for an additional 5 minutes, however, the solution was not permitted to boil. After the second heating, the sections were allowed to remain in the hot solution for 30 minutes (Shi et al., 1991). Sections were then rinsed in three changes of water for 5 minutes each and then allowed to air dry.

Tissue sections were then subjected to pepsin protease digestion to free the DNA from the cross-linked proteins (Evans et al., 2003). A 6000 U/ml stock solution of pepsin (Sigma P-7012, Sigma, St Louis, MO) was prepared with 0.2 M HCl, then aliquoted into 1 ml increments and stored at –20 ° C until needed. The working pepsin solution of 300 U/ml in 0.2 M HCl was applied to the tissues in a humid chamber at room temperature and sections were allowed to digest for 30 minutes at room temperature. The sections were then rinsed with water for 5 minutes; followed by 3 changes of a solution of PBS/0.05% Tween 20 (pH 7.0) for five minutes each; and rinsed again in water for 5 minutes.

Following protease unmasking, any endogenous peroxidase was quenched by immersing the tissue sections into a solution of methanol and 0.6% hydrogen peroxide for 30 minutes at room temperature. This step was followed by three water rinses for 5 minutes each. Sections were then permitted to air dry.

Endogenous cross-reactive streptavidin/biotin proteins were blocked prior to ISH or ISPCR. Blocking was accomplished by using a Streptavidin/Biotin Blocking Kit (Vector Labs. Inc., SP-2002, Burlingame, CA).

In situ hybridization: synthesis of biotinylated probes

Both random primer and specific primer directed probes were synthesized using the BD Biosciences Clontech SpotLight™ Random Primer Labeling Kit (BD Biosciences Clontech, cat# K1027-1, Palo Alto, CA). The kit produced probes between 50-300 base pairs in length. Random probes were generated using the random primers provided in the kit and the following DNA templates: PsHV 1 genotype 4 (whole virus); PsHV 1 genotype 1 (whole virus); a 537 bp amplicon encompassing a section of the PsHV 1 gene UL9 produced from a genotype 3 sample by primer set 11Rf8/11Rr4 (Table 4.2); a 667 bp amplicon spanning a section of PsHV 1 genes UL16/UL17 produced from a genotype 3 sample by primer set 23Ff5a/23Fr3 (Table 4.3); and a 634 bp amplicon encompassing a section of PsHV 1 gene UL30 also produced from a genotype 3 sample by primer set 39Ff/39Fr (Table 4.3). Specific primer-directed probes were generated using primers 23Ff5a/23Fr3 and 39Ff/39Fr using their respective amplicons as templates (Table 4.3).

The sensitivity and relative concentration of both random and specific primer-directed probes was assessed using a BD Biosciences Clontech SpotLight™ Chemiluminescent Hybridization & Detection Kit (BD Biosciences Clontech, cat# K1032-1, Palo Alto, CA) (Fig. 4.8).

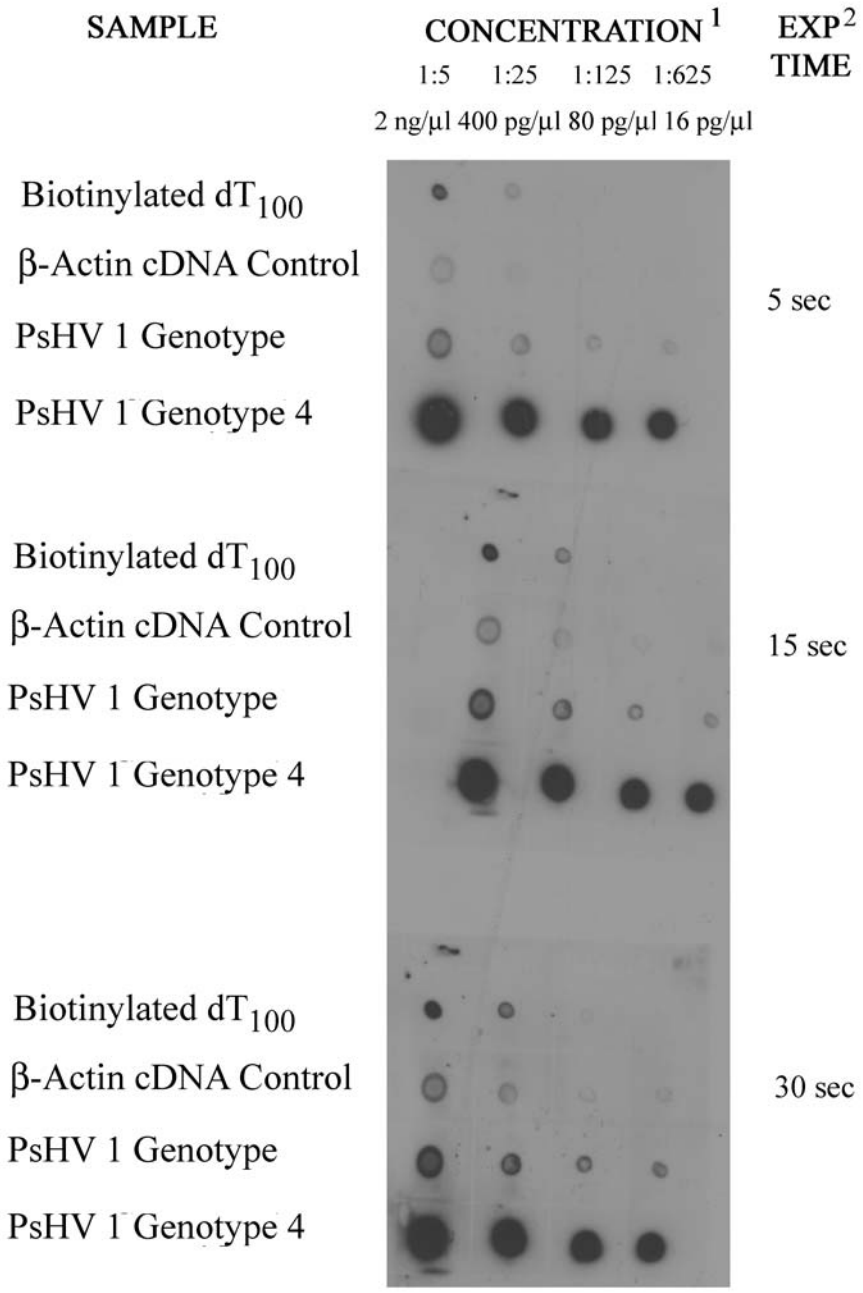

Fig. 4.8. Chemiluminescent film of relative intensities of signal of random probes to concentrations of known standards. [1]β-actin and PsHV concentrations are given as relative dilutions, and biotinylated dT_{100} is given in absolute concentration units. [2]Exposure time to radiographic film is given in seconds. A biotinylated 100mer of poly-T was used as a known standard. β-actin was used as an internal experimental positive control. Viral templates were PsHV 1 genotypes 1 and 4 whole purified virus.

In situ hybridization

Tissue sections were covered with sealable reaction chambers (HybriWell™, HBW22 or HBW1932, Grace Bio-Labs, Bend, OR). The chambers were then filled with a hybridization mixture that consisted of 40% 2X SSC buffer (Sigma, S-6639, St Louis, MO), 10% dextran sodium sulfate (ICN Biomedical Inc., cat# 160110, Aurora, OH), 50% *N, N*-dimethylforamide (Aldrich; cat # 22, 705-6; Milwaukee, WI) (Evans, M. et al., 2003). Sonicated salmon sperm DNA (Sigma, D-7656, St Louis, MO) that had been denatured by boiling for 5 minutes was added to the hybridization mix of some samples at concentration of 400 ng/µl to determine its utility as a blocking agent for non-specific binding of the PsHV probes to genomic DNA. Probes were added to the hybridization mix at a concentration of 1 µl of probe per 10 µl of hybridization mix. The reaction chamber port seals were placed over the ports and rubber cement was applied over the ports and around the edges of the chamber to secure it to the slide and prevent loss of liquid during heating.

The sections in the sealed chambers were then placed into a PTC-100 thermocycler (MJ Research Inc, Watertown, MA) equipped with a block that accommodated microscope slides. The thermocycler program was initial denaturation at 94° C for 5 minutes followed by a minimum of 12 hours hybridization at 37° C.

Following hybridization, the hybridization chambers were removed under a solution of 2X SSC with 0.05% Tween 20 (pH=7.0) at room temperature. The tissues were then washed in a high stringency bath of a solution of 0.2 SSC with 0.05% Tween 20 at 55° C for 15 minutes (Evans, M. et al., 2003).

In situ hybridization and direct in situ PCR: detection of biotin and chromagenic staining

IPD tissues used in this experiment were the FFPE tissues that had been shown by PCR to contain PsHV DNA (Table 4.4). The positive control tissue was an Amazon parrot that died of Pacheco's disease and exhibited PsHV intranuclear inclusion bodies in the crop and pancreas. The negative control was a near-hatch psittacine embryo (Table 4.4).

Tissues were washed in three changes of a solution of PBS with 0.05% Tween 20 (pH=7.0) for five minutes. The excess liquid was removed and biotinylated probes were detected in the tissue using a DakoCytomation GenPoint Tyramide signal amplification system (DakoCytomation Inc., product # K0620, Carpinteria, CA). The protocol for biotin detection was performed in accordance with the GenPoint directions except the primary streptavidin-HRP was used at a dilution of 1:5000 in PBS with 0.05% Tween 20 (pH=7.0) and left in contact with the tissue for 1 hour. The remainder of the procedure was completed as directed except that the wash buffer employed was PBS with 0.05% Tween 20 (pH=7.0) and 3-amino-9-ethyl-carbazole (AEC) was used as a chromagenic stain rather than diaminobenzidine (Evans, M. et al., 2003). All staining reactions were conducted within a sealed humid chamber at room temperature.

An aminotethylcarbazole (Sigma, cat# A-5474, St Louis, MO) solution was prepared in accordance with a protocol from Harlow (Harlow et al., 1988). Once the AEC was activated, the tissues were permitted to develop in contact with the reagent for about 15 minutes. The reaction was quenched by immersing in water for 5 minutes. The tissues were then counterstained with aqueous hematoxylin (Biomeda, cat# M10, Foster City,

CA), rinsed in water, air-dried, and sealed with an impervious resin (Crystal Mount, Biomeda, cat# M03, Foster City, CA).

Direct in situ PCR: blocking of non-specific amplification using S1 nuclease or 2', 3' dideoxythymidine 5'-triphophate (ddTTP)

Two methods were tested on the tissues in an attempt to inhibit non-specific amplification due to breaks in non-target (host genomic) DNA. S1 nuclease (Promega, cat# M5761, Madison, WI) was used to create blunt end DNA chains to inhibit binding of the DNA polymerase and ddTTP (Amersham, cat# 27-2081, Piscataway, NJ) was used as a DNA chain-terminator to prevent extension by DNA polymerase.

Treatment with S1 nuclease was accomplished by covering the tissues with sealable reaction chambers (HybriWell™) and filling the reaction chamber with 0.1 U/µl of S1 nuclease enzyme. The slides were incubated at 37° C for 30 minutes in a PTC-100 thermocycler (MJ Research Inc, Watertown, MA) (Ausbel et al., 1998). The reaction was stopped by the addition of 1µl of 0.5 M EDTA. Tissues were then rinsed in two changes of water for 5 minutes and allowed to air dry.

The chain terminator ddTTP was also tested as an inhibitor of non-specific amplification. Tissues were covered with sealable reaction chambers (HybriWell™). The protocol was performed in accordance with Nuovo, 1997. The reaction mixture contained [1X buffer A, 4.5 mM $MgCl_2$, 0.167 U/µl *Taq* polymerase (Promega, cat# M1861, Madison, WI)]; 200 µM dNTPs (Promega, cat# U1330, Madison, WI) , 1000 µM ddTTP, 0.067% bovine serum albumin (Sigma, cat# A3059, St Louis, MO) mixed in Nuclease-free water (Promega, cat# P1193, Madison, WI). The reaction chamber ports

were sealed and the slides were incubated at 55° C for 30 minutes in a PTC-100 thermocycler. The reaction chamber was removed from the slide and the tissues were then rinsed in two changes of water for 5 minutes and allowed to air dry.

Direct in situ PCR

IPD tissues used in this experiment were the FFPE tissues that had been shown by PCR to contain PsHV DNA (Table 4.5). The positive control tissue was an Amazon parrot that died of Pacheco's disease and exhibited PsHV intranuclear inclusion bodies in the crop and pancreas. The negative controls were the liver of an eclectus parrot that died of causes unrelated to PsHV and a near-hatch psittacine embryo (Table 4.4).

Direct *in situ* PCR was performed as described by Morel et al., 2003. Briefly, the tissues were covered with sealable reaction chambers (HybriWell™) and the reaction mixture was [1X buffer A, 1.5 mM $MgCl_2$, 0.1 U/µl *Taq* polymerase (Promega, cat# M1861, Madison, WI)]; [0.2 mM dATP, dGTP, dTTP; 0.1 mM dCTP (Promega, cat# U1330, Madison, WI)]; 0.1 mM biotin-14-dCTP (Invitrogen, cat # 19518-018, Carlsbad, CA); 0.3 µM sense primer; 0.3 µM anti-sense primer; 5% DMSO (HybriMax®, Sigma, St Louis, MO); made quantity sufficient for volume with nuclease-free water (Promega, cat# P1193, Madison, WI). Primers used to probe for PsHV 1 DNA were 23Ff-131D/23Fr+63 and 39RADFf/39Fr2 (Table 4.3). The reaction chamber port seals were placed over the ports and rubber cement was applied over the ports and around the edges of the chamber to secure it to the slide and prevent loss of liquid during heating.

The reaction was conducted on a PTC-100 thermocycler and the parameters were: 95° C for 5 minutes initial denaturation followed by 20-30 cycles of: 95° C for 1

minute, 60° C for 1.5 minutes annealing, and 72° C for 1.5 minutes extension. The reaction was completed with a final extension of 72° C for 5 minutes.

The reaction chambers were removed from the slides and the tissues were washed in PBS with 0.05% Tween 20 (pH=7.0) twice for five minutes. The slides were then air-dried and the amplicons fixed in absolute ethanol at a temperature of -20° C for five minutes. The tissues were then re-hydrated at room temperature in 95% ethanol for 3 minutes, then 70 % ethanol for 3 minutes.

Tissues were then processed to develop chromagenic stain as described in the section on detection of biotin and chromagenic staining above.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Discussion

Internal papillomatous disease is characterized by mucosal papillomas primarily affecting the epithelium of the oral cavity and cloaca of predominantly Neotropical parrots, but is also observed in the esophagus, crop, proventriculus, ventriculus, conjunctiva, nasolacrimal duct, and bursa (Graham, 1991). While these papillomas grossly and histologically resemble those caused by papillomaviruses, there is little evidence supporting the existence of papillomaviruses in these lesions (Johne et al., 2002; Latimer et al., 1997; Sundberg et al., 1986). However, strong evidence was presented suggesting that mucosal papillomatosis in Neotropical parrots is associated with PsHV 1 genotypes 1, 2, and 3, with the most prevalent genotype being 3 in the mucosal papilloma sample population of 30 parrots (Styles et al., 2004). Other tumors have also been associated with IPD including neoplasms of the bile and pancreatic ducts (Graham, 1991; Hillyer, 1991; Phalen et al., 1997). Data presented in Chapter 4 showed that only PsHV 1 genotype 3 DNA was detected in the bile and pancreatic duct tumors, and endoventricular carcinomas associated with IPD. This finding along with the results of the mucosal papilloma study showing that genotype 3 was the predominant PsHV genotype in the mucosal papilloma population suggests that genotype 3 may possess oncogenic properties that differ from other genotypes. It is possible that PsHV 1 genotype 3 may exert an oncogenic activity greater than other genotypes. Another possibility is that other PsHV genotypes have equivalent oncogenic activity to genotype 3, but genotype 3 displays a particular tropism for cells not observed with other

genotypes such as bile and pancreatic duct cells. For example, genotype 3 may be able to infect cells such as bile and pancreatic epithelium more efficiently than other genotypes which would provide the opportunity for oncogenesis in these tissues.

Development of tumors in birds infected with PsHV is thought to be associated with latent infection by the virus. PsHV latently infects parrots that are survivors of Pacheco's disease, and is shed intermittently or continuously at very low levels (Phalen et al., 1999). Latency has been established for the closely related alphaherpesvirus infectious laryngotracheitis, where the trigeminal ganglion was determined to be the site of latent infection (Hughes et al., 1987, Williams et al., 1992). It is unknown whether PsHV exists latently in nerve tissue. PsHV DNA could not be detected in peripheral nerve and ganglia by conventional PCR from birds with IPD, but it may be present in concentrations below the limit of detection of this method. In herpes simplex viruses, LAT (latency associated transcript) is believed to play a vital role in the maintenance of latency in the neuron (Howley and Lowy, 2001). Whether PsHV contains an LAT-like sequence is unknown, but alignment of the HSV 1 LAT sequence with the PsHV 1 genome resulted in no significant similarity.

The results of our investigations suggest that PsHV exists latently in the neoplastic epithelial cells. PsHV DNA could be consistently amplified from affected neoplastic mucosa, but not from adjacent or distant histologically normal mucosa from birds with IPD (Styles et al., 2004; Chapter IV). It is likely that through a currently unknown mechanism, PsHV induces the development of tumors and the type and severity of tumor depends on the genotype of PsHV and origin of the tissue.

Based on evidence from other oncogenic viruses, there are likely PsHV oncogenic proteins that interfere with the cell cycle, signal transduction, or other pathways that result in the development of mucosal papillomas. For example, the E5 protein found in bovine papillomavirus is responsible for the development of papillomas (Howley and Lowy, 2001). Each subunit of the E5 homodimer binds to a PDGF β receptor resulting in dimerization and activation of the receptors. The activated receptors then initiate a signal transduction pathway that leads to mitogenic stimulation. In cows infected with the group of bovine papillomaviruses that includes BPV 4, gastrointestinal tumors can be induced by a co-carcinogen (quercetin) found in a diet that includes bracken fern (Campo, 2002). It is possible that a similar initiating factor could be found in psittacine diets. However, when the E5 nucleotide and protein sequences of BPV 1 and 4 and two high-risk HPV viruses (16 and 18) were aligned against the PsHV, no similarities were observed. The oncogenic proteins of other herpesviruses such as the alphaherpesvirus Marek's disease target cell cycle activity within leukocytes and may be less suitable for comparison when trying to elucidate a mechanism for genotype 3. The Meq protein targets the cell cycle controls in B-lymphocytes, whether a Meq like protein in PsHV would have a similar effect is unknown. However, alignment of the Meq sequence with the PsHV genome resulted in no significant similarity. What advantage PsHV may gain by generating tumors is unclear. The papillomas that are caused by species-specific papillomaviruses are vehicles that permit continued replication of the virus. However, the replication rate in birds latently infected with PsHV appears to be low or not measurable using conventional methods (Phalen et al., 1999). It is possible that birds with IPD or tumors associated with PsHV are infected with a genotype to which they

may not be adapted. However, it is not clear what parrot species serve as hosts for PsHV and the host may vary with genotype. There is some evidence suggesting that hyacinth macaws (*Anodorhynchus hyacinthinus*) may a candidate species as hosts for genotype 3 since only one bird has been found to have a mucosal papilloma, but several hyacinth macaws were found to be asymptomatically infected (Phalen et al., 2004). Further studies need to be undertaken to quantify rates of viral shedding from birds with overt IPD and those that have no obvious lesions. This may give some indication that the development of tumors enhances viral survival. For example, if birds with overt papillomas, including papillomas that can only be visualized microscopically or with an endoscope, shed higher concentrations of PsHV as compared to those infected with the virus but appear grossly normal.

PsHV 1 genotype 3 DNA was found in the IPD lesions of two non-Neotropical species, an eclectus parrot and a Moluccan cockatoo. While the history of the Moluccan cockatoo is largely unknown, the eclectus parrot was a survivor of a Pacheco's disease outbreak that had been rescued with acyclovir treatment. The fact that only two non-Neotropical parrots exhibiting IPD were found in the data set including the entire tissue archive of the Schubot Exotic Bird Health Center collection, suggests that these two birds are exceptional and that IPD is predominantly a disease of Neotropical parrots.

PsHV 1 genotype 3 was amplified from all IPD tissues except the glottal papilloma and cloacal carcinoma of a Patagonian conure where a PsHV genotype 4 was detected. Patagonian conures have been speculated to be carriers of this genotype so it is possible that PsHV 1 genotype 4 may have been coincidental with these lesions (Phalen et al., 2004). While it is possible that genotype 4 was responsible for these lesions, the

dynamics of papillomas and cloacal carcinomas may be different and it must not be assumed they arise from a common set of mechanisms. IPD can develop in a wide age range of birds where they develop benign mucosal papillomas, and the tumors associated with IPD rarely metastasize. In contrast, cloacal carcinomas are malignant and may metastasize, and have thus far only been observed in older birds (Phalen, personal communication). However, the data set in this study was small (n=2) and while the tumors were malignant, there was no evidence of metastasis in these birds. However like IPD, cloacal carcinomas do appear to be associated with the presence of PsHV. It is also possible that this conure was co-infected with another PsHV genotype that caused the lesions (Tomaszewski, personal communication).

The discovery of PsHV 2 in African grey parrots suggests that there may be a diversity of PsHVs that are yet to be described in other psittacine species (Styles et al., 2005). Whether PsHV 2 causes IPD is unknown, but two African grey parrots were reported to have mucosal papillomas (Styles et al., 2005). It is unknown whether these lesions were caused by PsHV 2 or were coincidental with the infection. This is because PsHV 2 DNA was also amplified from the tissues of two other African grey parrots in the study that lacked any lesions or history of lesions (Styles et al., 2005). Infection by PsHV 2 may complicate the identification of PsHV 1 infected birds given that primers used in PCR diagnostic testing may detect both species of PsHV. Because PsHV-1 can cause Pacheco's disease in many species of psittacine birds including African grey parrots, infected parrots should be segregated from susceptible birds. However, infection with PsHV-2 may have less clinical significance, and differentiating these infections by the use of virus-specific PCR primers is indicated. The existence of PsHV

2 also opens the possibility that there may be other unrecognized PsHVs in other species. PsHV 1 has been associated with parrots from the neotropics, and now PsHV 2 with African grey parrots. Since the proto-continent that included Australia is believed to be the origin of psittaciforms (Cracraft, 2001), then species from that continent may be good candidates for future investigation for other PsHVs.

Future studies

Infectivity studies may be required to answer conclusively the question of whether PsHV 1 can cause IPD or other tumors. Efforts to transmit mucosal papillomas to clinically normal birds with homogenates failed (Sundberg et al., 1986). However in this transmission attempt, the birds were necropsied after 10 weeks and there may not have been sufficient time for lesions to develop, or there were insufficient numbers of infectious virions in the innoculum. Such an infectivity study would probably require a minimum of two years to identify and test the appropriate model species. In addition, these studies could be quite expensive due to the cost of test subjects, their maintenance, and the labor involved in maintaining them.

The pool of likely choices as test subjects would include Amazon parrots (*Amazona*), conures (*Aratinga*), and macaws (*Ara*). Each of these species has merit as a model, but *Ara* macaws, such as blue and gold macaws, may be best suited for test subject given the lower cost of these animals, their representation in the IPD population, and that these birds are susceptible to infection but rarely die of Pacheco's disease (PD) with genotype 3 (Phalen et al., 2004; Tomaszewski et al., 2003). These birds can be acquired as

incubator-hatched chicks at four weeks of age and could be hand-reared to completion to ensure a specific pathogen-free population.

Infecting the birds would require inoculating infectious PsHV 1 genotype 3 virions probably by an oral route. These macaws should become infected but are not likely to develop PD. If species more sensitive to genotype 3 are used in the study or if the macaws develop clinical signs, it may be possible to induce a latent infection by rescuing the birds with the anti-herpesviral drug acyclovir that might permit infection without resulting in PD.

The results of our investigations demonstrated that PsHV was present in the tumors associated with IPD, but not unaffected tissue. Efforts to localize PsHV in the neoplastic tissues failed due to the limit of detection of the methods used coupled with low concentrations and the poor integrity of the PsHV DNA. It may be possible to improve the detection rate of these methods by using frozen sections fixed briefly with paraformaldehyde rather than FFPE tissues (Chen et al., 2002). The use of frozen sections with LCM or in situ techniques would avoid the problems related to harsh fixation and is associated with a higher degree of success. If sensitivity can be improved, then it may be possible to employ another detection system such as digoxigenin, and avoid the non-specific staining associated with the chromagen-tyramide amplification system in those tissues that were problematic. Another approach would be to sample the frozen sections using LCM in conjunction and examined by nested PCR or possibly employ real-time PCR to quantify the concentration of PsHV in affected versus unaffected tissues. The negative controls for such an experiment would

include analogous tissues from birds known not to be infected with PsHV, and similarly positive controls from birds that died of Pacheco's disease.

The cutaneous papillomas found on the feet and legs of cockatoos and macaws contain herpesvirus virions (Lowenstine et al., 1983; Schmidt et al., 2003). Yet, these herpesviruses have yet to be characterized and do not amplify with known PsHV primers (Phalen et al., 1998). Four cockatoos with plantar papillomas were reported by Lowenstine et al. (1983). Three of these birds were most likely imported, and the fourth bird was documented as newly imported. Whether the herpesviruses found in these lesions are endemic to cockatoos or originate in an unknown host species is unclear because birds undergoing the quarantine process would have been exposed to parrots from other continents. Further studies elucidating these viruses will need to be conducted, probably starting with the use of degenerate primers for conserved herpesvirus sequences similar to those used in the discovery of PsHV 2. Another approach may be to use in situ hybridization using randomly generated probes to one or more genotypes of PsHV whole virus. Random probes would target more of the PsHV genome and perhaps enhance detection potential.

The discovery of PsHV 2 in African grey parrots has potential implications for the existence of a broader PsHV family. The sample size in our investigation was small (4 birds), and the prevalence of PsHV 2 is unknown. A PCR survey for the presence of PsHV 2 DNA in captive African grey parrots and other psittacines of African origin would provide some insight into the species range affected by this virus. Degenerate herpesvirus primers such as those used to amplify the UL30 gene (VanDevanter et al., 1996) of PsHV 2 could be used to survey Australian parrots. Ideally, the testing of wild

parrots in Africa may help to determine whether PsHV 2 originated in parrots on the African continent or came from other species. Investigating whether PsHV 2 is pathogenic is more challenging. Whether it is associated with papillomas could not be concluded from the results of our investigation.

The results of these investigations provide strong evidence linking PsHV to the development of IPD and other tumors in parrots. The findings show that mucosal papillomas are strongly associated with the presence of PsHV and suggest that bile and pancreatic duct tumors are manifestations of IPD and that proventricular/ventricular tumors may be related. Cloacal carcinomas appear to develop by a different pathway. With the exception of some mucosal papillomas, the development of all of these tumors is associated with the presence of PsHV 1 genotype 3. Further studies will need to be performed to elucidate the oncogenic mechanisms of this genotype.

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