Importance of the Pre-\(NH_2\)-Terminal Domain of HSV-1 DNA Polymerase for Viral Replication

Dissertation Advisor: Dr. Donald M. Coen **Shariya Louise Terrell**

Importance of the Pre-NH_2-Terminal Domain of HSV-1 DNA Polymerase for Viral Replication

Abstract

The catalytic subunit of the herpes simplex virus 1 DNA polymerase (HSV-1 Pol) has been extensively studied; however, its full complement of functional domains has yet to be characterized. The previously uncharacterized pre-NH_2-terminal domain (residues 1-140) within HSV-1 Pol is unique to the herpesvirus Pol family. We sought to investigate the importance of this domain for viral replication in cell culture and an animal model of infection.

We evaluated the enzymatic activity of purified pre-NH_2-terminal Pol mutant proteins in which conserved residues had been deleted or substituted. Subsequently, the corresponding *pol* mutant viruses were engineered for viral genetic analyses. We found that the extreme N-terminal 51 residues were not required for wild type 5'-3' polymerase activity *in vitro*. Interestingly, the extreme N-terminal 42 residues were dispensable for viral replication in cell culture while a conserved motif at residues 44-49 was necessary for efficient viral DNA synthesis and production of infectious virus.

Viral replication proteins have proven to be particularly important in the context of acute and latent infections in animals. Characterization of *pol* mutant virus replication in a mouse ocular model of infection revealed that the extreme N-terminal 42 residues

were not required for viral replication and reactivation from latency. The conserved motif, however, was shown to be required for robust acute ganglionic replication and efficient latency establishment.

We hypothesized that the conserved motif at residues 44-49 mediates a protein-protein interaction that positively impacts viral DNA synthesis during infection. Specific protein candidates were evaluated using purified proteins *in vitro*, and proteins that coprecipitated with wild type and mutant polymerases from infected cell lysates were analyzed. To date, we have yet to identify a protein whose binding was disrupted as a result of the mutation.

Ultimately, we have established a role for the pre-NH_2-terminal domain of HSV-1 Pol during viral replication that is distinct from 5'-3' polymerase activity. The conserved motif mediates a function that is required for efficient viral DNA synthesis in cell culture and is of even greater importance for acute ganglionic replication in mice. The mechanism of action more than likely reflects a conserved mechanism for herpesvirus replication.

Acknowledgements

I thank my Mom, Sandra Terrell, for being a wonderful mother, friend, mentor, and educator, among many other roles. I would not be who I am today without her unwavering love and support.

I thank my friends in the BBS program for their friendship and support. We have shared a lot of great memories throughout our graduate school years and I am grateful to have had them in my life. I also thank Kate Hodgins, Maria Bollinger, Steve Obuchowski, and Danny Gonzalez of the BBS office for their support throughout the years.

I thank my advisor Don Coen for his guidance and support. I am grateful for the patience and kindness he has shown me throughout my journey in graduate school. I would also like to thank current and former colleagues of Don Coen's laboratory for their advice, support, and friendship.

I thank my dissertation advisory committee David Knipe, Charles Richardson, and Sean Whelan for their willingness to assist me throughout my graduate career.

Attributions

The animal studies in Chapter 3 were performed in collaboration with Jean Pesola in the laboratory of Donald Coen at Harvard Medical School. Ping Bai, in the laboratory of Sandy Weller at the University of Connecticut Health Center, performed SPR analyses and contributed the corresponding Materials and Methods section in Chapter 4. Purified UL2 proteins were provided by Yaralid Sotomayor-Castro in the laboratory of Paul Boehmer at the University of Arizona College of Medicine-Pheonix.

Chapter 1

Introduction

Herpesviridae

The Herpesviridae family is comprised of icosahedral, enveloped, double stranded DNA viruses (reviewed in Pellet and Roizman, 2006). Herpesviruses are of ancient origin and have coevolved with their natural hosts, which is evidenced by the fine-tuned regulation of virus-host interactions that allow for efficient replication of the virus while promoting survival of the infected organism. All herpesviruses encode DNA replication machinery, replicate and assemble viral particles within the nucleus, and can establish latent infections. To date, the herpesviridae family contains more than 200 viruses that can infect a variety of vertebrate and invertebrate species. Eight herpesviruses have been identified as specific for humans: herpes simplex virus 1 and 2 (HSV-1, -2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), human herpesviruses 6 and 7 (HHV-6, -7), and Kaposi's sarcoma herpesvirus (KSHV).

Based on tropism and replication properties in animals and cell culture, herpesviruses can be divided into three major categories. Alpha herpesviruses (e.g. HSV-1,-2, VZV) exhibit the most diverse host cell range, a rapid and efficient replication cycle, and mainly establish latency within sensory ganglia. Beta herpesviruses (e.g. HCMV, HHV-6,-7) are characterized by a slow replication cycle, restricted host range, and latency establishment in glandular and lymphatic tissues. The lymphotropic gamma herpesviruses (e.g. EBV, KSHV) exhibit extended reproductive cycles and have been associated with cancer in humans.

The ability of herpesviruses to successfully manipulate cellular defense mechanisms, subvert host immune responses and persist for the lifetime of the host has posed a serious challenge to the development of effective treatments. Nucleoside analogs

(e.g. acyclovir), which target the viral DNA polymerase catalytic subunit, represent the main treatment option for herpesvirus infections (reviewed in Coen and Schaffer, 2003). However, the efficacy of these compounds is limited for immunocompromised patients, in which drug resistant infections readily develop. Investigations of the molecular mechanisms that govern viral replication processes within the host will provide valuable insight that may aid in the development of more successful treatments options. HSV-1 is a great model in which to study the biological characteristics of lytic and latent infections in both cell culture and in animal models of infection. Additionally, it has served as a valuable tool in the analysis of cellular processes such as eukaryotic DNA replication and translation. This dissertation focuses on the genetic analysis of the HSV-1 DNA polymerase catalytic subunit (HSV-1 Pol) and its importance to viral replication in cell culture and mice.

HSV-1

HSV-1, which is maintained within the human population, has an estimated seroprevalence of up to 70% among healthy adults in the United States (Coen and Schaffer, 2003). Infectious virus can be transmitted horizontally between individuals through close personal contact and vertically from mother to child (reviewed in Roizman et al., 2006). Symptoms of HSV-1 infection include oral and facial lesions that manifest during recurrent infections in affected individuals. Additionally, HSV-1 has been identified as a cause of herpetic genital lesions (Lafferty et al., 2000; Roberts et al., 2003). Occasionally, HSV-1 infection results in dehabilitating diseases such as encephalitis and ocular keratitis (Roizman et al., 2006). HSV-1 maintains a lifelong

association with the host without resulting in death in most cases, which contributes to the high prevalence of the virus throughout the population. However, newborns and immunocompromised individuals are at greater risk of developing disseminated infections that can result in fatality.

HSV-1 Replication

Genome

The HSV-1 genome is a linear, double stranded DNA molecule with a 68% G+C content (Kieff et al., 1971). The 150-kilobase pair genome can be divided into two elements, one unique long (U_L) and one unique short (U_S) region, that are flanked by inverted repeats (Wadsworth et al., 1976). Homologous sequences within internal and terminal repeat regions allows for the inversion of U_L and U_S during infection to yield four isomers at equimolar ratios (Delius and Clements, 1976; Hayward et al., 1975).

Structure

The basic structure of the mature viral particle is well conserved among herpesviruses (reviewed in Pellet and Roizman, 2006; Roizman et al., 2006). The HSV-1 virion is an enveloped, icosahedral capsid that is 100nm in diameter. The outer lipid bilayer, which is acquired from cellular membranes, contains viral glycoproteins that mediate attachment and entry. The core of the icosahedral capsid contains genomic viral DNA. The tegument comprises the area between the capsid and lipid bilayer and lacks defined structure. At least 20 viral proteins reside within the tegument and are delivered with the viral capsid into the cytoplasm of the infected cell.

HSV-1 Replication in Cell Culture

Glycoproteins on the surface of the virion mediate attachment and fusion of the viral envelope and cellular plasma membrane (Campadelli-Fiume et al., 2000; Spear et al, 2003). Tegument proteins are released into the cytoplasm, some of which act to prime the cell for infection by preventing activation of the cellular antiviral response and shutting down host mRNA translation (Cassady et al., 1998; Strom and Frenkel, 1987). The viral capsid is transported to the nucleus and releases viral DNA through nuclear pores (Batterson et al., 1983; Miyamoto and Morgan, 1971; Morgan et al., 1968). This event triggers the organized, temporal cascade of viral gene expression that is characteristic of HSV-1 infection in cell culture (Honess and Roizman, 1974; reviewed in Roizman et al., 2006). Prior to *de novo* protein synthesis, tegument protein VP16 associates with cellular transcriptional machinery to activate the expression of immediate early gene products that further attenuate host gene expression and antiviral responses (Hardy and Sandri-Goldin, 1994; LaBoissiere and O'Hare, 2000; York et al., 1994). Immediate early proteins also function as transcriptional activators that promote early viral gene expression (Roizman et al., 2006). Early viral proteins, which are typically involved in DNA replication and nucleotide metabolism, execute viral DNA synthesis and stimulate late gene expression. Late gene products are mainly structural proteins that are packaged and assembled into progeny virions. Late viral proteins also function in egress of the virus, a less well-defined process in which naked capsids exit the nucleus and translocate to the plasma membrane for release from the infected cell.

HSV-1 Replication in Mouse Models of Infection

A hallmark of HSV-1 replication is the ability of the virus to undergo both productive and latent infections within the mammalian host (reviewed in Roizman et al., 2006; Wagner and Bloom 1997). During primary infections of humans, HSV-1 replicates at a peripheral site of infection such as the eye or mucosal surfaces. Viral particles are transported to innervating sensory ganglia and establish latent infections. Upon stimulation such as stress or UV light exposure, the virus will reactivate from its latent state and initiate a recurrent infection at the original site of inoculation.

Although various *in vitro* latency models have been developed, animal models of infection remains the most physiologically relevant system in which to dissect the molecular aspects of acute and latent infections. The field has yet to identify an animal model of infection that reproduces every facet of HSV-1 infection as observed in humans. However, the mouse ocular model has been demonstrated to mimic specific stages of HSV-1 pathogenesis (reviewed in Wagner and Bloom, 1997). Virus inoculated on the eye via corneal scarification undergoes robust amplification at the periphery. The virus travels to the trigeminal ganglia (TG) from the cornea via anterograde transport and initiates a second round of productive infection (Lycke et al., 1984; Penfold et al., 1994). Latency is established in sensory neurons, wherein lytic gene expression is repressed and no infectious virus is produced (Reviewed in Efstathiou and Preston, 2005; Roizman et al., 2006). Latency associated transcripts and viral DNA molecules can be detected in latently infected TG (Efstathiou et al., 1986; Rock and Fraser 1983; Spivack and Fraser 1988; Stevens et al., 1987). One drawback for the mouse eye model is that infectious virus cannot be recovered from the original site of infection upon reactivation as is

observed in humans. The most widely-used assay for reactivation is the *in vitro* explant method where latently infected TG are cultured with susceptible feeder cells that serve as a readout for cytophathic effect and production of infectious virus (McLennan and Darby 1980; Stevens and Cook, 1971). The molecular mechanisms that govern the maintenance and reactivation from latency are poorly understood and remain under intense investigation.

HSV-1 DNA Replication

Viral Replication Proteins

The HSV-1 replisome components greatly resemble those found in *E.coli* and bacteriophage T4 systems (Boehmer and Lehman, 1997). HSV-1 encodes seven proteins that are required for viral DNA synthesis: origin binding protein (UL9), single-stranded DNA binding protein (ICP8), helicase-primase complex (UL8/5/52), and DNA polymerase holoenzyme (HSV-1 Pol (UL30)/UL42) (McGeoch et al., 1988; Wu et al., 1988). Immunofluorescence studies suggest that UL9, ICP8, and UL8/5/52 are necessary for viral replisome assembly at discrete foci within the nucleus called prereplicative sites (de Brun and Kops, 1994; Quinlan et al., 1984). The recruitment of the viral DNA polymerase holoenzyme and subsequent progression of viral DNA synthesis promotes fusion of prereplicative sites, which form replication compartments that eventually encompass the nucleus.

The viral genome contains three origins of replication: one copy of Ori_L located within the U_L region, and two copies of Ori_S that reside in the internal repeat short and terminal repeat short regions (Stow, 1982; Weller et al., 1985). Each origin contains an

A-T rich region that is flanked by inverted repeats containing DNA recognition sites for UL9 (Elias et al., 1990; Koff and Tegtmeyer, 1988). Presumably, UL9 initiates viral DNA synthesis through its ability to bind and activate origins of replication (Elias et al., 1986; Olivo et al., 1988). UL9 homodimers bind cooperatively to the inverted repeats and induce localized melting of the A-T rich spacers (Elias and Lehman, 1988; He and Lehman, 2001; Makhov et al., 1996). UL9 helicase activity, which is essential for viral replication, promotes DNA unwinding at the origin (Boehmer et al., 1993; Fierer and Challberg, 1992; Martinez et al., 1992; Malik et al., 1992).

The single stranded binding protein ICP8 is presumably recruited to the origin of replication through an interaction with UL9 (Boehmer and Lehman, 1993). This interaction stimulates UL9 helicase activity by enhancing the rate of DNA unwinding (Boehmer et al., 1993; Makhov et al., 1996). ICP8 molecules bind cooperatively to exposed single stranded DNA and this binding activity is essential for viral replication (Lee and Knipe, 1985; Ruyechan et al., 1983). ICP8 has been shown to stimulate HSV-1 Pol activity, which is a characteristic of single-stranded DNA binding proteins found in prokaryotic and eukaryotic systems (reviewed in Chase and Williams, 1986; Hernandez and Lehman, 1990; Ruyechan and Weir, 1984); however, studies have yet to determine whether this stimulation is a result of a direct interaction between HSV-1 Pol and ICP8.

Origin unwinding and stabilization through the activities of UL9 and ICP8 promote the recruitment of the DNA replication machinery to generate an active replication fork. The process of viral DNA synthesis will not commence without the generation of RNA primers, which is mediated by the heterotrimeric helicase-primase complex (Crute et al., 1988; Crute et al., 1989). Subunits UL5 and UL52 possess helicase

and primase activities that are essential for viral replication (Dracheva et al., 1995; Klinedinst et al, 1994; Zhu and Weller, 1992a,b). Thus, the UL5/UL52 heterodimer represents the core enzyme, while the UL8 subunit alone does not exhibit enzymatic or DNA binding activities (Calder and Stow, 1990; Dodson and Lehman, 1991; Parry et al., 1993). However, UL8 is necessary for the nuclear localization of UL5 and UL52 in infected cells and stimulates primase activity of the protein complex *in vitro* (Barnard et al., 1997; Marsden et al., 1996; Tanguy Le Gac et al., 1996; Tenney et al., 1994; Tenney et al., 1995). The helicase-primase complex can associate with UL9 and ICP8 via an interaction with the UL8 subunit, which may be necessary for recruitment to active origins of replication and coordination of viral replisome components during DNA replication (McLean et al., 1994; Tanguy Le Gac et al., 1996).

Primase activity is required for the recruitment of the DNA polymerase holoenzyme to prereplicative sites (Carrington-Lawrence and Weller, 2003). The catalytic subunit, HSV-1 Pol (UL30), possesses 5'-3' polymerase activity that is absolutely essential for replication of genomic viral DNA and production of infectious virus (Aron et al., 1975; Dorsky and Crumpacker, 1988). Reported HSV-1 enzymatic activities include 3'-5' exonuclease, RNase H and DNA lyase activities (Bogani and Boehmer, 2008; Crute and Lehman, 1989; Knopf and Weisshart, 1988; O'Donnell et al., 1987). The processivity subunit UL42 is a functional analog and structural homolog of the T4 bacteriophage gp45 protein, *E.coli* Pol III β subunit, and eukaryotic proliferating cell nuclear antigen (PCNA; Jarvis et al., 1989; Kong et al., 1992; Krishna et al., 1994; Zuccola et al., 2000). Unlike these DNA polymerase clamp proteins, UL42 can freely associate with DNA due to its intrinsic DNA binding activity (Vaughan et al., 1985;

Gallo et al., 1988). This activity serves to tether HSV-1 Pol to the primer-template and thereby increase processivity of the enzyme (Gottlieb et al., 1990; Hernandez et al., 1990; Randell et al., 2005).

HSV-1 expresses several nonessential replication proteins that have demonstrated roles in viral DNA synthesis processes. The viral thymidine kinase (TK) enzyme, a homodimer of the UL23 gene product, phosphorylates nucleosides in order to generate deoxynucleotide triphosphates (dNTPs) that serve as substrates for HSV-1 Pol 5'-3' polymerase activity (Chen et al., 1979; Brown et al., 1995). While viral TK activity is dispensable for WT levels of replication during infection of dividing cells, it is important for efficient viral DNA synthesis in resting cell cultures wherein cellular TK enzyme expression is reduced or absent (Field and Wieldy, 1978; Jamieson et al., 1974). Furthermore, TK^- mutants cannot replicate in ganglia and reactivate from latent infections in mice (Coen et al., 1989; Chen et al., 2004; Tenser et al., 1989; Thompson and Sawtell, 2000).

Another viral nucleotide metabolism enzyme is the ribonucleotide reductase (RR), which catalyzes the formation of dNTPs from ribonucleotide precursors (Huszar and Bacchetti, 1981; Ponce de Leon et al., 1977). The active enzyme is an $\alpha 2$-$\beta 2$ tetramer with two copies of both the large subunit, UL39, and small subunit, UL40 (McGeoch et al., 1988; Bacchetti et al., 1986; Ingemarson and Lankinen, 1987). Although a functional homolog is present in actively dividing cells, viral RR^- mutants exhibit a modest defect in viral DNA synthesis in actively dividing cells that is enhanced in resting cell cultures (Goldstein and Weller, 1988 a and b; Jacobson et al, 1989; Preston et al., 1988). The viral

RR is absolutely required for acute corneal and ganglionic replication in the mouse ocular infection model (Jacobson et al., 1989).

The viral uracil DNA glycosylase (UDG; UL2) participates in base excision repair processes by excising misincorporated uracils from viral DNA (Caradonna and Cheng, 1981; Mullaney et al., 1989). Most of the work regarding viral UDG function in cell culture has been performed in HCMV. Mutant viruses lacking UL114, the HCMV UDG, exhibit a delay in viral DNA production during infection that appears to be specific for resting cell cultures wherein the cellular UDG is absent (Courcelle et al., 2001; Prichard et al., 1996). Authors suggest that the inability of the UL114^{-} viruses to remove uracils from newly synthesized viral DNA prevented the rapid accumulation of progeny viral DNA molecules (Courcelle et al., 2001). In HSV, UL2 is dispensable for viral replication in cell culture, but is important for efficient acute replication and latency establishment in the mouse footpad model of infection (Pyles and Thompson, 1994). Although it is not absolutely required for reactivation, the UL2^{-} virus exhibited a ~3-fold in reactivation efficiencies following induced *in vivo* reactivation in mice via transient hyperthermia.

Cellular DNA Replication and Repair Proteins

A variety of cellular proteins that are involved in DNA replication, repair, and recombination are associated with viral proteins and localize to replication compartments during infection (Taylor and Knipe, 2004; Wilcock and Lane, 1991; Wilkinson and Weller, 2004). DNA replication proteins Polα, DNA ligase I, PCNA and replication protein A (RPA) colocalize with ICP8, and may play a functional role during viral DNA

synthesis processes (Wilock and Lane, 1991). For example, DNA ligase I can repair BER intermediates generated by HSV-1 Pol and UL2 *in vitro* (Bogani et al., 2009). Polα has been directly linked to viral DNA replication in that it can physically interact with UL9, which results in stimulated 5'-3' polymerase activity *in vitro* (Lee et al., 1995). Additionally, Polα can elongate RNA primers generated by the viral helicase-primase complex *in vitro* (Cavanaugh and Kuchta, 2009). The possibility of cellular replication proteins actively participating during viral DNA synthesis is an attractive one; however, their importance for viral replication in the infected cell has yet to be evaluated.

HSV-1 infection leads to the perturbation of cellular DNA damage and repair pathways. Ataxia-telangiesctasia-mutated (ATM) transduction pathways, which stimulate homologous recombination repair (HRR) of double-strand breaks (DSBs), are activated during HSV-1 infection (Abraham, 2004; Lilley et al., 2005; Shirata et al., 2005; Wilkinson and Weller, 2004). Activated MRN complexes (RAD50/Nbs1/Mre11), a downstream target of ATM, localize to viral replication compartments (Shirata et al., 2005; Wilkinson and Weller, 2004). Additionally, MRN components RAD50 and meiotic recombination 11 (Mre11) associate with ICP8 in infected cell lysates (Taylor and Knipe, 2004). HSV-1 replication is impaired in cell lines that are deficient for functional ATM and Mre11 proteins as compared to their corresponding complementing cell lines (Lilley et al., 2005). The loss of ATM signaling results in a defect in viral DNA synthesis, which implicates a role for the DNA damage response in HSV-1 DNA replication during infection. However, this conclusion was contradicted by Shirata et al. in that knockdown of ATM did not affect HSV-1 replication (Shirata et al., 2005). Further investigation is

necessary to clarify the functional role, if any, of ATM pathway proteins during HSV-1 infection.

Nonhomologous end-joining (NHEJ) repair proteins, which also respond to DSBs, are mobilized during HSV-1 infection (Abraham, 2004; Taylor and Knipe, 2004). Components of the NHEJ complex localize to replication compartments and associate with ICP8 in infected cell lysates (Taylor and Knipe, 2004). NEHJ repair protein Ku86 localizes to replication compartments but does not specifically associate with prereplicative sites, which suggests that NHEJ repair processes do not contribute to the early stages of viral DNA synthesis (Wilkinson and Weller, 2004). In contrast to HRR pathway proteins, HSV-1 replication was enhanced in the absence of NHEJ components Ku70 and DNA dependent protein kinase catalytic subunit (DNA PKc; Parkinson et al., 1999; Taylor and Knipe, 2004). Thus, it would appear that the NHEJ repair pathway is detrimental to HSV-1 replication although the exact mechanism of inhibition is unknown.

Proteins that participate in the ATM and Rad3 related (ATR) pathway, which is primarily triggered by stalled replication forks, are also recruited to viral replication compartments (Cimprich and Cortez, 2008; Mohni et al., 2010; Mohni et al., 2012). Interestingly, ATR pathway proteins are important for viral replication while the activation of ATR signaling negatively impacts HSV-1 recombination frequencies during infection (Mohni et al., 2010; Mohni et al., 2012). Activation of tumor suppressor protein p53 by ATR results in the downregulation of HRR repair of DSBs, possibly via p53-mediated sequestration of HRR proteins (Sirbu et al., 2011; Romanova et al., 2004; Sturzbecher et al., 1996; Linke et al., 2003). Interestingly, several cellular proteins

including p53 are redistributed upon HSV-1 infection and colocalize with ICP8 in replication compartments (Wilcock and Lane, 1991; Taylor and Knipe, 2004).

As with other replication processes, the virus must manipulate cellular machinery in order create an environment that is favorable for replication. Expression of viral replication proteins, and potentially viral DNA synthesis, can trigger ATM- and ATR-mediated repair pathways (Lilley et al., 2005; Shirata et al., 2005). It remains unclear as to whether the induction of cellular DNA damage pathways represents an active recruitment of HRR proteins to viral replication loci or if it is an inevitable response to viral infection. Further investigation is necessary in order to define the functional roles of specific cellular proteins during viral DNA synthesis.

Model of HSV-1 DNA Replication

HSV-1 genomic termini become fused upon infection, which has been proposed to represent circularization of the viral genome (Garber et al., 1993; Poffenberger and Roizman, 1985; Strang and Stow, 2005). Newly synthesized viral DNA within the infected cell is arranged as head-to-tail concatamers of unit-length viral genomes (Jacob et al., 1979). Originally, viral DNA synthesis was hypothesized to proceed by two distinct modes of replication (Figure 1.1.; Boehmer and Lehman, 1997). Initially, the ordered assembly of the viral replisome components at the origin results in theta replication with bidirectional fork movement and coordination of leading and lagging strand synthesis. Theta replication is then converted to a rolling circle mode by an unknown mechanism in order to generate the observed head-to-tail concatamers.

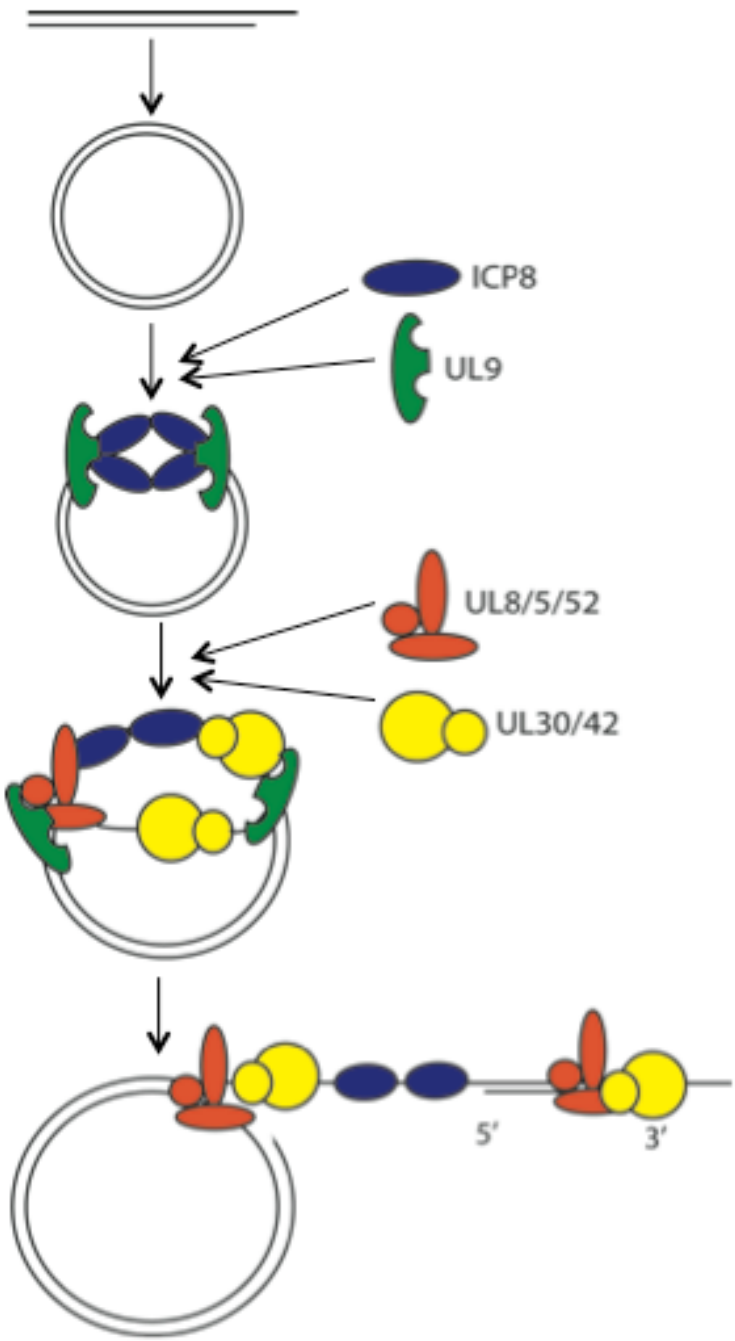

Figure 1.1. Model of HSV-1 DNA replication. Linear viral DNA is circularized during infection. UL9 binds origins of replication and induces localized melting of duplex DNA. ICP8 binds single-stranded DNA and promotes UL9-mediated DNA unwinding. Helicase-primase (UL8/5/52) activity recruits the DNA polymerase holoenzyme (UL30/42) and initiates DNA synthesis via theta replication mode. In an unknown mechanism, theta replication is converted to rolling circle mode in which head-to-tail concatamers of unit-length viral genomes are produced. Figure adapted from Roizman et al., 2006.

However, the rolling circle mode of replication does not account for the branched structure of viral DNA that is observed during HSV-1 infection (Severini et al., 1996).

Many lines of evidence suggest that DNA recombination events are an integral part of viral DNA synthesis processes, which could at least partially account for the production of concatameric and branched DNA. Once in the nucleus, genomic viral DNA appears to associate with promyelocytic leukemia nuclear bodies (PML; ND10) that contain cellular recombination and repair proteins (Maul et al., 1996; Carbone et al., 2002; Negorev and Maul, 2001). This arrangement may aid in the recruitment of specific DNA repair proteins and components of the HRR pathway that may be important for efficient HSV-1 replication (Lilley et al., 2005; Wilkinson and Weller, 2004). Cellular proteins RPA and the MRN complex promote strand invasion following DSBs, and these proteins are localized to viral replication compartments during infection (Alani et al., 1992; Sung, 1994; Wilcock and Lane, 1991; Wilkinson and Weller, 2004; Shirata et al., 2005). The exact mechanisms by which cellular repair proteins enhance viral replication have yet to be elucidated. As seen with other viral replication processes, it would be advantageous for the virus to hijack and redirect host proteins towards the production of infectious progeny virus.

Viral proteins can also directly participate or promote cellular proteins to engage in DNA recombination events during viral DNA synthesis. Replication of the episomal simian virus 40 (SV40) genomic DNA using HSV-1 replisome components in infected cells results in the production of complex branched structures that are similar to those observed during HSV-1 infection (Blumel et al., 2000). Viral proteins ICP8 and the alkaline nuclease UL12 catalyze strand exchange between a linear double-stranded DNA

molecule and circular, single-stranded DNA *in vitro*, which could contribute to the formation of concatameric DNA as seen with the bacteriophage lambda recombination system (Reuven et al., 2003). The exonuclease activity of UL12 is essential for promoting recombination via a strand annealing mechanism during infection, and this activity is enhanced in the presence of ICP8 or the cellular single strand annealing protein RAD52 (Schumacher et al., 2012). Additionally, UL12 binds directly to the MRN complex, which provides an additional link between viral and cellular recombination machinery (Balasubramanian et al, 2010). UL12^{-} mutants exhibit a dramatic decrease in the production of mature viral capsids without any meaningful alterations in viral DNA synthesis levels (Martinez et al., 1996; Shao et al., 1993). Based on these observations, authors suggest that UL12 aids in the processing of branched DNA structures that arise during viral DNA synthesis.

It has become increasingly evident that the process of viral DNA synthesis in the infected cell is more complex than originally perceived. These data suggest that viral proteins in conjunction with cellular proteins mediate recombination events that are important for the efficient production of viral DNA. These studies underscore the dynamic interplay between the virus and host cell during infection, which poses a serious challenge in our ability to recapitulate the process of viral DNA synthesis *in vitro*. However, the examination of HSV-1 replication provides a useful tool in dissecting the molecular mechanisms of eukaryotic DNA replication.

HSV-1 Pol

Structure

HSV-1 Pol is a member of the DNA polymerase family Pol B, which includes eukaryotic polymerases α, δ, and ε, in addition to viral and bacteriophage polymerases that are responsible for genomic DNA replication (Hubscher et al., 2002; Lehman and Kaguni, 1989; Rothwell and Waksman, 2005). HSV-1 Pol consists of 1235 amino acids and exhibits six regions of homology that are characteristic of the replicative DNA polymerase Polα (Knopf and Weisshart, 1988; Liu et al., 2006; Wang et al., 1989). Additionally, HSV-1 Pol shares significant structural homology with Polα family members, specifically bacteriophage RB69 (Hubscher et al., 2002; Liu et al., 2006). HSV-1 Pol is a single polypeptide that is comprised of six subdomains: palm, thumb, fingers, 3'-5' exonuclease, NH_2-terminal, and pre-NH_2-terminal domains (Figure 1.2; Liu et al., 2006; Weisshart and Knopf, 1988). The overall architecture of the enzyme is a ring-like structure in which double stranded DNA binds at the C-terminus with single stranded DNA threading through the central hole towards the N-terminus (Liu et al., 2006).

The palm, fingers, thumb, and 3'-5' exonuclease domains of HSV-1 Pol bear significant sequence and structural homology to corresponding domains in other Pol B enzymes, and are the best functionally characterized regions of the protein (Knopf and Weisshart, 1988; Liu et al., 2006; Rothwell and Waksman, 2005; Wang et al., 1989). The thumb, palm, fingers domains represent the prototypic catalytic center of polymerase activity (Hubscher et al., 2002, Wang et al., 1997). The thumb interacts with double stranded DNA while the palm and fingers domains coordinate primer-template binding

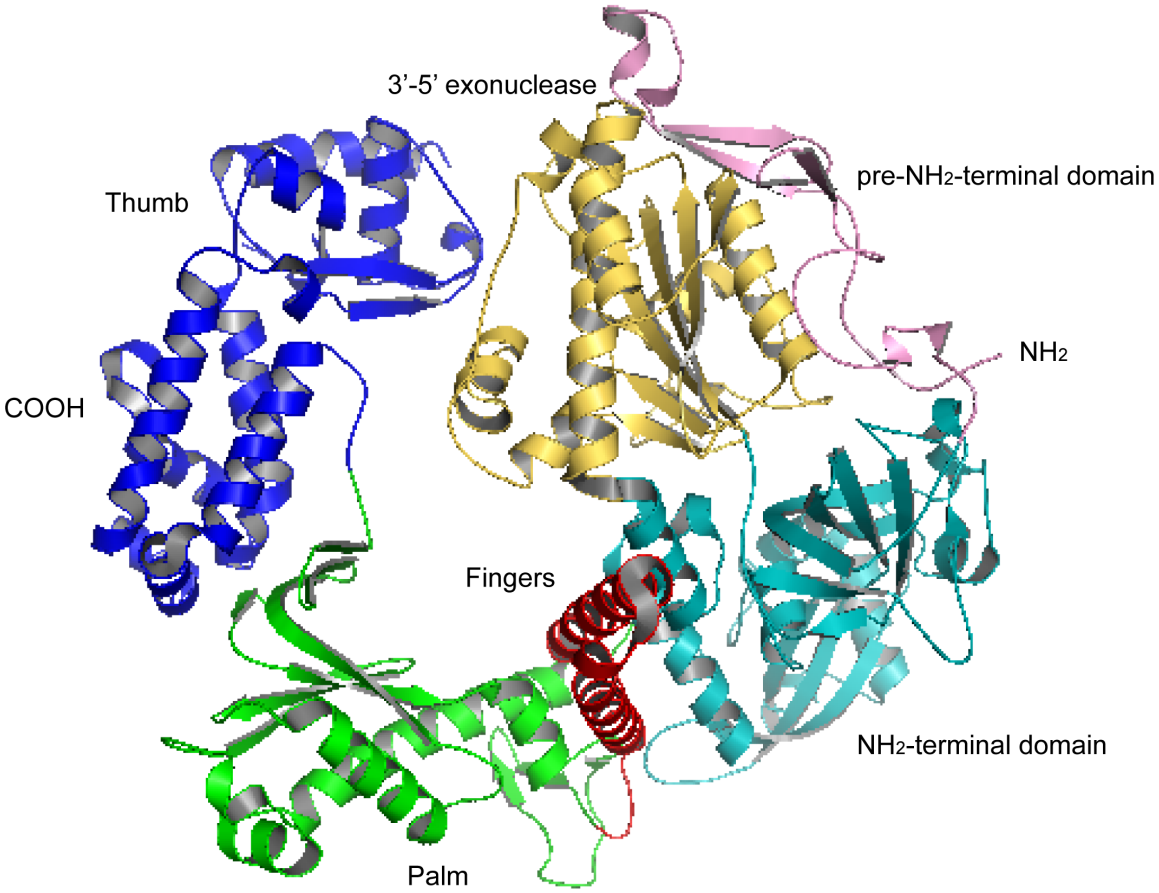

Figure 1.2. HSV-1 Pol crystal structure. HSV-1 Pol consists of 1235 residues that form a ring-like structure that is structurally homologous to other Family B polymerases. The enzyme is comprised of six subdomains: pre-NH_2-terminal domain (pink), NH_2-terminal domain (teal), 3'-5' exonuclease (yellow), fingers (red), palm (green), and thumb (blue). The crystal structure was solved by Liu et al., 2006.

and dNTP incorporation (Franklin, Wang, Steitz 2001; Steitz et al., 1994; Wang et al., 1997). Divalent metal ions can bind to residues located within the 3'-5' exonuclease domain, which serves as the active site for 3'-5' exonuclease activity. These four domains form a catalytic core that is well conserved among prokaryotic, eukaryotic, and viral DNA polymerases (Hubscher et al., 2002).

A crystal structure of HSV-1 Pol revealed a two domain architecture of the N-terminal portion of the enzyme (Liu et al., 2006). The architecture of the HSV-1 Pol NH_2-terminal domain resembles that of NH_2-terminal domains found in certain family B polymerases (Liu et al., 2006; Rodriguez et al., 2000; Swan et al., 2009; Wang et al., 1997; Wang et al., 1996).The second domain, at the extreme N- terminus of HSV-1 Pol, which was structurally distinct from the NH_2-terminal domain, was dubbed the pre-NH_2-terminal domain by Liu *et al* (Liu et al., 2006).

The NH_2-terminal domain of HSV-1 Pol consists of three motifs that are adjacent to the 3'-5' exonuclease domain (Liu et al., 2006). Based on the RB69 Pol structure, these two domains together form a putative ssDNA binding groove opposite from the dsDNA binding groove. Positioned at the putative ssDNA binding interface within the NH_2-terminal domain is a putative RNA binding motif (RNP motif) that is structurally similar to those found in pre-mRNA splicing factors (Birney et al., 1993; Liu et al., 2006); Burd and Dreyfuss, 1994). RNP motifs are conserved in 5'-3' exonucleases that exhibit RNase H activity and could potentially serve as the active site for such activity in HSV-1 Pol (Ceska and Sayers, 1998). Alternatively, the RNP motif could bind *pol* mRNA as suggested for Polα homologue T4, which has been shown to autoregulate its own expression (Pavlov and Karam, 1994; Rodriguez et al. 2000; Wang et al., 1996). The

proposed enzymatic activities of NH_2-terminal of HSV-1 Pol have yet to be characterized.

The pre-NH_2-terminal domain is comprised of the first 140 residues of HSV-1 Pol and is located at the surface of the enzyme (Liu et al., 2006). The first 58 residues of HSV-1 Pol are absent from the crystal structure and are presumably disordered. The extreme N-terminal 42 residues are conserved in HSV -1 and -2, while a motif at HSV-1 Pol residues 44-49 is highly conserved among all human herpesviruses (Di Tommaso et al., 2011; Liu et al., 2006; Notredame et al., 2000). HSV-1 Pol residues downstream of residue 59 engage in VanderWaals contacts with the adjacent 3'-5' exonuclease domain. The pre-NH_2-terminal domain is unique to herpesviruses polymerases and a homologous domain is absent from the related bacteriophage RB69 Pol and other Family B polymerase structures (Liu et al., 2006; Rodriguez et al., 2000; Swan et al., 2009; Wang et al., 1997; Wang et al., 1996). We have yet to identify a structural equivalent of the pre-NH_2-terminal domain in published protein structures and the function of this domain is unknown. Due to the conservation of the pre-NH_2-terminal domain among the herpesvirus Pol family, we hypothesized that it may be required for viral replication. The location of the pre-NH_2-terminal domain in the HSV-1 Pol structure, and absence of a structural equivalent in other DNA polymerases, suggested that the domain plays a specific role during herpesvirus replication that is distinct from 5'-3' polymerase activity.

Enzymatic Activities

HSV-1 Pol exhibits 5'-3' polymerase activity that is responsible for dNTP incorporation into nascent viral DNA strands (Marcy et al., 1990; Knopf ,1979). HSV-1

Pol 5'-3' polymerase activity is enhanced by an interaction with the viral processivity factor UL42 (Gottlieb et al., 1990; Hernandez et al., 1990). HSV-1 Pol is stimulated by high salt concentrations that inhibitory for cellular DNA polymerase activity (Powell and Purifoy, 1977; Weissbach et al., 1973). HSV-1 Pol requires a free 3'-OH in order to initiate dNTP incorporation and can utilize activated double-stranded DNA and primer-templates with a strong preference for DNA containing a high G-C content (Weissbach et al., 1973). Polymerase activity is absolutely essential for viral DNA synthesis and the production of infectious progeny virus in cell culture (Aron et al., 1975; Dorsky and Crumpacker, 1988).

The 3'-5' exonuclease activity of HSV-1 Pol serves as a proofreading function by removing misincorporated nucleotides during DNA polymerization (Hwang et al., 1999; Knopf and Weisshart, 1988; O'Donnell et al., 1987). HSV-1 Pol can degrade both single- and double- stranded DNA and exhibits enhanced activity on substrates with unpaired 3' termini (Knopf and Weisshart, 1988; Derse and Chen, 1981; O'Donnell et al., 1987). 3'-5' exonuclease activity is optimal under conditions that favor 5'-3' polymerase activity, but exonuclease activity is severely diminished in the presence of dNTPs (O'Donnell et al., 1987). Point mutations that abolish 3'-5' exonuclease activity have little effect on the intrinsic 5'-3' polymerase catalytic activity of the HSV-1 Pol enzyme (Hwang et al., 1997; Hall et al., 1995; Kuhn & Knopf, 1996). While 3'-5' exonuclease activity is not required for viral DNA synthesis or production of infectious virus in cell culture, it is important for replication fidelity as evidenced by increased mutagenesis frequencies for 3'-5' exonuclease deficient mutants (Tian et al., 2009; Hwang et al., 1997; Hwang and Hwang, 2003).

Several reports have demonstrated that HSV-1 Pol exhibits RNase H activity that degrades the RNA strand within an RNA-DNA hybrid (Marcy et al., 1990; Crute and Lehman, 1989; Weisshart et al., 1994). This activity could potentially be responsible for the removal of primers synthesized during lagging strand synthesis as observed with the PolI family of DNA polymerases (Ceska and Sayers, 1998; Hubscher et al., 2002). A 70-80kDa N-terminal fragment generated from proteolytic cleavage of HSV-1 Pol has been reported to exhibit both RNase H and 3'-5' exonuclease activities (Weisshart et al., 1994). However, some groups have attributed the observed RNase H activity to the 3'-5' exonuclease, but the results of such studies are inconclusive (Hall et al., 1996; Knopf and Weisshart, 1990). The possibility of whether or not HSV-1 Pol possesses an intrinsic RNase H activity that is separable from 3'-5' exonuclease activity remains under debate.

Most recently, HSV-1 Pol was found to possess lyase activities that correspond to those exhibited by the repair polymerase Polβ (Bogani and Boehmer, 2008). HSV-1 Pol exhibits 5'-deoxyribose phosphate and apurinic/apyrimidinic lyase activities that can execute steps in the base excision repair (BER) pathway. Furthermore, these cleavage events were demonstrated to proceed by a lyase mechanism that does not require metal cations. Although the active site has yet to be mapped, DNA lyase activity has been localized to a 63kDa C-terminal fragment of HSV-1 Pol. Authors speculate that HSV-1-mediated BER activities would be important for repair of the viral genome upon reactivation from latent infections in neurons. The contribution of HSV-1 Pol lyase activity to viral replication in cell culture and animal models of infection has yet to be determined.

Protein Interactions

The DNA polymerase holoenzyme is comprised of a stable 1:1 association between HSV-1 Pol and UL42 that readily copurifies and coimmunoprecipitates from infected cell lysates (Crute and Lehman, 1989; Vaughan et al., 1985; Gottlieb et al., 1990). The extreme C-terminal 18 residues of HSV-1 Pol are required and sufficient for UL42 binding (Digard et al., 1993a). The DNA binding activity of UL42 serves to tether HSV-1 Pol to the primer-template and thereby increases the processivity of dNTP incorporation and allows for long-chain DNA synthesis (Gottlieb 1990; Hernandez 1990; Randell et al., 2005). The association between the DNA polymerase holoenzyme subunits has been established as absolutely critical for viral DNA synthesis and production of infectious virus (Digard et al., 1993a, b). Thus, disruption of the HSV-1 Pol-UL42 complex has served as a basis for rational drug discovery (Pilger et al., 2004).

HSV-1 Pol copurifies with the viral helicase-primase complex from infected cell lysates (Strick et al., 1997). At least one report has demonstrated through several methods that the HSV-1 Pol binds directly to the UL8 subunit (Marsden et al., 1997). UL8 is required for efficient primer elongation by HSV-1 Pol *in vitro*; however, this effect did not appear to be species specific because the *E.coli* Pol I enzyme could substitute for HSV-1 Pol (Sherman et al., 1992; Tenney et al., 1994). Therefore, Marsden *et al* suggested that the observed interaction between UL8 and HSV-1 Pol served to recruit the viral polymerase to origins of replication for initiation of viral DNA synthesis. Consistent with this hypothesis is the fact that HSV-1 Pol is unable to localize to prereplicative sites in the absence of UL8 (Liptak et al., 1996). However, this reported observation was contradicted in a study that analyzed HSV-1 Pol localization with a virus that encoded a

truncated UL8 mutant (Marsden et al., 1996). To date, we have yet to directly evaluate the significance of the UL8-HSV-1 Pol interaction during viral replication.

The viral uracil DNA glycosylase UL2 was recently identified as an HSV-1 Pol binding partner and the two proteins colocalize to pre-replicative sites in transfected cells (Bogani et al., 2010). HSV-1 Pol can bind UL2 and UL42 simultaneously, which demonstrates that the UL2 interaction surface is outside of the UL42-binding site at the extreme C-terminus of HSV-1 Pol (Bogani et al., 2010). UL2 binding does not affect processive dNTP incorporation and is not required for the DNA lyase activities of HSV-1 Pol (Bogani and Boehmer, 2008; Bogani et al., 2010). UL2 and HSV-1 Pol can coordinate with cellular factors *in vitro* to excise and repair DNA substrates containing a misincorporated uracil (Bogani et al., 2009). HSV-1 Pol will stall upstream of a uracil in a primer extension assay only in the presence of UL2 (Bogani et al., 2000). This was presumably due to the excision of the uracil by UL2 and the resulting AP site cannot be processed by HSV-1 Pol. The HSV-1 Pol-UL2 interaction provides a link between DNA repair machinery and the viral replisome during viral DNA synthesis.

Host cell factor 1 (HCF-1) binds HSV-1 Pol in addition to viral replication proteins UL9 and UL52 (Peng et al., 2009). HCF-1 couples transcription factors with chromatin remodeling complexes in order to effectively regulate cellular gene expression (Kristie et al., 2010). Outside of its role as a transcriptional coactivator that promotes HSV-1 IE gene expression, HCF-1 has recently been shown to participate in viral DNA synthesis by recruiting the histone chaperone Asf1b to the viral replisome (Peng et al., 2009). Asf1 mediates chromatin reassembly during DNA replication, which is necessary for progression at the replication fork (Schulz and Tyler, 2006; Tyler et al., 1999;

Munakata et al., 2000). Interestingly, depletion of Asf1b prior to HSV-1 infection negatively impacted production of viral DNA and infectious virus (Peng et al., 2009). Asf1 interacts with the SWI/SNF chromatin remodeling complex, which was one of several chromatin remodeling proteins found to associate with ICP8 in infected cell lysates (Moshkin et al., 2002; Taylor and Knipe, 2004). These data implicate a role for host dependent chromatin remodeling during viral DNA synthesis.

Role of HSV-1 Pol in Disease Pathogenesis

Mutations that knock out HSV-1 Pol activity render the virus as replication incompetent in both cell culture and in mice, which is presumably due to the lack of 5'-3' polymerase activity that is responsible for production of progeny viral DNA molecules (Aron et al., 1975, Dorsky and Crumpacker, 1988; Katz et al., 1990). Hence, the viral polymerase has served as a prime target for antiviral therapies (reviewed in Coen and Schaffer). Compounds such as nucleoside analogs (e.g. acyclovir) that inhibit viral DNA synthesis prove to be an effective treatment for most individuals, but the efficacy of such drugs is significantly reduced in immunocompromised patients in which drug-resistant infections readily develop. Some drug-resistant mutant viruses contain lesions at the *pol* locus that alter the affinity of the enzyme for the antiviral compound and deoxynucleotides, and its ability to incorporate drug triphosphates or deoxynucleotides or both (Derse et al., 1982; Gibbs et al., 1988). Previous studies that have utilized drug resistant viruses in mouse models of infection have reported reduced neurovirulence in the peripheral nervous system and varying degrees of attenuation during acute and latent infections (Darby et al., 1984; Field and Coen, 1986; Larder and Darby, 1984; Pelosi et

al, 1998). These variations among drug resistant mutant phenotypes may be due to the fact that a number of independent mutations can confer resistance to one antiviral compound as demonstrated with PFA resistant HSV-1 Pol enzymes (Derse et al., 1982). Also, the question remains as to whether the observed phenotypes are a result of the mutation at the *pol* locus or a potentially unidentified mutation (Darby et al., 1984; Field and Coen, 1986; Larder and Darby, 1984; Pelosi et al, 1998).

Studies utilizing intertypic variants concluded that the N-terminal region of HSV-1 Pol was responsible for enhanced replication of recombinant HSV-2 viruses in mice and human peripheral blood mononuclear cells (Day et al., 1988, Lausch et al., 1990). However, the minimal HSV-1 sequence that conferred neuroinvasiveness also included the 3' end of Ori_L in addition to the *pol* 5' leader sequence (>200 nucleotides) that is upstream of the initiating methionine. Therefore, the exact contribution of the N-terminal half of HSV-1 Pol to viral replication in these previous studies is unclear. While HSV-1 Pol encodes multiple enzymatic functions that are distinct from 5'-3' polymerase activity, the importance of such activities for replication in animal models of infection has yet to be evaluated.

Chapter 2:

Characterization of Pre-NH_2-Terminal *pol* Mutant Viruses *in vitro*

Abstract

The catalytic subunit of herpes simplex virus 1 DNA polymerase (HSV-1 Pol) has been extensively studied; however, its full complement of functional domains has yet to be characterized. A crystal structure has revealed a previously uncharacterized pre-NH_2-terminal domain (residues 1-140) within HSV-1 Pol. Due to the conservation of the pre-NH_2-terminal domain within the herpesvirus Pol family and its location in the crystal structure, we hypothesized that this domain provides an important function during viral replication in the infected cell distinct from 5'-3' polymerase activity. We identified three pre-NH_2-terminal Pol mutants that exhibited 5'-3' polymerase activity indistinguishable from that of wild type Pol in vitro: deletion mutants PolΔN43 and PolΔN52 that lack the extreme N-terminal 42 and 51 residues, respectively, and mutant PolA_6, in which a conserved motif at residues 44-49 was substituted with alanines. We constructed the corresponding *pol* mutant viruses and found that *pol*ΔN43 displayed replication kinetics similar to those of wild type virus, while *pol*ΔN52 and *pol*A_6 infection resulted in an 8-fold defect in viral yield when compared to wild type and their respective rescued derivative viruses. Additionally, both *pol*ΔN52 and *pol*A_6 viruses exhibited defects in viral DNA synthesis that correlated with the observed reduction in viral yield. These results strongly indicate that the conserved motif within the pre-NH_2-terminal domain is important for viral DNA synthesis and production of infectious virus, and indicate a functional role for this domain.

Introduction

The efficiency of viral replication and spread of infectious progeny virus is dependent upon the expeditious and faithful replication of the parental genome. Herpes simplex virus-1 (HSV-1) encodes seven proteins that are essential for viral DNA synthesis: an origin binding protein (UL9), DNA polymerase holoenzyme (catalytic subunit UL30 (Pol) and processivity factor UL42), single stranded DNA binding protein (SSB; ICP8), and the helicase-primase complex (UL52, UL5, and UL8) (Boehmer and Lehman, 1997; Roizman and Knipe, 2001). The exact mechanisms by which these proteins act in concert to initiate and efficiently replicate the HSV-1 genome in the infected cell are poorly understood. HSV-1 Pol is the central enzyme for synthesis of viral DNA and is a target for antiviral drugs, however the efficacy of these treatments is limited, especially for immunocompromised patients with drug-resistant infections (Coen and Schaffer, 2003). Despite vigorous investigation into HSV-1 Pol function, all of the activities mediated by this enzyme have yet to be exhaustively characterized. Elucidation of conserved viral replication processes may identify factors that could serve as a target for new antiviral therapies.

HSV-1 Pol is a member of DNA polymerase family Pol B, which includes eukaryotic polymerases α, δ, and ε, in addition to viral and bacteriophage polymerases that are responsible for genomic DNA replication (Hubscher et al., 2002; Lehman and Kaguni, 1989; Rothwell and Waksman, 2005). The palm, fingers, thumb, and 3'-5' exonuclease domains of HSV-1 Pol bear significant sequence and structural homology to corresponding domains in other Pol B enzymes, and are the best characterized regions of the protein (Knopf and Weisshart, 1988; Liu et al., 2006; Rothwell and Waksman, 2005;

Wang et al., 1989). Accordingly, HSV-1 Pol exhibits 5'-3' polymerase and 3'-5' exonuclease activities that are characteristic functions of the replicative Pol B family (Knopf, 1979; Marcy et al., 1990; Rothwell and Waksman, 2005). Polymerase activity is essential for the production of infectious progeny virus (Aron et al., 1975; Dorsky and Crumpacker, 1988), while the 3'-5' exonuclease activity is not required for viral replication but is important for replication fidelity during viral DNA synthesis (Hwang and Hwang, 2003; Hwang et al., 1997). The extreme C-terminus of HSV-1 Pol is crucial for an interaction with processivity factor UL42 that is necessary for long-chain DNA synthesis and indispensable for viral replication (Digard et al., 1993). Most recently, HSV-1 Pol was found to possess apurinic/apyrimidinic and 5'-deoxyribose phosphate lyase activities consistent with base excision repair processes, which are typically functions of the repair polymerase family X (Bogani and Boehmer, 2008; Rothwell and Waksman, 2005). Although the active site has yet to be mapped, DNA lyase activity has been localized to a 63kDa C-terminal fragment of HSV-1 Pol (Bogani and Boehmer, 2008). Unlike the C-terminal half of HSV-1 Pol, the N-terminal half has yet to be functionally characterized (Liu et al., 2006). Thus, further investigation of this region may elucidate novel activities and better characterize functions of HSV-1 Pol.

A crystal structure of HSV-1 Pol revealed a two domain architecture of the N-terminal portion of the enzyme (Liu et al., 2006). One of the domains (NH_2-terminal domain) contains three structural motifs that closely resemble NH_2-terminal domain structures found in family B polymerases (Liu et al., 2006; Rodriguez et al., 2000; Swan et al., 2009; Wang et al., 1997; Wang et al., 1996). The second domain, at the extreme N-terminus of HSV-1 Pol, which was structurally distinct from the NH_2-terminal domain,

was dubbed the pre-NH_2-terminal domain by Liu *et al* (Liu et al., 2006). The pre-NH_2-terminal domain is comprised of the first 140 residues of HSV-1 Pol, of which only residues 59-140 are visible in the published structure (Liu et al., 2006). Although the pre-NH_2-terminal domain is well conserved among the herpesvirus Pol family, a structural equivalent has yet to be identified in other published polymerase structures (Di Tommaso et al., 2011; Liu et al., 2006; Notredame et al., 2000). The extreme N-terminal 42 residues, which are not present in the published crystal structure, are conserved in HSV -1 and -2 (Liu et al., 2006). Interestingly, a motif at HSV-1 Pol residues 44-49 is highly conserved among all human herpesviruses (Figure 2.1A; Di Tommaso et al., 2011; Liu et al., 2006; Notredame et al., 2000). The pre-NH_2-terminal domain is located at the periphery of the enzyme and outside of the catalytic center for 5'-3' polymerase activity (Dorsky and Crumpacker, 1988; Knopf and Weisshart, 1988; Liu et al., 2006), suggesting that it is unlikely to directly participate in polymerase activity. Due to its conservation among herpesviruses, we hypothesized that the pre-NH_2-terminal domain provides an important function for viral DNA synthesis and production of infectious virus distinct from 5'-3' polymerase activity. Accordingly, we generated pre-NH_2-terminal mutants for analysis of in vitro 5'-3' polymerase activity, viral DNA synthesis and production of infectious virus.

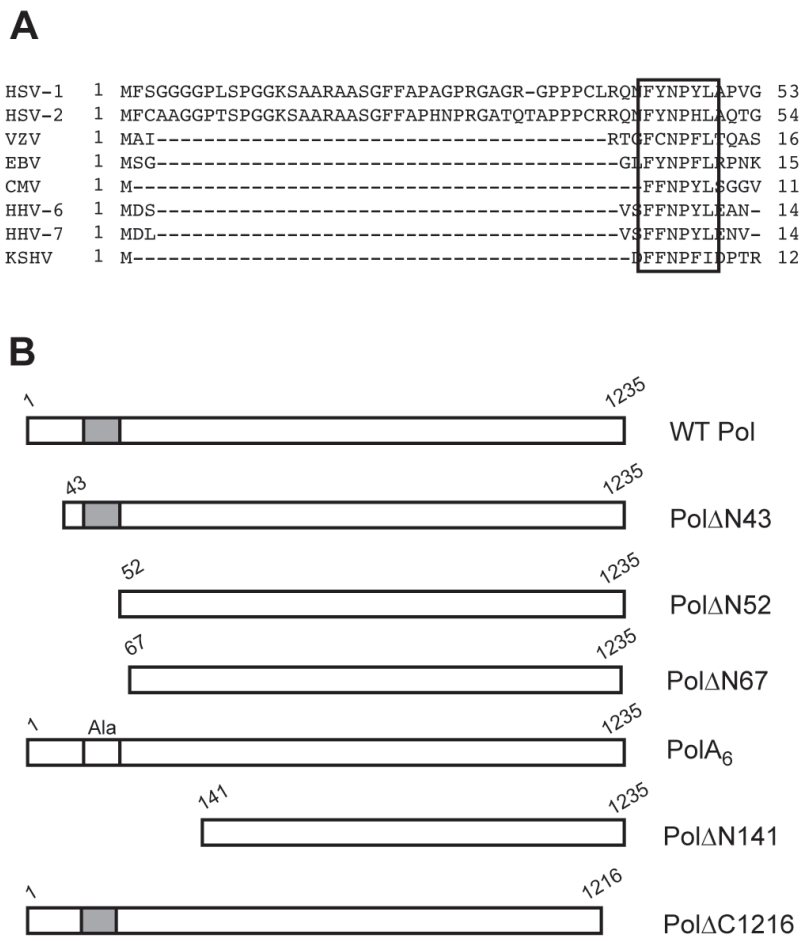

Figure 2.1. Sequence alignment and *pol* mutant constructs. (A) Sequence alignment of the eight human herpesvirus Pol sequences (DiTommaso et al., 2011; Notredame et al., 2000). A motif consisting of multiple hydrophobic and aromatic residues (FYNPYL) at 44-49 of HSV-1 Pol is conserved in the human herpesvirus Pol family (box). Polymerase sequences: HSV-1 (Gibbs et al., 1985), HSV-2 (Chibo et al., 2002), Varicella-Zoster virus (VZV; Davison and Scott, 1986), Epstein-Barr virus (EBV; de Jesus et al., 2003), Cytomegalovirus (CMV; Chou et al., 1999), human herpesvirus 6 (HHV-6; Isegawa et al., 2009), HHV-7 (Megaw et al., 1998), Kaposi's sarcoma –associated herpesvirus (KSHV; Neipel et al., 1997). (B) Schematic diagram of selected *pol* constructs analyzed in RRL assays. Amino-terminal deletions are numbered according to the first residue (downstream of initiating AUG codon) of the WT protein present in the mutant and carboxyl-terminal deletions are numbered according to the last amino acid present (upstream of terminating UGA codon). The conserved motif is depicted by the gray box (not drawn to scale), which was substituted with alanines (Ala) in mutant $PolA_6$.

Materials and Methods

Cells, viruses and antibodies. Vero (American Type Culture Collection) and polB3 cells, which inducibly express wild type (WT) HSV-1 Pol upon infection and were kindly provided by Charles Hwang (Hwang et al., 1997), were grown and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% newborn calf serum, 1% penicillin and streptomycin, and 1% amphotericin B. DNA harvested from WT HSV-1 strain KOS was included in restriction fragment length polymorphism (RFLP) analysis. Construction of mutant viruses from a bacterial artificial chromosome (BAC) clone of KOS is detailed below. *Spodoptera frugiperda* Sf9 cells (Invitrogen) were cultured under serum-free conditions with Sf-900II serum free medium (Invitrogen) supplemented with 10μg/mL gentamycin. The monoclonal anti-Pol antibody 1051c (Strick et al., 1997) was kindly provided by Robert Klemm (University of Heidelberg). Secondary antibody AlexaFluor 488-conjugated chicken anti-mouse was obtained from Invitrogen-Molecular Probes.

Plasmids and BACS. All of the indicated *pol* constructs used for in vitro transcription/translation studies were cloned into the SpeI/HindIII (New England Biolabs) sites of pBluescript II KS+ (pBS; Stratagene). The HSV-1 WT *pol* gene was shuttled from plasmid HTC-Pol (kindly provided by Gloria Komazin-Meredith) to the pBS vector to generate pBS-Pol. Truncation mutants were amplified from pBS-Pol by employing KOD Hot Start DNA polymerase (EMD Biosciences) and sequence specific primers that introduced a start codon (AUG) downstream of an SpeI site at the 5' terminus and a stop codon (UGA) upstream of a HindIII site at the 3' terminus. Plasmid pBS-PolA_6 was generated via two sequential rounds of site direction mutagenesis using

the QuikChange method (Stratagene) that introduced alanine substitutions F44A, Y45A, N46A, P47A, Y48A, L49A into pBS-Pol.

E. coli strain GS1783 (Tischer et al., 2010; kindly provided by Greg Smith, Northwestern University) harboring a BAC clone of HSV-1 strain KOS (I. Jurak *et al*., manuscript in preparation) was used to generate 'scarless' *pol* mutants via the two-step Red recombination techniques outlined by Tischer *et al* (Tischer et al., 2006). Plasmids pEP-KanaS (Tischer et al., 2006) and pBAD-I-*SceI* (Tischer et al., 2006) were kindly provided by Nikolaus Osterrieder and B. Karsten Tischer (Cornell University). Manipulations to generate deletions, substitutions, and insertions within the *pol* locus in the BAC were performed as previously described (Tischer et al., 2006). The presence of each mutation, and lack of unintended mutations, was verified by sequencing the *pol* gene in each mutant BAC clone. BAC-derived viruses were reconstituted via transfection of 2μg of purified BAC DNA into 3×10^5 polB3 cells using Lipofectamine reagent (Invitrogen). Five days post-transfection, the viral supernatant was harvested and titrated on polB3 cells. A single plaque was isolated and subjected to an additional two rounds of plaque purification prior to amplification of pure viral stocks. Viral DNA was harvested from infected polB3 cells and the *pol* locus was subjected to sequencing (data not shown). Purified DNA from BAC-derived virus and WT HSV-1 strain KOS was digested with BamHI (NEB) and electrophoresed on a 0.8% agarose gel overnight for RFLP analysis. Rescued derivative viruses were generated by restoring the WT *pol* sequence in BAC clones *pol*ΔN52 and *pol*A_6 via two-step Red recombination prior to reconstitution in polB3 cells.

In vitro transcription translation and DNA polymerase assays. HSV-1 Pol proteins were expressed via rabbit reticulocyte lysate (RRL) using the TNT Quick Coupled Transcription/Translation Systems kit (Promega). Six hundred nanograms of each plasmid DNA construct (or no DNA as a negative control) and other kit components were added to 30µl of RRL that had been supplemented with either [^{35}S]-methionine (Perkin Elmer) or cold methionine in parallel and incubated at 30°C for 90 min per the manufacturer's instructions. To assess the level of protein expression for each experiment, an aliquot from each radiolabeled reaction was analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. The unlabeled reactions were subjected to DNA polymerase assays similar to those that were previously described (Dorsky and Crumpacker, 1988) with some modifications: Individual RRL reactions were supplemented with a polymerase reaction mixture that brought the final concentration (in 100µl) to 50mM TrisHCl, pH 7.5, 100mM $(NH_4)_2SO_4$, 50µg/mL BSA, 0.5mM DTT, 7.5 $MgCl_2$, 10µg/mL activated calf thymus DNA, 5µM each of dCTP, dGTP, dATP, and 2.5µCi [α-^{32}P]-dTTP (Perkin Elmer). To assess stimulation of polymerase activity by UL42, reactions were supplemented with 2pmol MBP-UL42ΔC340 (Komazin-Meredith et al., 2008; kindly provided by Gloria Komazin-Meredith) and incubated at 37°C for 30 min prior to addition of the reaction mixture. Aliquots were removed at the indicated time points, mixed with EDTA, and incubated on ice to halt further enzymatic activity. Samples were spotted onto DE81 anion exchange filters (Whatman) and washed twice with 5% (w/v) Na_2HPO_4 buffer (dibasic), rinsed once with water, and once with 100% ethanol. Filters were dried and subjected to liquid scintillation counting.

Recombinant baculovirus, protein expression, purification, and polymerase activity. Bacmid donor plasmids were generated by shuttling *pol*ΔN52 and *pol*A_6 constructs from pBS-KS+ vectors to the pFastBac vector (Invitrogen) using SpeI and HindIII restriction sites. Sf9 cells were transfected with recombinant bacmid DNA to generate recombinant baculoviruses expressing N-terminal His_6-tagged PolΔN52 and $PolA_6$ proteins by using the Bac-to-Bac Baculovirus Expression System Kit (Invitrogen). His_6-WT Pol was expressed and purified from the corresponding recombinant baculovirus that was engineered in a similar fashion (kindly provided by Gloria Komazin-Meredith). Viral titers were determined using the BacPAK Baculovirus Rapid Titer Kit (Clontech). Mid-log phase Sf9 cells were infected at a multiplicity of infection (MOI) of 2 and cells were harvested at 65hpi. Cell pellets were washed with Dulbecco's phosphate-buffered saline (DPBS) and resuspended in buffer A (20mM HEPES, pH 7, 1mM DTT, 20% glycerol, 100mM guanidine HCl, 200mM NaCl, 20mM imidazole, and 1 Roche complete protease inhibitor tablet/100mL). His-tagged proteins were captured via batch purification with Ni^{2+}-NTA resin (Qiagen) for 30 min with gentle agitation at 4°C. The absorbed resin was loaded onto a column and washed extensively with buffer A. Pol was eluted from the column with buffer A containing 500mM imidazole. Fractions that contained His-tagged Pol were pooled and passed through a 1mL HiTrap Heparin HP column (GE Healthcare) that had been preequilibrated with buffer B (20mM HEPES, pH 7, 2mM DTT, 20% glycerol, 100mM guanidine HCl, 200mM NaCl). The column was washed with buffer B and protein was eluted with a linear NaCl gradient of up to 1M NaCl. Fractions containing Pol were pooled, concentrated with an Amicon Ultra-15 centrifugal unit (Millipore) and stored at -80°C.

Unless otherwise stated, polymerase assays using purified enzyme were conducted using 400fmol Pol in the presence and absence of 1pmol MBP-UL42ΔC340 and samples were processed as described above. For concentration dependent assays, basal polymerase activity was measured in individual reactions containing the indicated amount of purified enzyme following a 20 min incubation at 37°C. The amount of dTTP incorporation was calculated using a standard curve of known [^{32}P]-dTTP amounts.

Viral replication assays. Vero cells (2.5×10^5) were infected in triplicate at an MOI of 10 or 20 PFU/cell as indicated. After a 1 h adsorption period at 37°C, wells were washed with DPBS and replenished with 2mL of DMEM containing 2% NCS. At each time point, whole cell lysates were collected, frozen and subsequently thawed and sonicated. Cellular debris was pelleted by centrifugation and supernatants were titrated on polB3 cells in duplicate.

Indirect immunofluorescence. Vero cells (1×10^5) were seeded on glass coverslips and infected with the indicated virus at an MOI of 20. A 6 hpi, cells were fixed with 3.8% formaldehyde for 15 min and permeabilized with 1% Triton X for 10 min. Samples were incubated with blocking buffer (10% normal goat serum in PBS) at 4°C overnight. Cells were stained with 1051c (0.1mg/mL) for 1 hr at room temperature. Samples were washed with PBS and reacted with secondary antibody (1:1000) for 1 hr at room temperature. Coverslips were washed with PBS and mounted onto glass slides using ProLong Gold AntiFade Reagent (Invitrogen). Fluorescence microscopy was performed with a Yokogawa spinning disk confocal on a Nikon Ti inverted microscope using a 60× Plan Apo NA 1.4 objective lens. Sequential optical sections of 0.5 micron

step size were collected with a Hamamatsu ORCA ER cooled CCD camera. Images are presented as the median of the acquired z-series sections using MetaMorph 7 software.

Real-time PCR assay of viral DNA synthesis. Vero cells (2.5×10^5) were infected with virus at an MOI of 20 in triplicate and cell lysates were harvested at 12 and 16 hpi. DNA from mock- and HSV- infected Vero cells was isolated and processed as described previously for murine trigeminal ganglia (Pesola et al., 2005). Viral DNA standards were generated by spiking ten-fold serial dilutions of purified HSV-1 DNA into mock-infected Vero cell lysate. Cellular DNA standards were prepared by making serial three-fold dilutions of mock-infected lysate. Viral and cellular DNA standards were processed along with experimental samples as a control for the efficiency of DNA recovery from infected cell lysate. Real time PCR assays for viral (Pesola et al., 2005) and cellular (J M Pesola, unpublished results) DNA were conducted in 20µl reactions with 2µl of the experimental samples or standards (~1/20 of entire sample), using 0.1µM primers and SYBR green PCR master mix (Applied Biosystems). Each primer set selectively amplified the viral *thymidine kinase* gene or the cellular α-*galactosyltransferase* gene resulting in ≥96% PCR amplification efficiency in each assay. Real-time reactions were performed on the Applied Biosystems StepOnePlus Real-Time PCR System per the manufacturer's instructions. Absolute and relative amounts of viral or cellular DNA, respectively, in each experimental sample were interpolated from the generated standard curve (linear regression performed on a plot of the threshold cycle versus log quantity DNA; all R^2 values ≥ 0.98). Viral DNA was normalized to the measured cellular DNA content and values are reported as viral DNA copies per reaction

for each experimental sample. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, Calif.).

Results

Analysis of HSV-1 Pol mutant protein activity in RRL. We hypothesized that the pre-NH_2-terminal domain is not directly involved in 5'-3' polymerase activity due to its location outside of the previously characterized active site (Dorsky and Crumpacker, 1988; Knopf and Weisshart, 1988; Liu et al., 2006). However, further examination of the crystal structure revealed an extensive hydrophobic network between the 3'-5' exonuclease domain and anti-parallel beta sheets within the pre-NH_2-terminal domain located downstream of HSV-1 Pol residue 70 (Liu et al., 2006). Therefore, we anticipated that extensive deletions within these regions could lead to misfolding or destabilization of the Pol enzyme. A previous study had generated HSV-1 Pol mutant proteins via RRL for analysis of 5'-3' polymerase activity (Dorsky and Crumpacker, 1988). In order to avoid engineering viruses with mutations that could indirectly abrogate enzymatic activity and thereby result in viral lethality (Aron et al., 1975; Dorsky and Crumpacker, 1988), we utilized this expression system to test 5'-3' polymerase activity of Pol mutants in the presence and absence of viral processivity factor UL42.

Selected *pol* constructs utilized in this study are depicted in Figure 2.1B. Truncation mutant PolΔN43 was constructed to investigate the importance of the extreme N-terminal 42 residues. In an effort to explore the potential role of the conserved motif FYNPYL, we generated deletion mutant PolΔN52 that lacks the extreme N-terminal 51 residues, and substitution mutant $PolA_6$, in which residues 44-49 were substituted with alanines. Additional mutants were tested in order to identify the most extensive truncation mutant that encoded an enzymatically active polymerase. One set of programmed reticulocyte lysates was supplemented with [^{35}S]-methionine in order to

visualize the relative amounts of synthesized protein in each reaction via SDS-PAGE and autoradiography. Unlabeled lysates in which cold methionine was substituted for [^{35}S]-methionine were used to analyze enzymatic activity of HSV-1 Pol mutants. Basal polymerase assays were conducted by measuring the incorporation of [^{32}P]-dTTP into activated calf thymus DNA as previously described (Dorsky and Crumpacker, 1988). In vitro reactions supplemented with purified MBP-UL42ΔC340, which is capable of stimulating HSV-1 Pol (Digard et al., 1993; Komazin-Meredith et al., 2008), were used to assess processive polymerase activity. Two controls were included in each assay: a reaction that lacked a *pol* construct in order to assess background activity of endogenous RRL protein and another that expressed Pol truncation mutant PolΔC1216, which cannot interact with UL42 and thereby is not stimulated by it (Digard et al., 1993).

The results from the in vitro analysis of each pre-NH_2-terminal mutant are summarized in Table 2.1. The two most conservative truncation mutants, PolΔN43 and PolΔN52, and substitution mutant $PolA_6$ reproducibly exhibited time dependent 5'-3' polymerase activity similar to WT Pol in the presence and absence of UL42 (Figure 2.2A and B and data not shown). The enhanced level of activity detected in reactions containing PolΔN43 is most likely due to increased protein expression in this experiment (Figure 2.2C). PolΔN141, in which the entire pre-NH_2-terminal domain was deleted, did not exhibit detectable enzymatic activity (Figure 2.2A and B). Although a previous study had reported that a mutant lacking the extreme N-terminal 66 residues exhibited polymerase activity (Dorsky and Crumpacker, 1988), we found that truncation mutant PolΔN67 reproducibly lacked polymerase activity above the negative controls in both the basal and processive polymerase assays (Table 2.1). Thus, removal of up to 51 residues at

Table 2.1. Summary of *in vitro* polymerase activity of selected Pol mutants

Polymerase mutant construct	Basal DNA polymerase activity[a]	Stimulated polymerase activity via UL42[b]
PolΔN43	+	+
PolΔN52	+	+
PolΔN67	−	−
PolΔN141	−	−
$Pol A_6$	+	+
PolΔC1216	+	−

[a] + , wild type levels of activity; − , activity at or below that of background

[b] + , wild type levels of activity; − , activity at or below that of UL42-binding mutant PolΔC1216

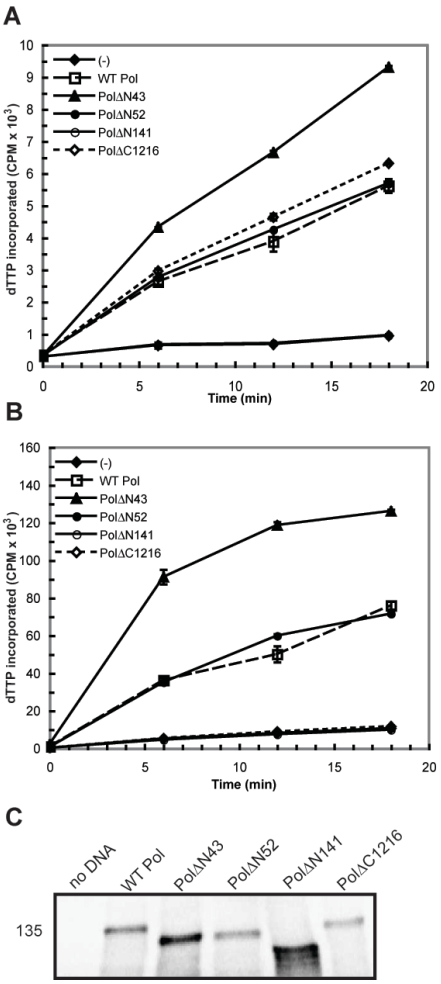

Figure 2.2. 5'-3' polymerase activity and Pol mutant protein expression in RRL. HSV-1 Pol constructs were engineered and expressed in RRL for evaluation of 5'-3' polymerase activity. DNA was omitted from one in vitro reaction (-) in order to assess background levels of nucleotide incorporation. (A) Basal polymerase activity. Individual RRL reactions containing the DNA constructs indicated in the key were supplemented with polymerase reaction mixture and analyzed for enzymatic activity. The level of [α-^{32}P]-dTTP incorporation at each time point is reported as counts per min (CPM) and graphed as the mean of duplicate samples. (B) Processive polymerase activity. Parallel RRL reactions were supplemented with HSV-1 viral processivity factor UL42 prior to the addition of polymerase reaction mixture in order to assess stimulated polymerase activity. Mutant PolΔC1216 cannot bind UL42 and thereby served as a negative control in this assay. (C) In vitro expression of Pol mutant proteins. Aliquots of RRL reactions supplemented with [^{35}S]-methionine were electrophoresed on a 5% SDS-PAGE gel, dried, and exposed to a phosphorimager screen overnight. The position of a molecular weight marker is included on the left side of panel.

the extreme N-terminus of HSV-1 Pol yielded an enzymatically active protein while more extensive deletions negatively impacted 5'-3' polymerase activity.

5'-3' polymerase activity of purified HSV-1 Pol enzyme. A previous study had reported that a purified HSV-1 Pol truncation mutant lacking the extreme N-terminal 42 residues retained 5'-3' polymerase activity (Liu et al., 2006). In order to more quantitatively evaluate the activity of the remaining Pol mutants that maintained detectable activity in the RRL studies, recombinant baculoviruses were constructed for the generation of His-tagged fusion proteins PolΔN52 and $PolA_6$. WT Pol, PolΔN52 or $PolA_6$ proteins were purified to homogeneity from insect cell lysates infected with the appropriate recombinant baculovirus. We assayed the ability of each enzyme to incorporate [^{32}P]-dTTP into activated calf thymus DNA in the presence or absence of purified MBP-UL42ΔC340.

In accordance with the results generated from the polymerase assays conducted in RRL (Table 2.1), we found that both purified mutant proteins displayed basal polymerase activity similar to that of WT Pol (Figure 2.3A). The enhancement of polymerase activity in the presence of UL42 demonstrated a functional interaction with the viral processivity factor (Digard et al., 1993; Figure 2.3B). Lastly, we evaluated basal polymerase activity as a function of protein concentration for each enzyme (Figure 2.3C). The average rates of dTTP incorporation for WT, PolΔN52 and $PolA_6$ were very similar: 620, 570 and 680 fmol/min/nmol, respectively. Therefore, the engineered mutations in HSV-1 Pol had little or no impact on 5'-3' polymerase activity or association with UL42.

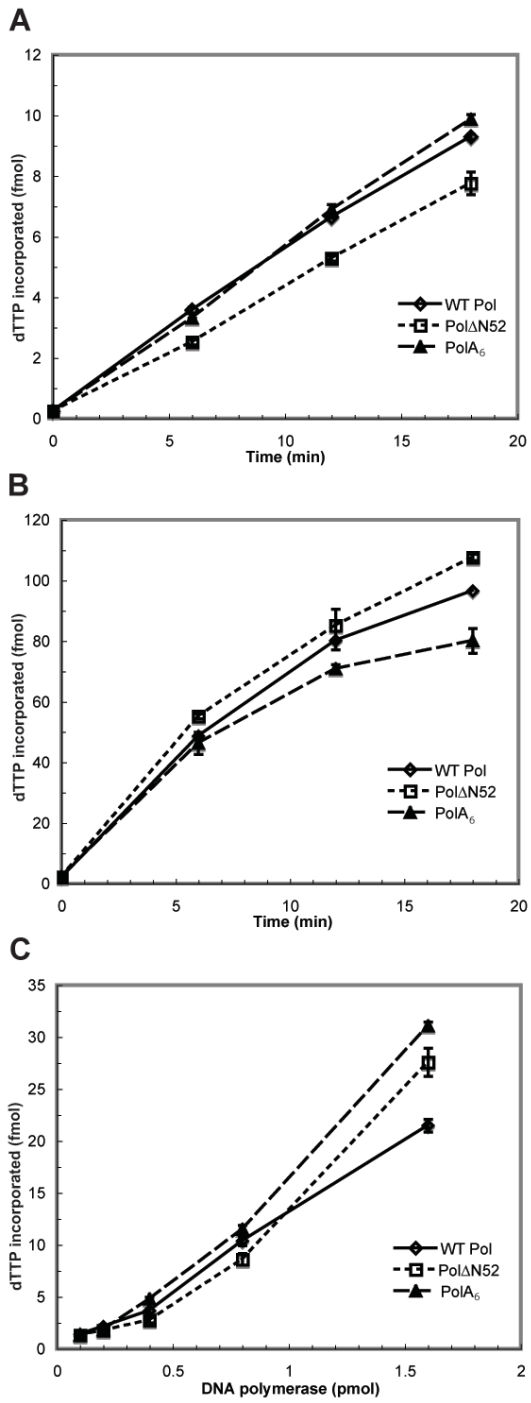

Figure 2.3. In vitro 5'-3' polymerase activity of purified Pol protein. WT Pol and mutant Pol proteins were expressed and purified from insect cells infected with recombinant baculovirus. Purified protein was subjected to basal (A) and processive (B) polymerase assays as described in the legend of Figure 2.2. Additionally, basal activity was measured and plotted as a function of enzyme concentration (C). The amount of dTTP incorporation (fmol) was calculated at each time point or enzyme concentration and graphed as the mean of duplicate samples.

Replication of BAC-derived HSV-1 *pol* mutant viruses. In order to assess the biological significance of *pol* mutations within the context of infection, *pol* mutant viruses were engineered via manipulation of an infectious bacterial artificial chromosome (BAC) clone of HSV-1 strain KOS (I Jurak *et al.*, in preparation). Using BAC recombineering techniques (Tischer et al., 2006), *pol* coding sequences were deleted in order to produce viruses that expressed pre-NH_2-terminal Pol mutants corresponding to the proteins included in our RRL analyses (Table 2.1). Mutant viruses *pol*ΔN43 and *pol*ΔN52 were analyzed to determine whether the extreme N-terminal 42 or 51 residues of HSV-1 Pol were essential for viral replication. Mutant virus *pol*ΔN141, in which the entire pre-NH_2-terminal domain was deleted, was included to verify that a mutant lacking detectable polymerase activity in the in vitro RRL studies would result in a non-viable virus. Due to the potential for replication defects as a result of the introduced mutations, each BAC, including WT, was introduced into and propagated in polB3 cells that inducibly express WT HSV-1 Pol upon infection (Hwang et al., 1997). Each BAC clone and corresponding virus was sequenced at the *pol* locus in order to confirm the presence of the engineered mutation and verify the lack of adventitious mutations. To confirm the overall integrity of the viral genome, we performed RFLP analysis on purified viral DNA from WT and mutant BAC-derived viruses in comparison with WT HSV-1 strain KOS. Digestion with BamHI revealed that the restriction pattern of WT BAC-derived viral DNA was similar to that of KOS (Figure 2.4). A 3.3kb band, which corresponds to the 5' end of the *pol* gene, underwent a mobility shift that was respective to the deletions present in mutants *pol*ΔN43, *pol*ΔN52, and *pol*ΔN141 (Figure 2.4).

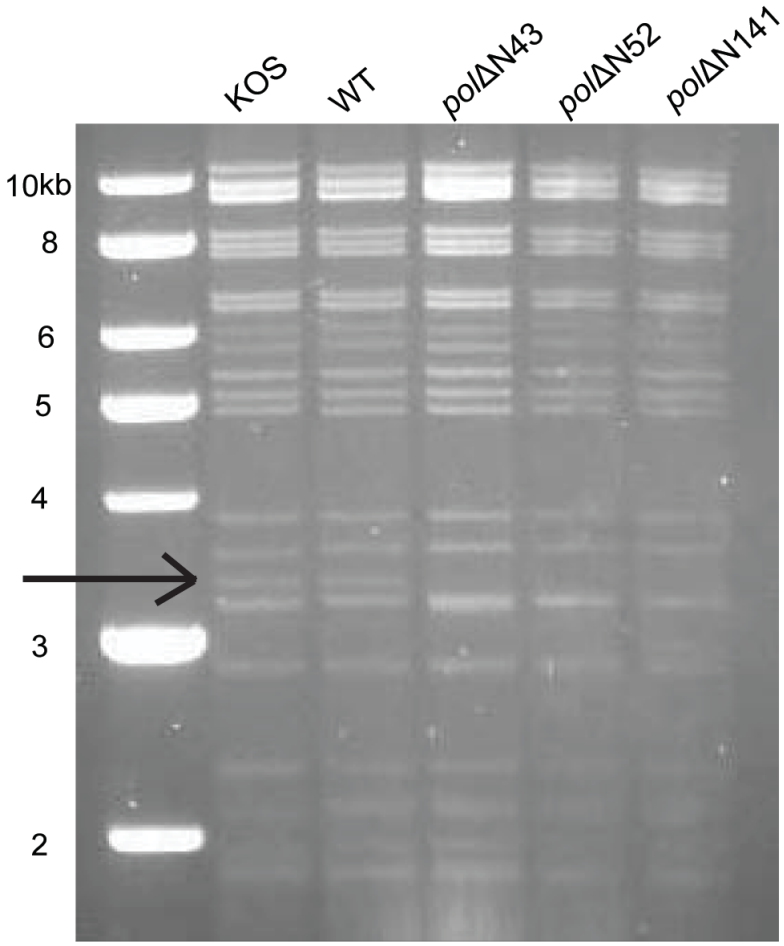

Figure 2.4. Restriction enzyme analysis of genomic viral DNA. Purified viral DNA from KOS and the indicated reconstituted BAC-derived virus was digested with BamHI and electrophoresed on a 0.8% agarose gel. The arrow indicates a 3.3kb band in the KOS and WT lanes that undergoes a mobility shift respective to the deletion present at the 5' end of the HSV-1 *pol* gene in each mutant virus. The left-hand panel indicates the sizes (kb) of bands from a DNA ladder.

To assess the viability of reconstituted virus harvested from complementing polB3 cells, supernatant from cells transfected with BAC DNA was titrated on polB3 cells and non-complementing Vero cells and scored for the ability to form plaques on Vero cells relative to polB3 cells (plating efficiency) compared to WT virus (Table 2.2). As expected, WT-BAC derived virus exhibited a plating efficiency of 100%, while *pol*ΔN141 was unable to form plaques on Vero cells. Mutant virus *pol*ΔN43 exhibited a plating efficiency similar to that of WT (93%). Interestingly, mutant *pol*ΔN52 exhibited a lower plating efficiency (73%) and a small plaque phenotype compared to WT and *pol*ΔN43 (data not shown). This result suggested that *pol*ΔN52 could not replicate as well as WT in Vero cells. Analysis of single cycle replication kinetics validated these initial observations: *pol*ΔN141 failed to replicate; *pol*ΔN52 exhibited a 5-fold and 7-fold decrease in viral yield at 12 and 16 hours post infection (hpi), respectively; while *pol*ΔN43 replication kinetics were indistinguishable from those of WT (Figure 2.5). Thus, the extreme N-terminal 42 residues of HSV-1 Pol were dispensable for viral replication in cell culture, while a mutant in which the extreme N-terminal 51 residues were removed exhibited notably decreased viral replication.

Conserved motif FYNPYL is important for efficient viral DNA synthesis and production of infectious virus. We hypothesized that the absence of the conserved motif accounted for the *pol*ΔN52 replication defect. To test this possibility, we constructed mutant virus *pol*A_6 in which the six residue motif was substituted with six alanines. Additionally, we wanted to test whether the observed replication defects were due to the engineered mutations. Accordingly, the *pol*ΔN52 and *pol*A_6 BACs were used as templates to restore the WT *pol* sequence and generate rescued derivative viruses

Table 2.2. Plating efficiencies of BAC-derived viruses

Virus[a]	Titer (PFU/mL) on:		Plating Efficiency (%)[b]
	Vero	polB3	
WT	2.7×10^7	2.7×10^7	100
*pol*ΔN43	4.2×10^7	4.5×10^7	93
*pol*ΔN52	4.9×10^6	6.7×10^6	73
*pol*ΔN52R	3.4×10^7	3.3×10^7	100
polA_6	5.2×10^6	6.5×10^6	80
polA_6R	1.6×10^7	1.6×10^7	100
polΔN141	0	7.5×10^6	0

[a] The resulting viral supernatant that was generated from transfection of polB3 cells with BAC DNA was harvested and subsequently titrated on the indicated cell line

[b] Calculated as the ratio of viral titers on Vero cells and polB3 cells

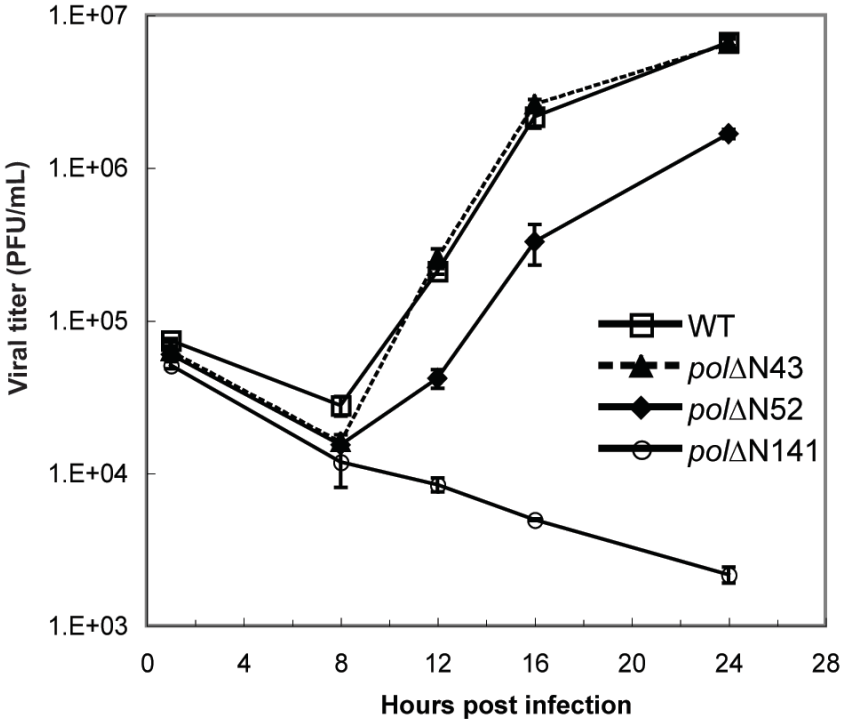

Figure 2.5. Single cycle replication kinetics of *pol* mutant viruses. Vero cells were infected with the indicated BAC-derived viruses at MOI=10 and whole cell lysate was harvested at the indicated time points. Lysates were freeze-thawed and sonicated prior to titration on polB3 cells. Viral yield is reported as PFU/mL with each data point respresenting the mean ± SD of triplicate samples.

*pol*ΔN52R and *pol*A_6R. WT, mutant, and rescued derivative viruses were tested for their ability to replicate in Vero cells. Interestingly, *pol*A_6 mimicked *pol*ΔN52 in forming small plaques (data not shown), and exhibiting a diminished plating efficiency (Table 2.2) and production of infectious virus (Figure 2.6). We also found that WT-like plating efficiencies (Table 2.2) and replication kinetics in Vero cells (Figure 2.6) were restored in both rescued derivatives *pol*ΔN52R and *pol*A_6R. Each mutant virus exhibited a 6-fold and 8-fold defect at 12 and 16 hpi, respectively, when compared to their respective rescued derivative virus (Figure 2.6). WT and rescued derivative viruses reached peak viral production at 24 hpi and remained up to 6-fold higher than that of the mutant viruses.

We sought to determine whether there were any affects of the engineered mutations on HSV-1 Pol localization during infection. During viral DNA synthesis in WT virus-infected cells, HSV-1 Pol localizes to replication compartments – large globular structures within the nucleus, which can be seen as early as 5.5 hpi (Bush et al., 1991; Liptak et al., 1996). Using indirect immunofluorescence, we observed that HSV-1 Pol staining was, as expected, predominantly found in large replication compartments that encompassed most of the nucleus in cells infected with WT, *pol*ΔN52R, and *pol*A_6R viruses (Figure 2.7A to C). Pol staining was also mainly found in the nuclei of cells infected with the *pol*ΔN52 and *pol*A_6 mutants but was concentrated in smaller structures than in cells infected with the other viruses (Figure 2.7E and F). No staining was observed in mock-infected cells (Figure 2.7D). Localization of PolΔN52 and PolA_6 to replication compartments rather than punctate prereplicative sites indicated that viral

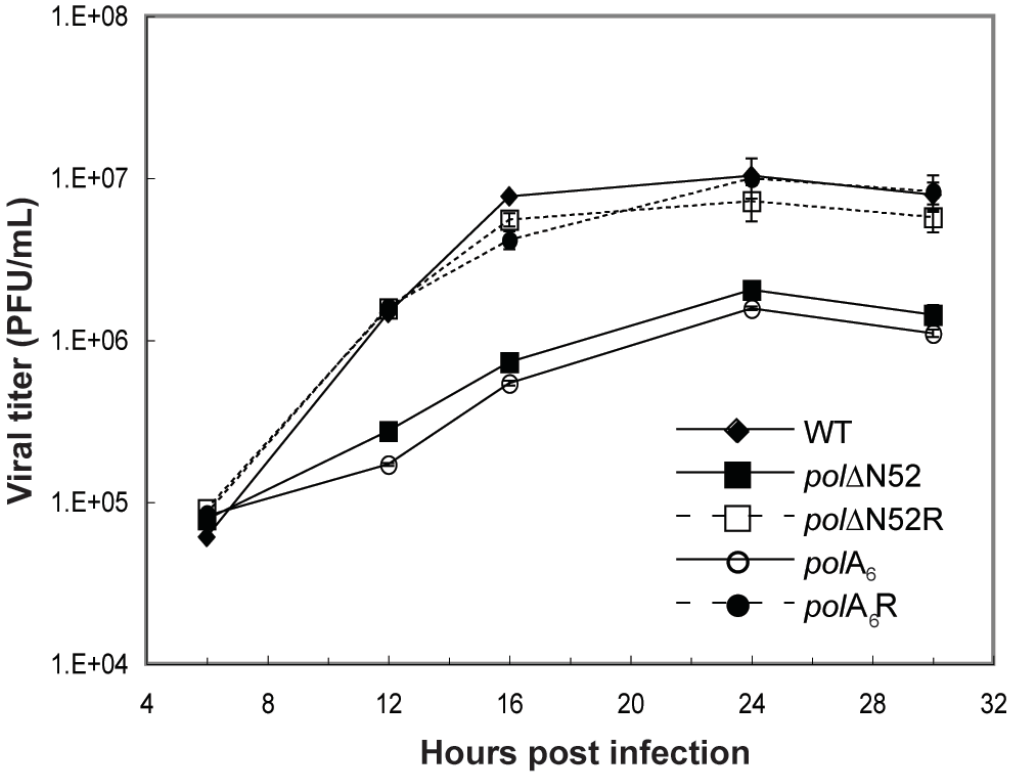

Figure 2.6. Replication kinetics of defective pre-NH_2-terminal *pol* mutant viruses. Vero cells were infected with BAC-derived virus at an MOI of 20. Whole cell lysate was harvested at the indicated time points and titrated on polB3 cells. Viral yield is reported as PFU/mL with each data point representing the mean ± SD of triplicate samples.

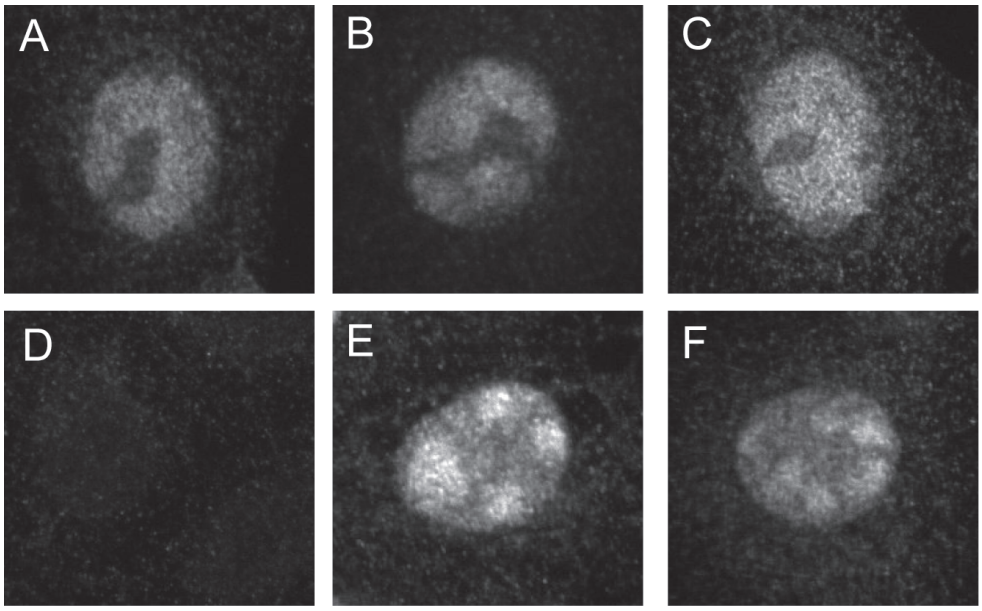

Figure 2.7. Localization of HSV-1 Pol in infected cells. Vero cells were either mock infected (D) or infected with BAC-derived virus at an MOI of 20 (A to C, E, F). Samples were fixed at 6 hpi and processed for indirect immunofluorescence with anti-Pol antibody. (A) WT virus; (B) *pol*ΔN52R virus; (C) *pol*A_6R virus; (D) mock; (E) *pol*ΔN52 mutant; (F) *pol*A_6 mutant.

DNA synthesis had not been drastically inhibited (Liptak et al., 1996). Thus, the conserved motif is not required for nuclear localization of HSV-1 Pol. The presence of smaller replication compartments during *pol*ΔN52 and *pol*A_6 mutant infection suggested that the motif is required for WT-like levels of DNA synthesis.

We then analyzed levels of viral DNA synthesis during the course of infection for each virus. DNA was isolated from mock- and HSV- infected cell lysates at 12 and 16 hpi. Viral and cellular DNA standards as well as experimental samples were subjected to real time PCR with primers that targeted viral and cellular genes *thymidine kinase* (*tk*) and *1,3-alpha-galactosyltransferase*, respectively. Standard curves were generated in order to quantify the number of viral DNA copies per reaction, which was normalized to cellular DNA content. Both *pol*ΔN52 and *pol*A_6 exhibited a decrease in viral DNA production that corresponded to the observed defects in viral yield with a 6-fold and ~10-fold defect at 12 and 16 hpi, respectively, when compared to the appropriate rescued derivative (Figure 2.8). These differences were statistically significant, while the differences in viral DNA content between *pol*ΔN52 and *pol*A_6 were not, indicating that substitution of the conserved motif recapitulated the deletion mutant phenotype. There was no apparent instability of HSV-1 Pol mutant polypeptides PolΔN52 and PolA_6 as indicated by Western blot analysis of infected cell lysate (S.L. Terrell and D.M. Coen, unpublished results). These data indicate that the loss of conserved motif FYNPYL is responsible for the observed defects in viral yield that correlate with decreased viral DNA production.

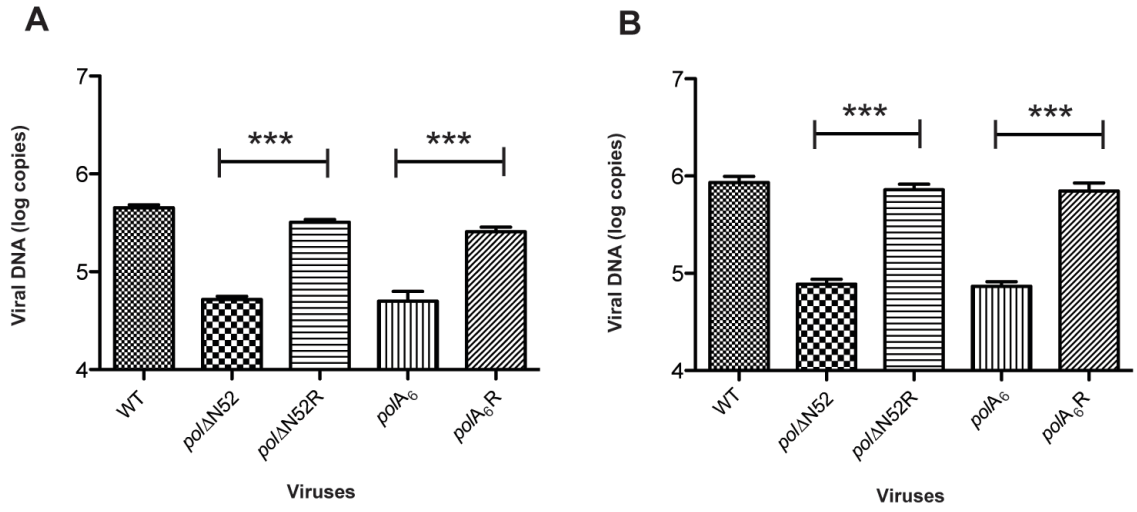

Figure 2.8. Viral DNA synthesis during *pol* mutant virus infection. Vero cells (2.5×10^5) were infected at an MOI of 20 and DNA was harvested from infected cell lysates at 12 (A) and 16 (B) hpi. The viral *thymidine kinase* was quantified via real-time PCR and normalized to cellular *1,3-alpha-galactosyltransferase* as described in the Materials and Methods and values are reported as log viral DNA copy number. Means ± SD of triplicate samples are plotted. ***, $p<0.0001$ (by one way ANOVA analysis with Bonferroni's multiple comparison post tests).

Discussion

We have shown in this report that three pre-NH_2-terminal HSV-1 Pol mutants retained polymerase activity similar to that of WT Pol in vitro, while two of the corresponding mutant viruses exhibited decreased virus production. Our efforts identified a conserved motif at HSV-1 Pol residues 44-49 as necessary for the efficient production of viral DNA during infection. This decrease in viral DNA synthesis correlated with the reduced production of infectious viral progeny. Taken together, our data strongly suggest that the pre-NH_2-terminal domain includes a function that is not important for 5'-3' polymerase activity, yet is crucial for efficient viral DNA synthesis during infection.

Enzymatic activity of pre-NH_2-terminal Pol mutants in vitro. Analysis of 5'-3' polymerase activity in RRL, as pioneered by Dorsky and Crumpacker (Dorsky and Crumpacker, 1988), provided a rapid method for identification of catalytically active Pol mutants that warranted further analysis. The dynamic nature of the HSV-1 Pol protein made it difficult to predict how each mutation would affect protein function. In our studies, only mutants that contained deletions or substitutions within the extreme N-terminal 51 residues retained 5'-3' polymerase activity similar to WT. Truncation mutant PolΔN67, in which the extreme N-terminal 66 residues were removed, was found to be catalytically inactive in our assays, which is in contrast with a previous report (Dorsky and Crumpacker, 1988). This discrepancy may be due to the previously reported construct containing eight codons of non-native sequence upstream of residue 67 (Dorsky and Crumpacker, 1988). The pre-NH_2-terminal domain engages in an extensive hydrophobic network with the adjacent 3'-5' exonuclease domain (Liu et al., 2006), disruption of which may indirectly lead to a catalytically inactive protein. However, we

cannot conclusively determine whether each inactive Pol mutant was misfolded or destabilized as a result of the mutation. Although our in vitro studies cannot exclude the possibility that any of the engineered mutations affected HSV-1 Pol activities such as 3'-5' exonuclease or lyase activity, we suggest that these possibilities are unlikely. The 3'-5' exonuclease and lyase active sites have been mapped within the interior of the 3'-5' exonuclease domain (Hwang et al., 1997; Kuhn and Knopf, 1996) and a 63-kDa C-terminal fragment (Bogani and Boehmer, 2008), respectively, and are separate from our engineered mutations within the extreme N-terminal 51 residues at the surface of the enzyme. Additionally, a previous study had reported that a mutant lacking detectable 3'-5' exonuclease activity exhibited a 50-fold decrease in viral yield with only a 3-fold decrease in viral DNA production (Tian et al., 2009). The defect in viral yield observed in the previous study largely reflected an alteration in replication fidelity rather than a defect in viral DNA synthesis as seen with our Pol mutants (Tian et al., 2009). Regardless, purification and assay of PolΔN52 and PolA_6 protein relative to WT Pol confirmed that the mutations did not induce any global effects on protein folding as the mutant proteins exhibited robust 5'-3' polymerase activity.

Importance of the pre-NH_2-terminal domain during viral DNA synthesis. Viral genetic analyses allowed us to evaluate the effect of each mutation within the context of infection. Despite 76% protein sequence identity in the extreme N-terminal 42 residues of Pol in HSV-1 and -2, our studies have shown that these were dispensable for viral replication in cell culture. There is a possibility that the extreme N-terminal 42 residues may be necessary for replication or pathogenesis in animal models of viral infection. We have demonstrated that the conserved motif FYNPYL contributes to the

efficient synthesis of viral DNA and production of progeny virus. It seems reasonable based on our results that the defect in production of infectious virus is due to a defect in viral DNA synthesis.

Although the pre-NH_2-terminal domain is conserved in the herpesvirus Pol family, a homologous domain is absent from the related bacteriophage RB69 Pol and other published Family B polymerase structures (Liu et al., 2006; Rodriguez et al., 2000; Swan et al., 2009; Wang et al., 1997; Wang et al., 1996). The conserved motif that we have identified as being important for viral DNA is a cluster of mostly aromatic and hydrophobic residues that are absent from the HSV-1 Pol crystal structure (Liu et al., 2006), suggesting that this motif is located within a disordered region of the protein. One could envision a scenario where this flexible segment near the extreme N-terminus of HSV-1 Pol interacts with a factor that actively recruits the polymerase to the replication fork or otherwise positively impacts viral DNA production. UL8, a component of the viral helicase-primase complex, has been shown to interact with HSV-1 Pol in vitro (Marsden et al., 1997). This interaction has been proposed to serve as a molecular tether for HSV-1 Pol at the leading strand of the replication fork (Liu et al., 2006). Another potential candidate is the viral SSB protein ICP8, which has been reported to stimulate HSV-1 Pol 5'-3' polymerase activity (Ruyechan and Weir, 1984). Protein complexes containing the HSV DNA polymerase holoenzyme, ICP8, and viral alkaline nuclease UL12 have been captured from infected cell lysate via immunoaffinity chromatography (Vaughan et al., 1984). Although a direct interaction has yet to be demonstrated, evidence supporting such an interaction includes a study that found specific ICP8 mutants conferred altered sensitivity to viral DNA synthesis inhibitors (Chiou et al., 1985).

Additionally, HSV-1 Pol was unable to localize to prereplicative sites within the nucleus in the absence of a functional ICP8 protein (Bush et al., 1991). As yet, binding sites for ICP8 and UL8 have not been mapped on HSV-1 Pol, so the implications of disrupting such interactions during viral infection are unknown. Presumably, any other viral or cellular protein that participates in viral genome synthesis and maintenance is a potential candidate. For example, a recent study reported that cellular transcriptional regulator HCF-1 can interact simultaneously with HSV-1 Pol and histone chaperone Asf1b (Peng et al., 2010). Depletion of Asf1b prior to infection with HSV-1 resulted in a 5- and 10-fold decrease in viral DNA and virus production, respectively, at 18 hpi, which suggested that HCF-1 and Asf1b are necessary for efficient viral DNA synthesis (Peng et al., 2010). These possibilities are currently under investigation. As of yet, we have been unable to identify a binding partner whose association with Pol is disrupted as a result of the deletion or substitution mutation (unpublished results). More than likely, the presumed protein-protein interaction would represent a conserved replication mechanism exhibited by human herpesviruses.

The exact mechanism in which HSV-1 DNA replication is carried out within the infected cell has yet to be fully elucidated. Following DNA melting at the origin of replication, HSV-1 DNA synthesis is hypothesized to begin on a circular template via theta replication and is converted by an unknown mechanism to an exponential rolling circle replication mechanism, which is responsible for the bulk of viral DNA synthesis (Boehmer and Lehman, 1997; Roizman and Knipe, 2001; Strang and Stow, 2005). Interestingly, we observed that WT virus exhibited exponential viral growth between 6 and 12 hpi while mutant viruses *pol*ΔN52 and *pol*A_6 lacked this burst and maintained a

diminished level of virus production throughout the course of infection. If viral yield were a direct function of viral DNA synthesis in this case, it could potentially represent a compromise in late phase DNA replication. However, our studies do not clearly indicate whether the defect in viral DNA synthesis is due to a decrease in the rate of DNA production or a perturbation in the recruitment and retention of HSV-1 Pol to active replication forks. Identification of the mechanism responsible for the observed defect would lead to enhanced characterization of the functional HSV-1 replisome and provide valuable insight into processes that are essential for efficient HSV-1 replication in cell culture.

Acknowledgments

We thank Charles Richardson, Sharmistha Ghosh, Dave Filman, Jennifer Baltz, Blair Strang and Jeremy Kamil for helpful discussions. We are grateful to Gloria Komazin-Meredith for providing purified HSV-1 WT Pol (with corresponding recombinant baculovirus) and MBP-UL42ΔC340, as well as technical expertise and support. We thank Can Cui for generation of the infectious KOS BAC clone, Greg Smith for *E. coli* GS1783, Nikloaus Osterrieder and B. Karsten Tischer for providing plasmids pEP-KanaS and pBAD-I-*SceI*, Jean Pesola for designing the real-time PCR assay and performing statistical analyses, and to Blair Strang and Jean Pesola for helpful comments on the manuscript. This work was supported by NIH award F31-AI084490 (SLT) and NIH grant R01-AI019838 (DMC).

Chapter 3

Replication of Pre-NH_2-Terminal *pol* Mutant Viruses in a Mouse Ocular Model of Infection

Abstract

The catalytic subunit of the herpes simplex virus 1 (HSV-1) DNA polymerase (HSV-1 Pol) is essential for viral DNA synthesis and production of infectious virus in cell culture. While it has been established that 5'-3' polymerase activity is absolutely essential for viral replication in all contexts, non-lethal mutations that abrogate other functions of HSV-1 Pol have yet to be evaluated in animal models of infection. In a previous report, we utilized bacterial artificial chromosome technology to generate defined *pol* mutations and investigate the role of the previously uncharacterized pre-NH_2-terminal domain of HSV-1 Pol. We found that the extreme N-terminal 42 residues (deletion mutant *pol*ΔN43) were dispensable for replication in cell culture, while residues 44-49 (alanine-substitution mutant *pol*A_6) were required for efficient viral DNA synthesis and production of infectious virus. In this study, we sought to address the importance of these conserved elements in viral replication in a mouse ocular infection model. Mutant virus *pol*ΔN43 exhibited a wild type-like phenotype with no significant impact in acute or latent infection. While mutant *pol*A_6 exhibited a modest defect at the peripheral site of infection, we found that ganglionic replication was severely impaired during acute infection as compared to wild type and its rescued derivative virus *pol*A_6R. Additionally, both viral DNA copy number in latently infected ganglia and reactivation rates were diminished during *pol*A_6 infection. These results implicate a role for the conserved motif at residues 44-49 in acute ganglionic infection and latency establishment in mice.

Introduction

Herpes simplex virus 1 (HSV-1) replication in the mouse eye model of infection mirrors the pattern of disease progression observed in humans (reviewed in Efstathiou and Preston, 2005; Wagner and Bloom, 1997). Following HSV-1 replication on the cornea, viral particles enter nerve axon terminals and travel to neuronal cell bodies within the trigeminal ganglia (TG) via retrograde transport, wherein a second round of productive infection is initiated. Latent infections are characterized by restricted lytic gene expression and maintenance of episomal viral DNA molecules within the innervating sensory neurons. Infectious virus can be reactivated from this latent state and spur recurrent acute infections at the original site of infection.

Previous studies have established that specific viral DNA synthesis proteins that are not required for viral replication in cell culture are in fact necessary for acute infection and reactivation in an animal host. Thymidine kinase negative (TK^-) mutants replicate like wild type (WT) virus in dividing cells although they exhibit growth defects in resting cell cultures due to repression of cellular TK expression (Field and Wildy, 1978; Jamieson et al., 1974). However, viral TK activity is absolutely essential for acute ganglionic replication and reactivation from latency of well-studied HSV-1 strains (Chen et al., 2004; Coen et al., 1989; Tenser et al., 1989; Thompson and Sawtell, 2000). Viral ribonucleotide reductase negative (RR^-) mutants exhibit a modest defect in viral DNA synthesis in actively dividing cells, which is further enhanced during infection of resting cells (Goldstein and Weller 1988a, b; Jacobson et al., 1989; Preston et al., 1988). Unlike the TK enzyme, RR is required for viral replication in mouse eyes during acute infection and cultured mouse cells at 38°C (Jacobson et al., 1989). Utilization of alternative

nucleotide metabolism pathways can compensate for the loss of either viral or cellular RR activity during infection of actively dividing cells (Goldstein and Weller 1988a; Nutter et al., 1985). In contrast, the catalytic subunit of the viral DNA polymerase (HSV-1 Pol) is absolutely essential for viral DNA synthesis and production of infectious virus (Aron et al., 1975; Dorsky and Crumpacker, 1988). Mutations that knock out HSV-1 Pol activity are replication incompetent in both cell culture and in mice, which is presumably due to the lack of 5'-3' polymerase activity that is responsible for production of progeny viral DNA molecules (Aron et al., 1975; Dorsky and Crumpacker, 1988; Katz et al., 1990). Drug resistant viruses containing point mutations at the *pol* locus that alter 5'-3' polymerase activity exhibit varying degrees of attenuation during acute and latent infections in mice (Darby et al., 1984; Field and Coen,1986; Larder and Darby, 1984; Pelosi et al., 1998). Outside of 5'-3' polymerase activity, the importance of other specific HSV-1 Pol functions has yet to be evaluated in an animal model of infection.

Using bacterial artificial chromosome (BAC) technology, we generated recombinant viruses with specific alterations at the 5' end of the *pol* gene in order to assess the role of the previously uncharacterized pre-NH_2-terminal domain of HSV-1 Pol (Chapter 2 − Terrell and Coen, 2012). The extreme N-terminal 42 residues, which exhibit 76% protein sequence identity between HSV -1 and -2, were deleted to generate mutant virus *pol*ΔN43 (DiTommaso et al., 2011; Notredame et al., 2000; Chapter 2 − Terrell and Coen, 2012). HSV-1 Pol residues 44-49 (FYNPYL), which are strongly conserved within the human herpesvirus *pol* family, were substituted with six alanines in mutant virus *pol*A_6 (Chapter 2 − Terrell and Coen, 2012). Characterization of *pol* mutant virus phenotypes in cell culture revealed that *pol*A_6 infection resulted in an 8-fold defect in

viral yield with a concurrent 10-fold decrease in viral DNA copies while *pol*ΔN43 exhibited WT-like growth kinetics. Additionally, we demonstrated that the deletion and substitution mutations did not impact 5'-3' polymerase activity of the corresponding pre-NH_2-terminal HSV-1 Pol mutant enzymes *in vitro*. Thus, our previous work established a role for the conserved motif FYNPYL in viral DNA synthesis processes during cellular infection that was distinct from 5'-3' polymerase activity. In this present study, we sought to investigate the biological significance of these conserved HSV-1 Pol residues in acute viral replication, latency establishment and reactivation in a mouse model of infection.

Materials and Methods

Cells and viruses. Vero cells (American Catalog of Cell Culture) and polB3 cells (Hwang et al., 1997; kindly provided by Charles Hwang) were maintained as previously described (Chapter 2 − Terrell and Coen, 2012). Human foreskin fibroblasts (American Catalog of Cell Culture) were maintained in Dubelcco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% amphotericin B. Viruses that were generated from an infectious bacterial artificial chromosome (BAC) clone of KOS (Jurak et al., unpublished) included the virus derived from the wild type BAC (WT), *pol*ΔN43, *pol*A_6, and *pol*A_6R (Chapter 2 − Terrell and Coen, 2012). All BAC-derived viruses were propagated in and titrated on polB3 cells, which inducibly express WT HSV-1 Pol. Virus *dl*sptk, which contains a 360-base pair deletion in the HSV-1 *thymidine kinase* locus (outside of the UL24 coding sequence) that abolishes enzymatic activity (Coen et al., 1989), was amplified in Vero cells.

Assays of acute and latent infections in mice. Seven-week old CD-1 mice were anesthetized with ketamine-xylazine and 2×10^6 plaque forming units (PFU) were administered to each eye following corneal scarification as previously described (Pesola et al., 2005). Infectious virus was harvested from each mouse eye with pre-moistened cotton swabs that were resuspended in 1mL of culture media (DMEM supplemented with 5% newborn calf serum, 1% penicillin/streptomycin, and 1% amphotericin B) and stored at -80°C until titration. Acutely infected ganglia were harvested and stored at -80°C in 1mL of culture media. TG were thawed, dounce-homogenized, frozen, thawed, and sonicated prior to titration. For any samples that were suspected to contain very low amounts of infectious virus, the entire lysate was plated onto confluent polB3 cells in 6-

well plates. Reactivation assays were performed by dissociating latently infected TG as previously described (Leib et al., 1991) and the single cell suspension was added into individual wells on a 6-well plate containing polB3 cell monolayers. Analysis of reactivation kinetics was performed similarly to that previously described (Balliet et al., 2007). Aliquots (150µl) of the viral supernatant were harvested from each well and stored at -80°C before plating on confluent 24-well plates containing polB3 cells for detection of infectious virus. For samples in which virus had not reactivated by ten days post-dissociation, whole cell lysates were harvested, frozen, thawed and, plated onto confluent polB3 wells (6-well plate), and monitored for an additional four days prior to fixing and staining.

Latent viral DNA detection via quantitative real-time PCR. Latently infected TG were harvested from mock and HSV-1 infected mice at 30 days post infection (dpi) and processed for DNA isolation as previously described (Pesola et al., 2005). Real time PCR assays were performed as previously described (Chapter 2 − Terrell and Coen, 2012) with primers that targeted the viral *thymidine kinase* gene (Chapter 2 − Terrell and Coen, 2012) or the murine *adipsin* gene (Kramer et al., 2011) and resulted in ≥92% mean PCR amplification efficiency in each assay. Viral and mouse DNA standards used for quantitation of recovered DNA in latently infected TG were prepared as previously described (Pesola et al., 2005). R^2 values for both viral and mouse DNA standard curves were ≥0.99. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, California) with one-way ANOVA analysis and Bonferroni's multiple comparison post tests unless otherwise indicated.

Replication kinetics in resting cells. Analysis of viral replication in actively dividing and resting cell cultures was performed as previously described with modifications (Field and Wildy, 1978; Jamieson et al., 1974). HFFs (1×10^6) were seeded into 6-well plates and maintained in DMEM/10% FBS for 24 hr or less prior to infection. Resting cells were produced by maintaining cell cultures in DMEM/0.5% FBS for four days prior to infection. Resting and dividing HFFs were infected at an MOI of 0.01 and whole cell lysates were harvested at 48 hpi and titrated on polB3 cells.

Results

Acute replication of *pol* mutant viruses on the eye. We sought to evaluate the role of conserved residues within the pre-NH_2-terminal domain of HSV-1 Pol in a mouse ocular model of infection and latency. The generation and characterization of pre-NH_2-terminal domain *pol* mutant viruses has been described previously (Chapter 2 − Terrell and Coen, 2012). Deletion mutant *pol*ΔN43, which lacks the extreme N-terminal 42 residues that are conserved in HSV-1 and -2, exhibited WT replication kinetics in cell culture. The conserved motif at HSV-1 Pol residues 44-49 was substituted with six alanines in mutant *pol*A_6, which resulted in decreased viral DNA production during infection. Rescued derivative virus *pol*A_6R, in which the WT *pol* ORF was restored in the background of the mutant, recapitulated the WT phenotype and demonstrated that the replication defect was specifically attributable to the engineered mutation.

Mutations that abolish either viral thymidine kinase (TK) or ribonucleotide reductase (RR) activity and resulted in only modest defects in cell culture have deleterious effects on acute phase viral replication in mice (Chen et al., 2004; Coen et al., 1989; Jacobson et al., 1989; Thompson and Sawtell, 2009). Therefore, we wished to determine whether conserved elements within the pre-NH_2-terminal domain of HSV-1 Pol were important for viral replication *in vivo*. Our analyses included the virus derived from the parental WT BAC (WT), *pol*ΔN43, *pol*A_6, and its rescued derivative *pol*A_6R. Using the mouse ocular infection model, CD-1 mice were infected with 2×10^6 PFU of virus via corneal scarification. Virus was swabbed from the mouse eye and titrated on the complementing cell line polB3, which inducibly expresses WT Pol (Hwang et al., 1997). At the peak of acute replication in the eye at 1 dpi, the rescued derivative *pol*A_6R as well

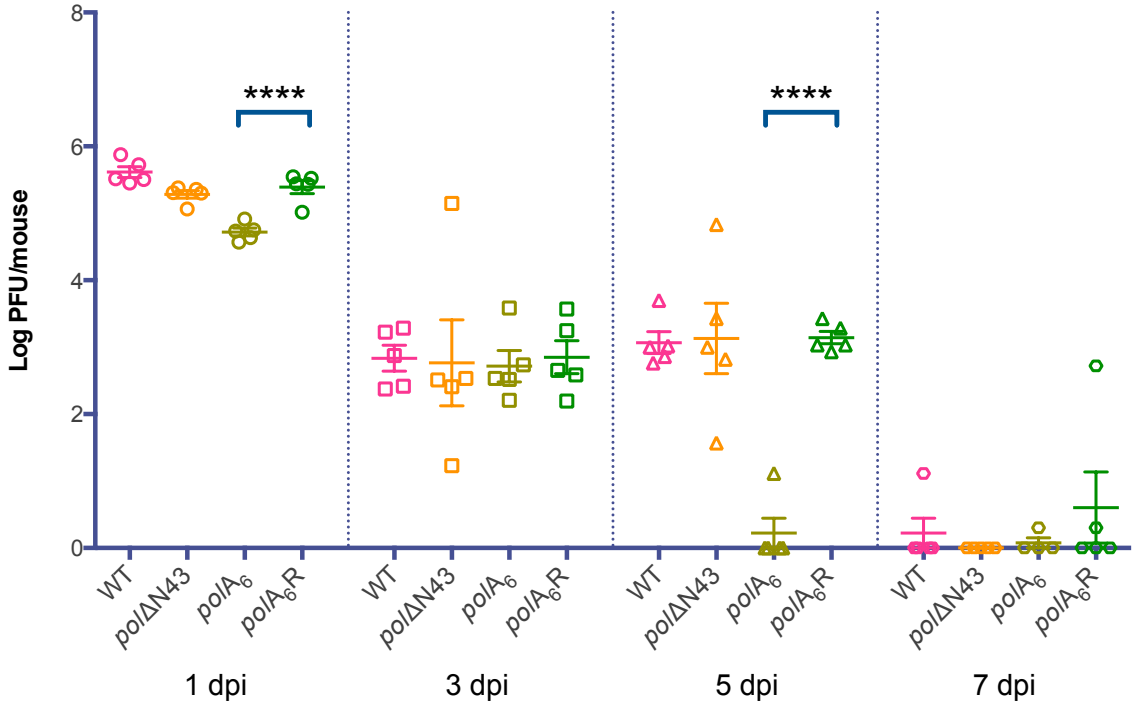

Figure 3.1. Acute viral replication in mouse eye. Mice were infected with 2×10^6 PFU per eye via corneal scarification. Infectious virus was swabbed from the eyes of infected mice at 1, 3, 5, and 7 dpi. The log PFU detected from each mouse (both eyes) and error bars representing the mean ± SEM for each data set are plotted. All viruses were analyzed by one-way ANOVA, followed by Bonferonni post-tests comparing WT, *pol*A_6 and *pol*A_6R to one another, accounting for multiple comparisons. ****, $p<0.0001$

as mutant *pol*ΔN43 generated infectious virus roughly as efficiently as WT (Figure 3.1). However, mutant virus *pol*A_6 reproducibly exhibited a 5-fold defect in viral replication at 1 dpi, which was statistically significant ($p<0.0001$; Figure 3.1). The magnitude of this defect was similar to that observed in cell culture (Chapter 2 − Terrell and Coen, 2012). At 3 dpi, *pol*A_6 viral yield was comparable to those of WT and *pol*A_6R despite the decreased level of replication at 1 dpi. Ganglionic replication of *pol*A_6 virus at 5 dpi was significantly attenuated while the other viruses continued to exhibit robust replication ($p<0.0001$; Figure 3.1). Although the initial replication defect exhibited by *pol*A_6 was restored to WT levels by 3 dpi, this mutant was unable to replicate as efficiently as WT and *pol*A_6R on the mouse eye through 5 dpi.

Replication of *pol* mutant viruses in TG during acute infection. We wished to evaluate *pol* mutant virus replication during acute infection of the TG. At 3 dpi, *pol*A_6 replication was significantly reduced with a ~3-log defect in infectious virus production as compared to its rescued derivative virus *pol*A_6R (Figure 3.2A). Although entire homogenates of ganglia from mice infected with *pol*A_6 were plated onto polB3 cells, a number of samples failed to yield detectable virus. This result was in stark contrast to *pol*ΔN43 infection that produced viral titers comparable to WT virus (Figure 3.2A). Interestingly, mutant *pol*A_6 also did not display robust levels of replication at 5 dpi with titers remaining three orders of magnitude below *pol*A_6R (Figure 3.2B). Mutant *pol*ΔN43 exhibited a 20-fold decrease in viral yield at 5 dpi, but this difference was not found to be statistically significant (Figure 3.2B). Infectious virus became nearly undetectable in the TG by 7 dpi for all viruses, including WT (Figure 3.2C). Therefore, *pol*A_6 exhibited

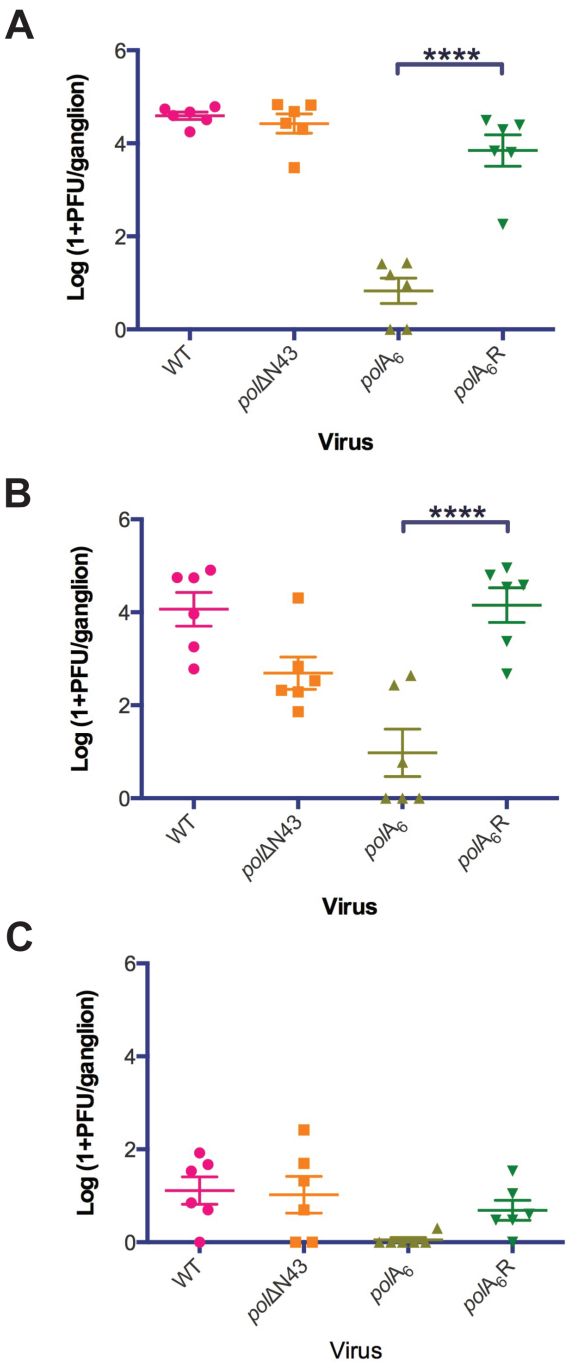

Figure 3.2. Replication kinetics of *pol* mutant viruses in acutely infected TG. Following virus inoculation, mouse TG was harvested at 3 (A), 5 (B), and 7 (C) dpi. TG were processed and assayed for detection of infectious virus as described in Materials and Methods. One PFU was added to each value and log PFU per TG ± the SD of mean values are plotted. All viruses were analyzed by one-way ANOVA, followed by Bonferonni post-tests comparing WT, *pol*A_6 and *pol*A_6R to one another, accounting for multiple comparisons. ****, $p<0.0001$

severely impaired ganglionic replication throughout the acute phase of infection, while *pol*ΔN43 and *pol*A_6R displayed WT-like replication levels in TG.

Latency establishment and reactivation. We hypothesized that the acute replication defect exhibited by *pol*A_6 would compromise the efficiency of latency establishment. To investigate this possibility, we quantified the number of viral DNA copies in latently infected TG via real-time PCR. Mutant virus *pol*A_6 reproducibly exhibited a 6-fold decrease in latent viral DNA as compared to its rescued derivative *pol*A_6R ($p<0.0001$; Figure 3.3). As expected, latency establishment during *pol*ΔN43 infection was indistinguishable from that of WT and no significant differences were found between WT and *pol*A_6R viral DNA copy number (Figure 3.3).

Previous reports have demonstrated that specific viral DNA synthesis proteins that are dispensable for replication in cell culture are absolutely essential for reactivation (Chen et al., 2004; Coen et al., 1989; Jacobson et al, 1989; Thompson and Sawtell, 2000). Therefore, we sought to determine whether the conserved HSV-1 Pol residues absent in mutants *pol*ΔN43 and *pol*A_6 were required for reactivation from latent infection. Reactivation assays were performed using the dissociation method in which latently infected TG are digested into single cell suspensions and plated individually onto polB3 cells in a 6-well plate. Wells that did not yield infectious virus by ten days post explant were harvested, frozen, thawed and plated onto a fresh polB3 monolayer as a final test of reactivation. As expected, 100% of WT- and *pol*A_6R- latently infected TG had reactivated by three days post explant (Figure 3.4). Mutant virus *pol*ΔN43 displayed a comparable level of reactivation (96%), which demonstrated that the extreme N-terminal 42 residues were not required for reactivation. The reactivation efficiency of *pol*A_6 was

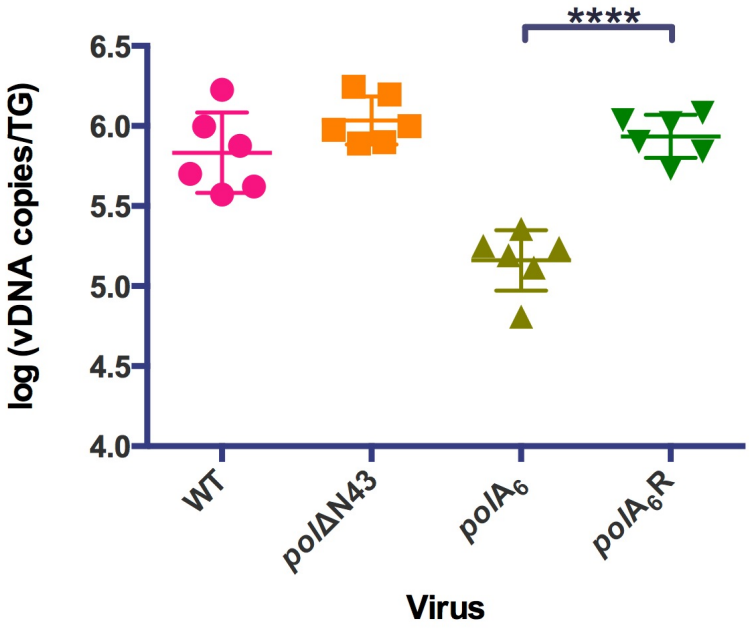

Figure 3.3. Latency establishment of *pol* mutant viruses. Latently infected TG were harvested from mice at 30 dpi and processed for DNA isolation. Viral DNA (vDNA) was quantified via real-time PCR and was normalized to mouse DNA. Log vDNA copies ± SD of mean values are plotted. All viruses were analyzed by one-way ANOVA, followed by Bonferonni post-tests comparing WT, *pol*A_6 and *pol*A_6R to one another, accounting for multiple comparisons. ****, $p<0.0001$

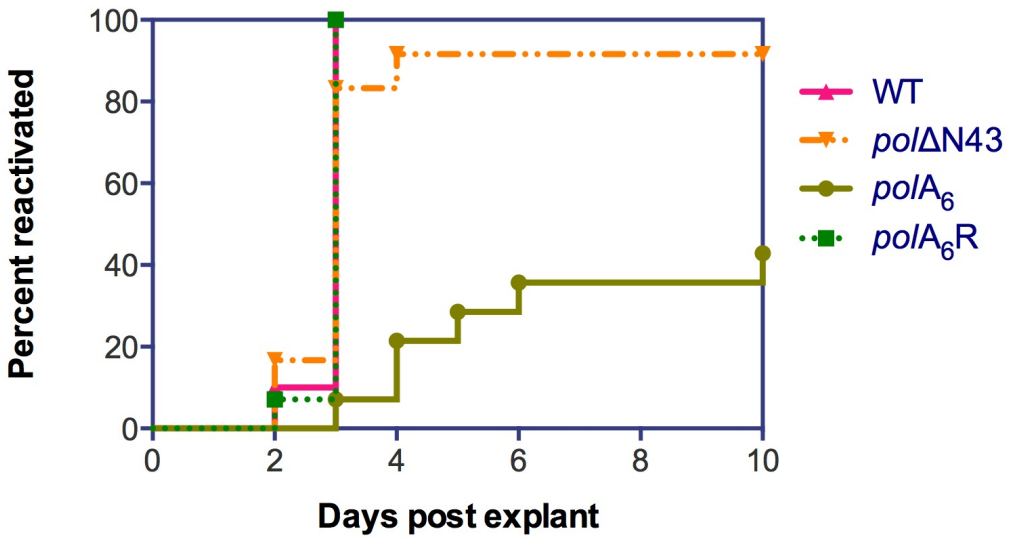

Fig 3.4. Kinetics of *pol* mutant virus reactivation from explanted TG *in vitro*. Latently infected TG harvested at 30 dpi were enzymatically dissociated and plated onto polB3 cells in a 6-well plate. Aliquots of viral supernatant from individual wells were analyzed directly for detection of infectious virus.

only 43% and this reduction was found to be statistically significant when compared to *pol*A_6R (p=0.0019, Fisher's exact test). In addition to diminished reactivation efficiencies, *pol*A_6 displayed slower reactivation kinetics as compared to WT, *pol*ΔN43, and *pol*A_6R (Figure 3.4). These results suggest that the conserved motif FYNPYL is necessary for WT latency establishment and reactivation efficiencies.

Replication kinetics of *pol*A_6 in resting cells. A number of mutant viruses that exhibit decreased viral replication during the acute phase of infection also display replication defects in resting cell cultures (Bolovan et al., 1994; Brown et al., 1994; Field and Wildy, 1978; Goldstein and Weller, 1988a; Jacobson et al., 1989; Jamieson et al., 1974). In order to determine whether the metabolic state of the cell enhanced the *in vitro* growth defect observed with *pol*A_6, we analyzed multi-cycle replication kinetics in resting HFFs. We included *tk* null virus *dl*sptk as a positive control in this experiment, as it has been well established that such viruses specifically exhibit impaired replication in resting cells in which cellular TK expression is repressed (Field and Wildy, 1978; Jamieson et al., 1974). We found that *dl*sptk replication was similar to that of WT in actively dividing cells at 48 hpi and was reduced by threefold in resting cell cultures (Table 3.1). As expected, *pol*A_6 exhibited reduced viral yield in dividing cells with a 7-fold drop in infectious virus as compared to *pol*A_6R (Table 3.1). Interestingly, the *pol*A_6 replication defect was enhanced in stationary cell cultures to a 57-fold defect in viral yield at 48 hpi when compared to *pol*A_6R (Table 3.1). These results suggest that *pol*A_6 replication is further restricted in resting cells and this effect may contribute to the severity of the acute ganglionic replication defect observed in mice.

Table 3.1. Replication of *pol*A_6 in resting cell cultures

Virus	Titer (PFU/mL)	
	Dividing cells	Resting cells
WT	2.1×10^8	9.1×10^7
*dls*ptk	2.5×10^8	3.1×10^7
polA_6	6.4×10^7	2.0×10^6
polA_6R	4.3×10^8	1.1×10^8

Discussion

In this study, we sought to specifically address the biological significance of conserved residues within the pre-NH_2-terminal domain of HSV-1 Pol in a mouse model of infection. In accordance with the previously reported *in vitro* phenotypes (Chapter 2 − Terrell and Coen, 2012), we found that *pol*ΔN43 exhibited an *in vivo* replication profile similar to WT. Despite 76% protein sequence identity between HSV -1 and -2 (DiTommaso et al., 2011; Notredame et al., 2000), the extreme N-terminal 42 residues appeared to be dispensable for replication and reactivation from latent infection in mice. Only viruses within simplexvirus genera of the alphaherpesvirus family retain these extreme N-terminal 42 residues, yet their conservation among these related viruses is low with just 14% protein sequence identity (DiTommaso et al., 2011; Notredame et al., 2000). However, we cannot rule out the possibility that these conserved residues confer an advantage that is specific for simplexvirus replication. In contrast, mutant *pol*A_6 displayed significant defects in both acute and latent infections that were restored to WT-like levels with rescued derivative *pol*A_6R, with a particularly severe defect in acute ganglionic replication. These studies have demonstrated an especially important role for the conserved motif FYNPYL in viral replication in mice. The implications of these observations are discussed below.

Mutant *pol*A_6 exhibits acute replication defects in the mouse eye and TG. Previous characterization of the *pol*A_6 growth phenotype in cell culture revealed a defect in viral yield that corresponded with a comparable decrease in viral DNA synthesis (Chapter 2 − Terrell and Coen, 2012). We have demonstrated that mutant *pol*A_6 also exhibited a similar defect in viral replication in the mouse eye while replication at the

secondary site of infection was notably attenuated. Overall, *pol*A_6 infection failed to produce a robust level of viral replication in the TG during the acute phase with the highest yield in any ganglia being only 400 PFU versus ~1×10^5 PFU from WT and *pol*A_6R infections at 5 dpi. While we cannot completely rule out the possibility that the ganglionic replication defect is an amplification of the 5-fold defect observed at 1 dpi, there does not appear to be a direct correlation between acute replication defects in the eye and TG in the literature. For example, virus *in1814* that encodes a mutant VP16 exhibits defects of up to 30-fold in the eye at 2 dpi with only a 17-fold decrease in the ganglia at 4 dpi (Thompson and Sawtell, 2009). Additionally, drug resistant mutant PFA^r5 exhibits a 13-fold decrease in the mouse eye at 1 dpi that remains a 13-fold decrease in the TG at 3 dpi as compared to its WT (Pelosi et al., 1998). Our data would suggest that the observed ganglionic replication defect reflects a specific block during *pol*A_6 infection, which is supported by the observation of restricted *pol*A_6 replication in resting cell cultures. Therefore, the function mediated by the conserved motif FYNPYL is of greater importance in non-dividing cells and especially in neurons. Further investigation is needed in order to elucidate the exact mechanism by which *pol*A_6 exhibits this replication defective phenotype.

Reduced latency establishment and reactivation efficiencies during *pol*A_6 infection. Replication incompetent *pol* null viruses have been previously shown to establish latency in animal models of infection as measured by the amount of viral DNA, albeit at a much reduced level compared to WT virus (Katz et al., 1990). Thus, we anticipated that the reduced level of ganglionic replication during *pol*A_6 infection would negatively impact latent viral load. Despite the severity of the phenotype, the number of

viral DNA copies in latently infected TG was only decreased by 6-fold as compared to *pol*A_6R, which was a reproducible and statistically significant difference. The decrease in latent viral DNA did not equal the magnitude of the replication defect observed in acutely infected ganglia; rather, it was more consistent with the 5-fold corneal replication defect at 1 dpi. These observations are similar to those of a previous report that demonstrated that amplification of virus at the original site of inoculation was important for efficient latency establishment (Thompson and Sawtell, 2000). Decreased latency establishment in addition to the previously described viral DNA synthesis defect during *pol*A_6 infection (Chapter 2 − Terrell and Coen, 2012) contributed to diminished reactivation efficiency and kinetics. It is possible that, in addition to these two deficiencies, reactivation is further restricted in this mutant by a specific defect in neuronal replication.

Given the severity of the acute ganglionic replication defect for *pol*A_6, we were surprised that its reactivation efficiency was only twofold lower than that of *pol*A_6R. Interestingly, a VP16 mutant that displayed WT reactivation efficiencies from *in vitro* explant reactivation assays was unable to produce detectable infectious virus and lytic viral proteins in TG following hyperthermic stress *in vivo* (Thompson and Sawtell, 2009). Thus, recovery of infectious *pol*A_6 virus from latently infected TG reactivated *in vivo* could potentially be even less efficient than what was observed with the *in vitro* explant model. While the conserved motif is not necessary for reactivation from explanted TG, it appears to play a role in the efficient establishment of latency.

The *pol*A_6 phentoype is very similar to that of TK^- virus replication in a mouse ocular model of infection (Chen et al., 2004; Thompson and Sawtell, 2000). A previous study reported that the *tk* null virus 17/tBTK^- exhibited a 12-fold decrease in both corneal

replication and latent viral genome copy number (Thompson and Sawtell, 2000). Mutant 17/tBTK$^-$ was unable to replicate in TG during acute phase infection, which suggested that viral DNA in latently infected TG originated from replication on the eye rather than ganglionic replication (Thompson and Sawtell, 2000). Some interesting points regarding this previous study include the fact that the 15-fold defect in infectious virus production was mirrored in viral DNA copy number in eye homogenates with 17/tBTK$^-$ infection as compared to its rescued variant 17/tBTK$^+$. Additionally, the defects in viral DNA copies present in eye and TG homogenates when comparing 17/tBTK$^-$ and 17/tBTK$^+$ infection at 4 dpi appear to be comparable (Thompson and Sawtell, 2000). This would suggest that viral replication at the periphery directly impacted the number of viral genomes that enter the ganglia during acute phase replication. Both *pol*A$_6$ and *tk* null viruses specifically exhibit defects in viral DNA synthesis during cellular infection in addition to displaying similar defects during productive and latent infection in mice (Chen et al., 2004; Field and Wildy, 1978; Jamieson et al., 1974; Chapter 2 − Terrell and Coen, 2012; Thompson and Sawtwell, 2000). Despite the differences with viral dosage and selected time points used in these studies, the outcome of reduced latency establishment following decreased corneal replication remains consistent. However, examples exist in which mutant viruses that are defective for corneal replication sustain WT-like reactivation efficiencies and latent viral DNA loads (Perng et al., 1996; Thompson et al., 2009). In combination, these data would support the possibility that the level of viral DNA synthesis rather than infectious virus production at the peripheral site of infection is important for latency establishment.

Identification of the mechanistic aspect that is responsible for the *pol*A_6 replication defect remains elusive. Based on *in vitro* growth characteristics, we concluded that the *pol*A_6 enzyme is unable to produce WT-like levels of viral DNA that correlated with a defect in infectious virus production (Chapter 2 − Terrell and Coen, 2012). Interestingly, this inability became more detrimental during infection of resting cells and especially in the ganglia. Due to the nature of the mutation, we hypothesize that the defect is due to a disrupted protein-protein interaction that is dispensable for viral replication in cell culture and is essential for robust ganglionic replication in mice. One possible explanation is that a protein encoding a redundant function, which can partially compensate for the loss of function during *pol*A_6 infection in actively dividing cells, is reduced or absent in resting cells and neurons. Alternatively, the mutation may have only diminished the binding affinity of HSV-1 Pol rather than completely abolishing the protein-protein interaction. In this scenario, the ability of the HSV-1 Pol enzyme to form a stable complex would be of greater importance in neurons due to limited expression of the protein binding partner. These possibilities are currently under investigation. The mechanism by which the conserved motif FYNPYL mediates efficient viral DNA synthesis and production of infectious virus may very well reflect a conserved replication mechanism in HSV-1 replication as well as for the family of human herpesviruses.

Acknowledgements

We thank Jean M. Pesola at Harvard Medical School in the laboratory of Donald Coen, who provided training for the animal care and handling, collaborated in the execution of these studies, conducted statistical analyses, and provided helpful comments and discussions.

Chapter 4

Protein Interactions of Pre-NH_2-Terminal Pol Mutants

Abstract

Successful viral replication is dependent upon the faithful execution of viral DNA synthesis processes. Herpes simplex virus 1 (HSV-1) encodes a DNA polymerase that directly mediates the production of progeny viral DNA molecules and is essential for production of infectious virus. In a previous study, we sought to engineer mutations within the pre-NH_2-terminal domain of the catalytic subunit of the viral DNA polymerase (HSV-1 Pol) that did not disrupt 5'-3' polymerase activity. We identified two HSV-1 Pol mutant enzymes that retained WT-like 5'-3' polymerase activity *in vitro* yet exhibited decreased levels of viral DNA synthesis during cellular infection: deletion mutant PolΔN52 in which the extreme N-terminal 51 residues were deleted, and substitution mutant $PolA_6$ in which residues 44-49 were substituted with alanines. We hypothesized that these mutations disrupted a protein-protein interaction that was necessary for efficient viral DNA production in the infected cell and wished to identify the lost binding partner. Using a candidate-based approach, we examined the association of reported HSV-1 Pol binding partners with our mutants via coimmunoprecipitation studies with recombinant purified proteins *in vitro*. Alternatively, we took a global approach by incorporating a FLAG tag at the extreme N-terminus of the WT and mutant *pol* locus in order to identify interactions with potential viral or cellular binding partners from infected cell lysates. Thus far, we have yet to identify a protein interaction that was lost as a result of the mutation.

Introduction

Herpesviruses exhibit similar mechanisms by which viral proteins direct the process of viral genome amplification and production of infectious virus. Herpes simplex virus 1 (HSV-1) encodes seven proteins that are absolutely essential for viral DNA synthesis: DNA polymerase holoenzyme (Pol (UL30)/UL42), helicase primase complex (UL8/UL5/UL52; H/P complex), single stranded DNA binding protein (SSB; ICP8), and origin binding protein (UL9) (Boehmer and Lehman, 1997). While the process of viral DNA synthesis has yet to be recapitulated *in vitro*, protein-protein interactions appear to lie at the heart of the process. UL9 binds DNA sequences within the origin of replication and subsequently recruits ICP8 via a direct protein-protein interaction (Elias and Lehman, 1988; Koff and Tegtmeyer, 1988; Olivo et al., 1988). ICP8 binding enhances the inherent helicase and ATPase activity of UL9, which is responsible for unwinding double stranded DNA and further promotes the assembly of the viral replisome (Boehmer et al., 1993; Boehmer and Lehman, 1993). An interaction between UL9 and UL8 is thought to facilitate the recruitment of the H/P complex to the origin (McLean et al., 1994). H/P complex subunits UL5 and UL52 possess helicase and primase activity, which is enhanced upon association with the UL8 subunit (Calder and Stow 1990; Dodson and Lehman, 1991; Falkenberg et al., 1997; Tanguy Le Gac et al., 1996; Tenney et al., 1994; Tenney et al., 1995). Activity of the H/P complex can also be stimulated by ICP8, which was shown to be dependent upon the presence of the UL8 subunit (Crute and Lehman, 1991; Tanguy Le Gac et al., 1996; Hamatake et al., 1997). UL42 enhances HSV-1 Pol activity by tethering the holoenzyme to DNA and thereby increasing the processivity of nucleotide incorporation during DNA synthesis (Gallo et al., 1989; Gottlieb et al., 1990).

While these activities have been demonstrated with purified proteins *in vitro*, the exact mechanism by which HSV-1 DNA is synthesized within the context of the infected cell has yet to be elucidated.

HSV-1 Pol can associate with a multitude of viral proteins, but the significance of these reported interactions have yet to be demonstrated. The C-terminus of UL8 directly interacts with HSV-1 Pol and has been proposed to associate with the viral polymerase holoenzyme during leading strand synthesis (Liu et al., 2006; Mardsen et al., 1996). A previous report demonstrated an interaction between HSV-1 Pol and the viral DNA glycosylase UL2, which was suggested to serve as a mechanism by which DNA repair machinery is coordinated with the viral replisome (Bogani et al., 2009; Bogani et al., 2011). Complexes containing the viral single stranded DNA binding protein ICP8 and HSV-1 Pol coprecipitate from infected cell lysate (Strick et al., 1997; Vaughan et al., 1984), but this association may represent an interaction with DNA rather than a specific protein-protein interaction. Evidence supporting a functional interaction between ICP8 and HSV-1 Pol includes a study that found specific ICP8 mutants conferred altered sensitivity to viral DNA synthesis inhibitors (Chiou et al., 1985). Binding sites for the aforementioned proteins have yet to be mapped on HSV-1 Pol and the result of disrupting such an interaction is unknown.

The viral processivity factor UL42 binds at the extreme C-terminus of HSV-1 Pol and the two polymerase subunits predominantly exist as a heterodimer in infected cell lysates (Digard et al., 1993; Gallo et al., 1988; Vaughan et al., 1984). Given the ubiquitous presence of UL42 at the extreme C-terminus of HSV-1 Pol, we anticipated that additional interactions could be mediated by the extreme N-terminus. Previously, we

incorporated two independent mutations within the HSV-1 *pol* locus that resulted in decreased viral DNA synthesis and production of infectious virus during cellular infection without negatively impacting 5'-3' polymerase activity of the purified enzyme *in vitro*: deletion of the extreme N-terminal 51 residues and substitution of residues 44-49 with alanines. We chose to directly investigate whether or not deletion mutant PolΔN52 or substitution mutant $PolA_6$ proteins could retain interactions with specific candidate proteins *in vitro*. To undertake an unbiased approach, we engineered FLAG-tagged *pol* viruses in order to identify any potential viral or cellular protein binding partner.

Materials and Methods

Cells and viruses. Vero cells (American Type Culture Collection) and polB3 cells, a kind gift from Charles Hwang (Hwang et al., 1997), were maintained as previously described (Chapter 2 − Terrell and Coen, 2012). HeLa (American Type Culture Collection) were grown and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% newborn calf serum, 1% penicillin/streptomycin, and 1% amphotericin B.

Protein expression and purification. Purified wild type (WT) Pol, PolΔN52 and $PolA_6$ proteins were purified from baculovirus-infected insect cell lysates as previously described (Chapter 2 − Terrell and Coen, 2012).

Surface plasmon resonance analysis. SPR measurements were performed using a Biacore T100 (Biacore). Purified WT Pol, PolΔN52 and $PolA_6$ proteins were immobilized on a CM5 sensor chip in separate channels as ligands. The amount of each ligand bound corresponded to approximately 2000 Resonance Units (RUs). Purified protein analytes (UL8, ICP8, UL12 and UL8/5/52 (H/P complex)) in HBS buffer (10mM HEPES [pH 7.4], 3mM EDTA, 0.15M NaCl, 0.05% Surfactant P20) were injected over the sensor surface at a flow rate of 30 µl/min for 180 sec. Post injection dissociation was monitored in HBS buffer without analyte for 180 sec at the same flow rate. The surface was regenerated between injections using 2.5M NaCl at a flow rate of 100µl/min for 20 sec. For kinetics assays, sensorgrams were fitted to a 1:1 Langmuir binding model using Biacore T100 Evaluation software.

HSV-1 Pol and UL2 coimmunoprecipitation. Full length UL2 and truncation mutant $UL2_{M91\text{-}V334}$ that were expressed as C-terminal V5-His_6 fusion proteins were

kindly provided by Paul Boehmer (University of Arizona; Bogani et al., 2009). Each HSV-1 Pol protein (1.2 pmol) was incubated either alone or in combination with UL2 or $UL2_{M91\text{-}V334}$ (19 pmol) in 300µl binding buffer (20mM HEPES [pH 7.5], 10% glycerol, 175mM NaCl, 0.5% NP-40, 10mg/mL BSA, 1mM DTT, 10ul/mL HALT protease inhibitor cocktail (Sigma) for 1 hr at 4°C. Reactions were supplemented with 3µg of anti-V5 antibody (Invitrogen) and incubated for an additional 2 hr prior to the addition of pre-equilibrated EZView Red Protein A affinity resin (Sigma), followed by a 1 hr incubation at 4°C with rotation. The protein/antibody complexes were spun down at 8200×g and washed three times with wash buffer (50mM TrisCl [pH 8], 175mM NaCl, 0.5% NP-40, and HALT protease inhibitor cocktail). Proteins were eluted with 30µl of a 1:1 mix of wash buffer and 2x Laemmli buffer and boiled at 90°C. Ten microliters of the eluate were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were detected via Western blot analysis. Pol detection: polyclonal PP5 antibody (Yager et al., 1990; 1:2500), goat α rabbit (Southern Biotech; 1:2000). UL2 detection: mouse α-His (Novagen; 1:2000), TrueBlot α mouse IgG (eBioscience; 1:1000).

Generation of FLAG-tagged *pol* viruses. *E. coli* strain GS1783 harboring the bacterial artificial chromosome (BAC) clone of HSV-1 strain KOS (Jurak et al., in preparation) or mutant $PolA_6$ (Chapter 2 − Terrell and Coen, 2012) were utilized in two-step Red recombination techniques (Tischer et al., 2006) to generate tagged virus as previously described (Chapter 2 − Terrell and Coen, 2012). Briefly, a FLAG epitope (DYKDDDDK) was inserted downstream of the initiation codon (AUG) of the *pol* coding sequence in each parental clone using primers FLAG-Pol For (5'- TCC CCC CTC TTT AGG GGT TCG GGT GGG AAC AAC CGC GAT GGA CTA CAA GGA TGA

CGA CGA TAA GTA GGG ATA ACA GGG TAA TCG ATT T -3') and FLAG-Pol Rev (5'- ACT TTC CTC CGG GGG ACA GCG GGC CGC CGC CAC CGG AAA ACT TAT CGT CGT CAT CCT TGT AGT CCA TCG CGG TTG TTC CCA CCC GCC AGT GTT ACA AC CAA TTA ACC -3') to generate BACs FLAG*pol* and FLAG*pol*A_6. Presence of the insertion, and lack of adventitious mutations, was verified by sequencing the resulting FLAG-tagged BAC clones. BAC derived viruses were harvested and propagated as previously described (Chapter 2 − Terrell and Coen, 2012).

Viral replication assays. Vero cells (4×10^5) were infected in triplicate a multiplicity of infection (MOI) of 1 with parental and FLAG-tagged viruses. After a 1 hr adsorption period at 37°C, wells were washed with Dulbecco's phosphate buffered saline (DPBS) and replenished with 2mL of DMEM containing 2% NCS. At each time point, whole cell lysates were collected, frozen and subsequently thawed and sonicated. Cellular debris was pelleted by centrifugation and supernatants were titrated on polB3 cells in duplicate.

Western blot analysis. Vero cells (6×10^5) were either mock infected or infected at an MOI of 20 with parent and FLAG-tagged viruses. At 8 hpi, monolayers were washed with DPBS and harvested with 330μl Laemmli buffer. Protein lysates were boiled at 90°C for 7 min and resolved by SDS PAGE. Membranes were blocked in 5% milk/TBST and probed with PP5 α-Pol (1:2500), α-FLAG M2- horseradish peroxidase (HRP) (Sigma-Aldrich; 1:1000), and α-β-actin (Abcam; 1:10000). Blots were exposed to secondary antibodies goat α-rabbit-HRP and goat α-mouse-HRP (Southern Biotech) and detected with chemiluminescence solution (Pierce).

FLAG coimmunoprecipitation. HeLa cells ($\sim2\times10^7$) were infected with WT,

FLAG*pol* or FLAG*pol*A_6 at an MOI of 10. At 8 hpi, cells were washed twice with cold DPBS and harvested with 3mL lysis buffer (50mM HEPES-KOH [pH 7.4], 1% Triton X-100, 150mM NaCl, 10% glycerol, 2mM EDTA, and one Complete EDTA-free protease inhibitor tablet (Roche) per 50mL). Cells were incubated in lysis buffer for 30 min and spun down at 10K RPM for 10 min at 4°C. Clarified lysate was incubated with 3ug of Mouse IgG1 Isotype control antibody (Sigma) at 4°C overnight in the presence or absence of 400U of benzonase (Novagen). To evaluate the efficiency of benzonase treatment, 25µl of treated and untreated samples were diluted 1:1 with 6x gel loading buffer and analyzed on a 0.8% agarose gel stained with ethidium bromide. Subsequently, lysates were added to 80µl settled of EZ-View Protein G affinity resin (Sigma) for 2 hr at 4°C with rotation. Clarified lysates were removed and added to 80µl settled EZ-view anti-FLAG M2 resin (Sigma) and incubated at 4°C for 2 hr. Beads were washed three times with 750µl of lysis buffer for 15 min with rotation. Subsequently, beads were washed with tris-buffered saline (TBS; 50mM Tris-Cl [pH 7.5], 150mM NaCl) twice and proteins were eluted with 300µl of 3xFLAG peptide (Sigma; 200ng/µl in TBS). Following a 1 hr incubation at 4°C, the supernatant was removed and concentrated with a Vivaspin 500 ultrafiltration device (GE Healthcare), according to the manufacturer's instructions, to a 30µl volume. The final eluate was diluted 1:1 with 2× Laemmli buffer and boiled at 90°C for 7 min. Immunoprecipitated proteins (25µl) were electrophoresed on an any KD mini-protean precast polyacrylamide gel (BioRad) and detected with SilverQuest staining kit (Invitrogen) according to the manufacturer's instructions. Bands were excised and submitted to the Taplin Mass Spectrometry Facility (Harvard Medical School) for liquid chromatography-tandem mass spectrometry analysis (LC/MS/MS). For

Western blot analysis, 5µl of input fractions and eluted proteins were resolved by SDS-PAGE. Viral proteins were detected with PP5 (as described above) and αICP8 antibody 3-83 (Knipe et al., 1987; 1:10,000) with goat α-rabbit-HRP and (1:5,000; Southern Biotech).

Results

SPR analysis of HSV-1 Pol protein interactions. HSV-1 Pol has been reported to associate with several viral DNA synthesis proteins, yet the effect of disrupting such interactions remains largely unknown. The best characterized interaction is with the viral processivity factor UL42, which binds at the extreme C-terminus of HSV-1 Pol and is necessary for processive DNA synthesis and viral replication (Digard et al., 1993a, b). Therefore, we anticipated that additional proteins could bind at the extreme N-terminus of HSV-1 Pol. Previously, we engineered two HSV-1 Pol mutant enzymes that exhibit WT 5'-3' polymerase activity and retain a functional interaction with UL42 *in vitro*: PolΔN52 in which the extreme N-terminal 51 residues were deleted, and $PolA_6$ in which the conserved residues 44-49 (FYNPYL) were substituted with alanines (Chapter 2 − Terrell and Coen, 2012). When these mutations were incorporated into the viral genome, the resulting mutant viruses exhibit up to a 10-fold defect in viral DNA synthesis during infection. We hypothesized that residues within the extreme N-terminal 51 residues of HSV-1 Pol could mediate a protein-protein interaction that may or may not impact viral DNA synthesis during infection. We sought to address this hypothesis by employing multiple methods to evaluate specific candidates *in vitro*.

Previous studies have demonstrated a direct interaction between HSV-1 Pol and UL8 via enzyme-linked immunosorbent assays with purified protein *in vitro* (Marsden et al., 1996). An interaction between HSV-1 Pol and the viral single stranded DNA binding protein (SSB) ICP8 has been suggested previously, but has yet to be confirmed (Strick et al., 1997; Vaughan et al., 1984). We wished to determine whether these viral replication proteins could directly associate with HSV-1 Pol and if this interaction would be

disrupted as a result of the deletion or substitution mutations within the pre-NH_2-terminal domain.

Interactions between HSV-1 Pol and viral protein binding candidates ICP8, UL8, and the H/P complex were evaluated using surface plasmon resonance assays performed by Ping Bai in Sandy Weller's lab (University of Connecticut Health Center). Purified WT Pol, PolΔN52, or $PolA_6$ were immobilized to a biosensor chip and protein analytes were applied as described in Material and Methods. The viral alkaline nuclease UL12 does not bind WT Pol and served as a negative control in this assay (Figure 4.1A). An interaction between WT Pol and the H/P complex was easily detected via SPR analysis, while an association with the UL8 subunit alone produced a rather modest signal (Figure 4.1 A). In contrast, we were unable to detect an interaction between WT Pol and ICP8 (Figure 4.1A). The SPR sensorgrams for both PolΔN52 and $PolA_6$ were similar to that of WT Pol (Figure 4.1B and C). We sought to quantify the binding kinetics of the H/P complex in order to identify any potential alteration in the binding affinities for mutants PolΔN52 and $PolA_6$. Over a range of 5 to 160nM of the H/P complex, SPR sensorgrams for WT and mutant polymerases exhibited dose-dependent binding of the H/P complex (data not shown). Ultimately, we found no meaningful differences in the association and dissociation rates of the WT and mutant polymerases with the H/P complex (Table 4.1). Therefore, we would conclude that the H/P complex binding site is outside of the extreme N-terminal 51 residues of HSV-1 Pol.

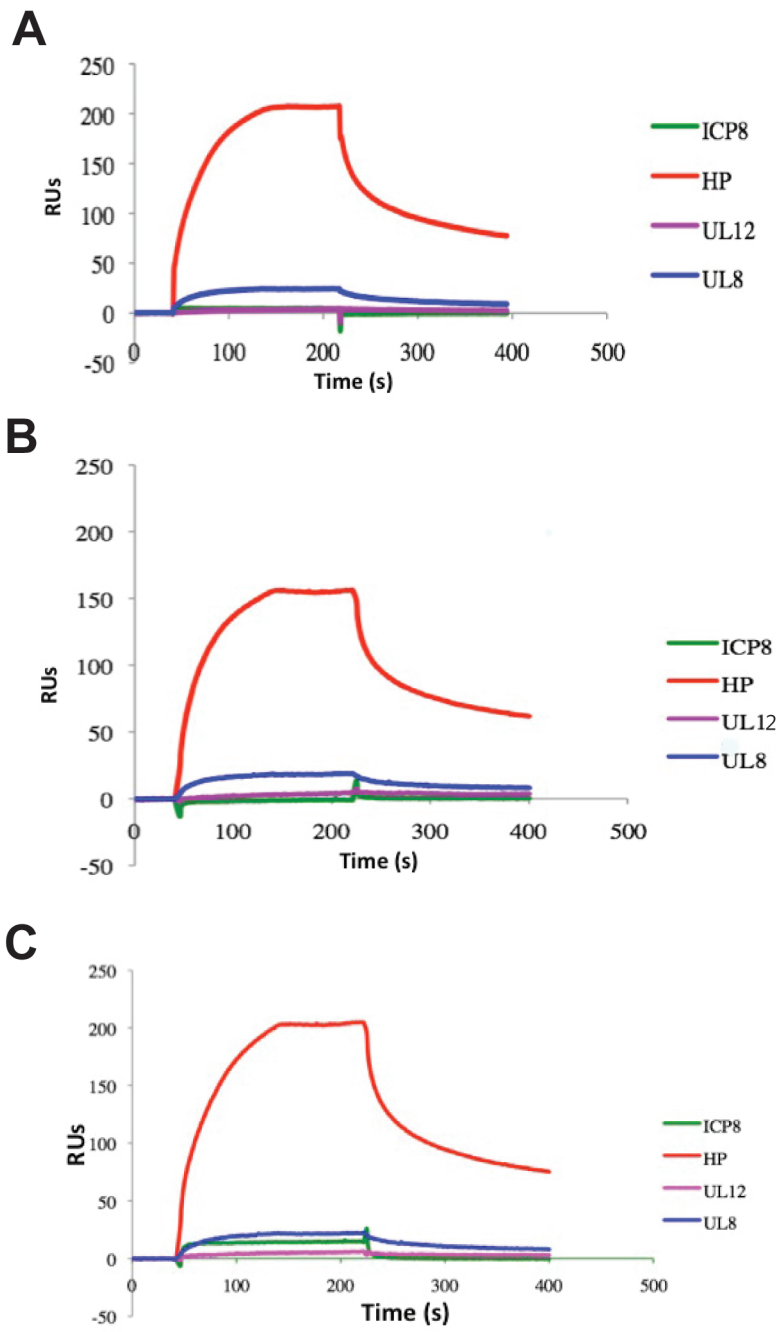

Figure 4.1. HSV-1 Pol interactions with viral DNA replication proteins. SPR analyses of protein interactions were performed using a Biacore T100. Sensorgrams of viral DNA replication proteins binding immobilized WT Pol (A), PolΔN52 (B), or $PolA_6$ (C) protein are shown. Signals are reported in resonance units (RUs).

Table 4.1. Binding kinetics of HSV-1 Pol and H/P complex

Protein	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
WT Pol	5.2×10^{4}	4.6×10^{-3}	88.3
PolΔN52	5.0×10^{4}	3.3×10^{-3}	65.1
$PolA_6$	4.2×10^{4}	4.1×10^{-3}	96.8

HSV-1 Pol and UL2. A previous study demonstrated a direct interaction between HSV-1 Pol and the viral uracil DNA glycosylase (UDG) UL2 that was localized to the extreme N-terminal 25kDa of UL2 (Bogani et al., 2010; P. Boehmer, personal communication). HSV-1 Pol can bind UL2 and UL42 simultaneously, which demonstrates that the UL2 interaction surface is outside of the UL42-binding site at the extreme C-terminus of HSV-1 Pol (Bogani et al., 2010). Although UL2 is dispensable for HSV-1 replication in cell culture (Mullaney et al., 1989; Pyles and Thompson, 1994), the HCMV UDG UL114 appears to play a role in viral DNA synthesis and production of infectious virus at early times postinfection (Courcelle et al., 2001; Prichard et al., 1996). Additionally, UL114 binds an N-terminal 100kDa fragment of the HCMV DNA polymerase catalytic subunit UL54 (Strang and Coen, 2010). Therefore, we wished to determine whether mutants PolΔN52 and PolA_6 could retain an interaction with UL2. Coimmunoprecipitation studies were performed with purified HSV-1 Pol proteins and V5-tagged recombinant proteins UL2 (full length) and truncation mutant $UL2_{M91\text{-}334}$ that is unable to bind WT Pol (P. Boehmer, personal communication). Both mutants PolΔN52 and PolA_6 coprecipitated with full length UL2, but were not recovered when incubated alone or in the presence of mutant $UL2_{M91\text{-}334}$ (Figure 4.2A and B). These results indicated that the UL2 binding site is outside of the extreme N-terminal 51 residues of HSV-1 Pol and that, presumably, the mutant polymerases retain an interaction with UL2 during infection.

Characterization of FLAG-tagged *pol* viruses. Previously, we demonstrated that the substitution mutant *pol*A_6 recapitulated the replication defect exhibited by

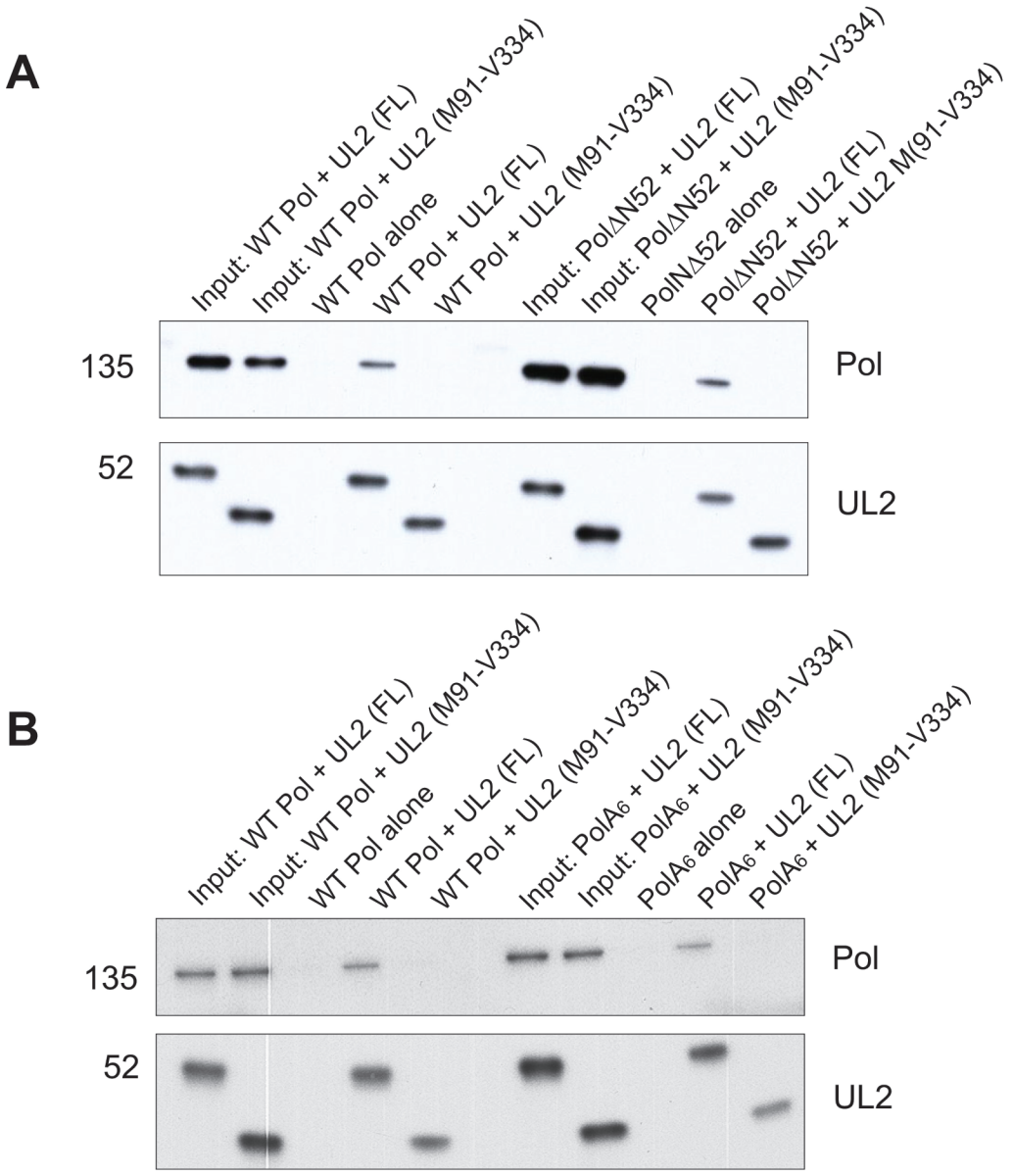

Figure 4.2. Coimmunoprecipitation of UL2 and HSV-1 Pol. Purified WT, PolΔN52 (A), and PolA_6 (B) proteins were incubated in the presence and absence of V5, His_6-tagged WT UL2 (full length) and Pol binding mutant UL2(M91-V334). Aliquots of input fractions and αV5 immunoprecipitated proteins were resolved by SDS-PAGE and probed with αPol and αHis antibodies for the detection of HSV-1 Pol and UL2, respectively. The positions of molecular weight markers are indicated on the left.

deletion mutant *pol*ΔN52, while deletion mutant *pol*ΔN43 replicated like WT virus (Chapter 2 − Terrell and Coen, 2012). We hypothesized that the conserved motif at HSV-1 Pol residues 44-49 could mediate a protein-protein interaction that is required for efficient viral DNA synthesis in the infected cell. Due to the vast number of possible interaction partners, we sought to address this hypothesis by employing an unbiased approach for analysis of HSV-1 Pol interactions in infected cell lysates. We anticipated that the addition of an epitope at the *pol* locus would allow for the efficient capture of HSV-1 Pol and hopefully prevent the disruption of potential interaction partners. Given that the UL42 binding site is at the extreme C-terminus of HSV-1 Pol, we chose to insert a FLAG epitope (DYKDDDDK) at the extreme N-terminus immediately downstream of the initiation codon (AUG) in the parental WT and *pol*A_6 BAC clones to generate FLAG*pol* and FLAG*pol*A_6 BACs, respectively. Addition of the FLAG epitope, and the absence of adventitious mutations, was confirmed by sequencing. Infectious virus was reconstituted via transfection of polB3 cells with BAC DNA as previously described (Chapter 2 − Terrell and Coen, 2012). In order to confirm that the BAC-derived FLAG viruses expressed a FLAG-tagged Pol protein, infected Vero cell lysates was harvested and analyzed for viral protein production via Western blot. Using a polyclonal α-HSV-1 Pol antibody, we observed comparable protein expression in all viruses at 8 hpi (Figure 4.3A). FLAG-tagged proteins at the corresponding molecular weight of HSV-1 Pol were detected in both FLAG*pol* and FLAG*pol*A_6 samples, which were not expressed by either parental virus (Figure 4.3A).

Our next task was to determine whether the addition of the FLAG epitope at the extreme N-terminus of HSV-1 Pol would reduce viral replication. Accordingly, we

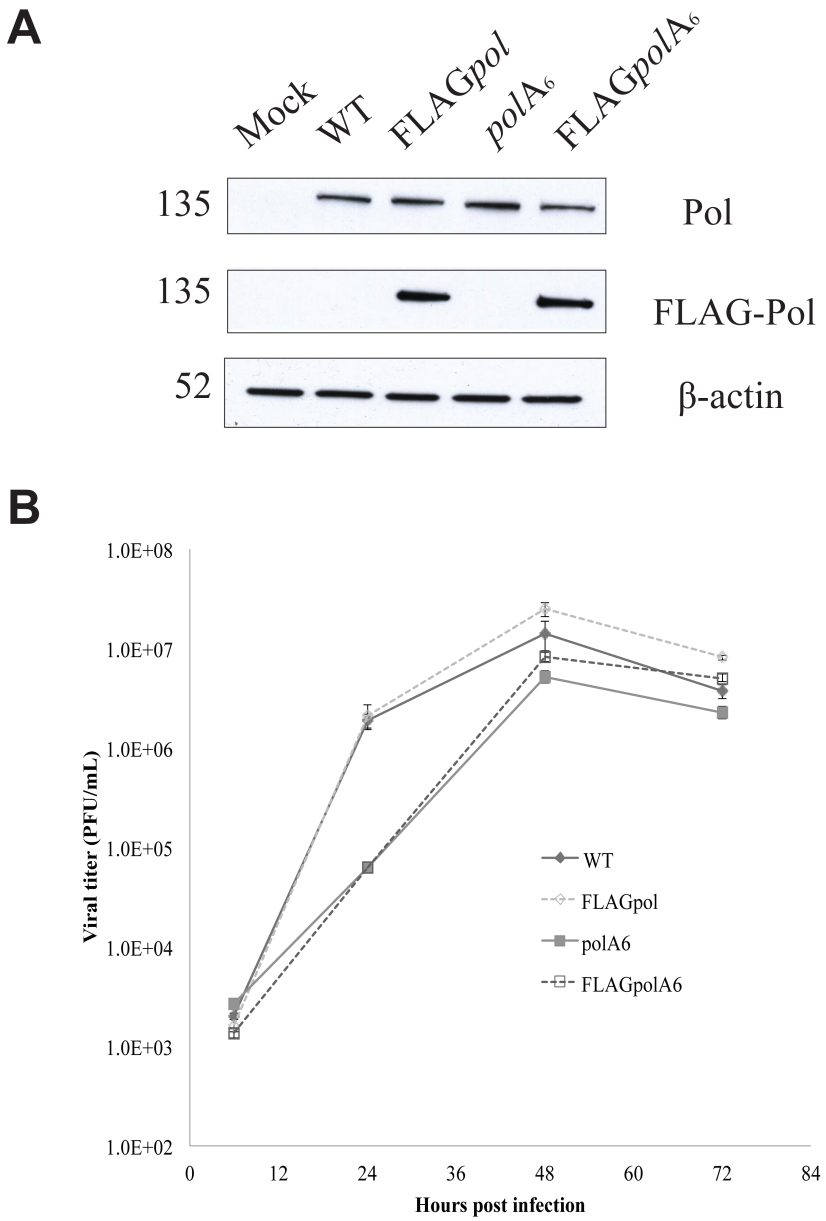

Figure 4.3. Characterization of FLAG-tagged *pol* viruses. (A) Expression of FLAG-tagged Pol protein in infected cell lysates. Vero cells were either mock infected or infected with the indicated virus at an MOI of 20. Infected cell lysates were harvested at 8 hpi and resolved via SDS-PAGE. Antibodies recognizing HSV-1 Pol, the FLAG peptide, and β-actin were used for Western blot analysis. The positions of molecular weight markers (kDa) are indicated on the right. (B) Replication kinetics of FLAG-tagged viruses. Vero cells were infected with parental and FLAG-tagged *pol* viruses an an MOI of 1. Whole cell lysates were harvested at the indicated time points and titrated on polB3 cells. Viral yield is reported as PFU/mL with each data point representing the mean ± SD of triplicate samples.

analyzed the multicycle replication kinetics (MOI of 1) of the FLAG-tagged viruses in comparison with its respective parental virus in Vero cells. WT virus reached peak viral titer at 48 hpi and its replication kinetics were recapitulated with FLAG*pol* infection (Figure 4.3B). As expected, FLAG*pol*A_6 yielded viral titers that were comparable to *pol*A_6 and both viruses exhibited up to a 35-fold defect in viral yield at 24 hpi compared WT virus (Figure 4.3B). Therefore, we conclude that the addition of the FLAG epitope at the extreme N-terminus did not significantly alter viral replication.

Identification of HSV-1 Pol binding partners via FLAG coimmunoprecipitation. In order to identify potential differences between WT and PolA_6 binding partners, we performed immunoprecipitations using both FLAG-tagged viruses. We included the untagged WT virus as a control in an effort to aid in the identification of specific associations with HSV-1 Pol. Infected HeLa cells were harvested at 8 hpi and proteins were precipitated with αFLAG antibody in the presence and absence of the nuclease benzonase in order to determine whether any potential interactions were indirectly mediated by nucleic acids. Aliquots of cell lysates were electrophoresed on an ethidium bromide-stained agarose gel in order to confirm the degradation of nucleic acids in the presence of benzonase (Figure 4.4A). Captured proteins were eluted with 3×FLAG peptide, boiled in Laemmli buffer, and analyzed by SDS-PAGE and silver stain. For samples that were precipitated in the presence of benzonase, we were able to detect protein bands at approximately 130 and 60 kDa in both FLAG*pol* and FLAG*pol*A_6 lanes, but not in the untagged WT lane (Figure 4.4B). SDS-PAGE protein profiles of eluates from untreated cell lysates were visually indistinguishable from that of benzonase-treated samples (data not shown).

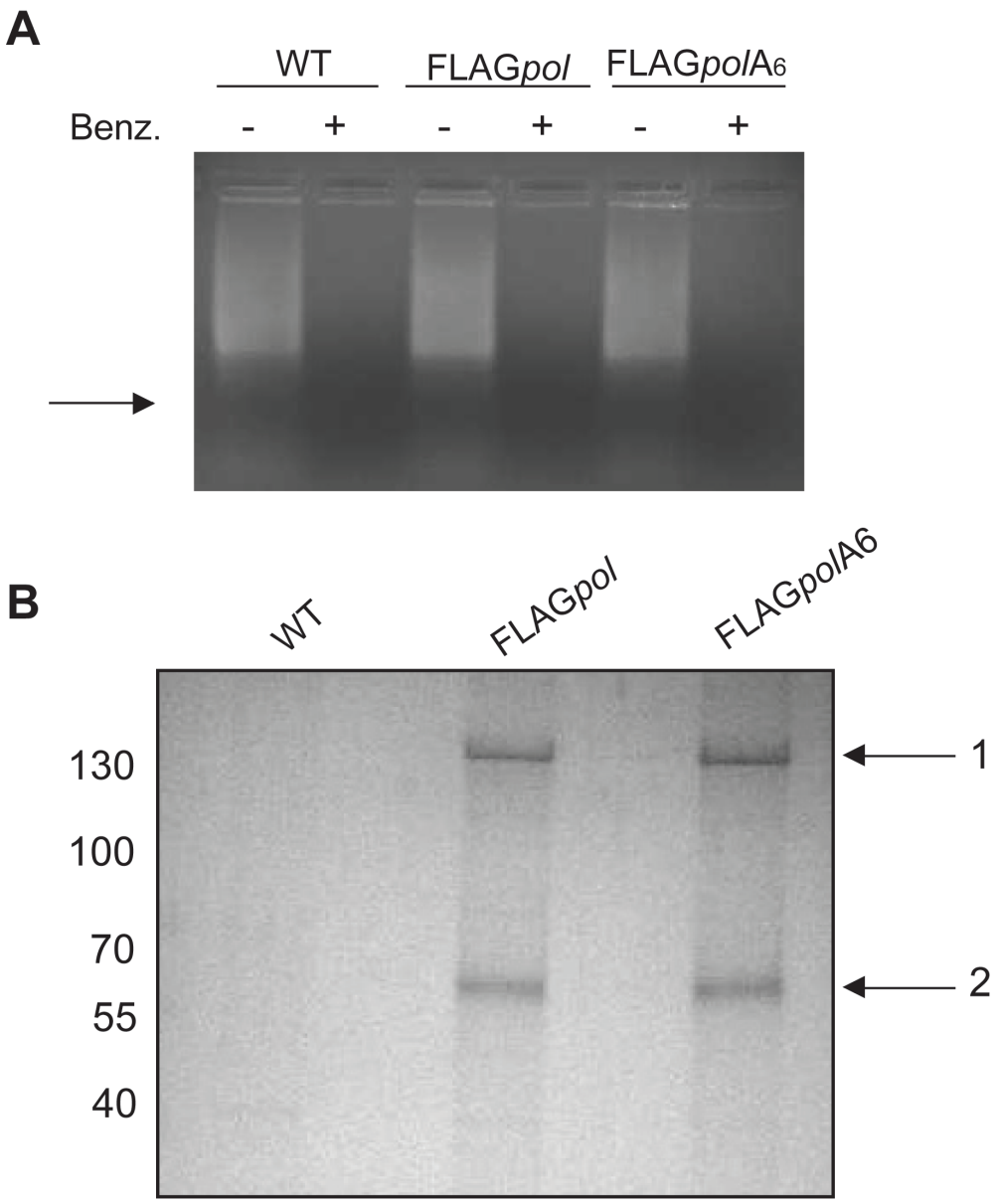

Figure 4.4. Immunoprecipitation of HSV-1 Pol from infected cell lysates in the presence of benzonase. HeLa cells were infected at an MOI of 10 with the indicated viruses and harvested at 8 hpi. Clarified cell lysates were incubated overnight in the presence and absence of benzonase. Following benzonase treatment, aliquots of each sample were diluted with gel loading buffer and analyzed on a 0.8% agarose gel and stained with ethidium bromide (A). The left hand arrow indicates the position of the dye front. Proteins were immunoprecipitated with αFLAG antibody, resolved by SDS-PAGE and detected with silver stain (B). The positions of molecular weight markers are indicated on the left. Bands that were excised and submitted for MS analysis are indicated on the right (arrows).

Proteins that are visible by silver stain tend to approach the lower limit of detection of LC/MS/MS analysis, as communicated by the Taplin MS facility at Harvard. As per their recommendations, we excised and submitted bands corresponding to 130 and 60 kDa (Bands 1 and 2) from the WT, FLAG*pol* and FLAG*pol*A_6 lanes for analysis (Figure 4.4). Table 4.2 includes a list of proteins detected by MS analysis in which at least 3 unique peptides were identified in eluates from the FLAG-tagged viruses and not the untagged WT virus. We were able to detect multiple HSV-1 Pol peptides that were present in Band 1 from both FLAG*pol* and FLAG*pol*A_6 samples and not in WT (Figure 4.4; Table 4.2). UL42 served as a positive control in this assay, as it associates with HSV-1 Pol in a 1:1 fashion and the heterodimer is readily precipitated from infected cell lysates (Gallo et al., 1988; Vaughan et al., 1985). As expected, UL42 was highly represented in terms of the number of peptides detected in both FLAG*pol* and FLAG*pol*A_6 eluates, irrespective of benzonase treatment (Band 2, Figure 4.4; Table 4.2). Peptides from the large subunit of the viral ribonucleotide reductase (UL39) were also detected in Band 1 from both samples (Figure 4.4; Table 4.2). Ribonucleotide reductase is a viral DNA synthesis protein that catalyzes the synthesis of deoxyribonucleotides during viral infection. The number of UL39 peptides was not diminished upon benzonase treatment, which suggested that the association was not dependent upon nucleic acid binding (Table 4.2). Viral transactivator ICP27 also associated with WT Pol and PolA_6 in both untreated and benzonase-treated samples (Band 2, Figure 4.4; Table 4.2). ICP27 has been shown to localize to replication compartments wherein it can interact with transcription machinery and direct early and late viral gene expression (de Bruyn Kops & Knipe, 1988). We were unable to identify any cellular proteins that coprecipitated with WT Pol or PolA_6 in the

Table 4.2. Proteins identified from HSV-1 Pol immunoprecipitation by MS analysis

Band[a]	Protein identified	No. of peptides[b]: FLAG*pol*	FLAG*pol*A_6
1	HSV-1 Pol (UL30)	210,226	124,199
	Ribonucleotide Reductase (UL39)	26,51	12,42
2	UL42	88,89	73,83
	ICP27	8,9	4,5

[a] indicates the bands that were isolated from the silver stained gel (Figure 4.4)
[b] total number of peptides identified for each protein (− , + benzonase treatment)

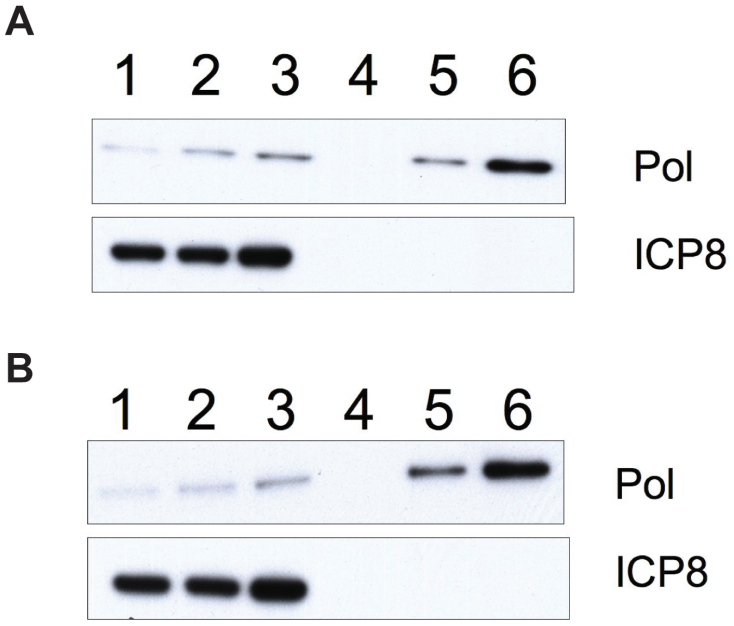

Figure 4.5. Western blot analysis of HSV-1 Pol immunoprecipitation from infected cell lysates. Aliquots from clarified lysates (input) and proteins isolated from FLAG immunoprecipitations in the absence (A) and presence (B) of benzonase were resolved via SDS-PAGE and analyzed for the presence of HSV-1 Pol and ICP8. Lane 1, WT input; Lane 2, FLAG*pol* input; Lane 3, FLAG*pol*A_6; Lane 4, WT IP; Lane 5, FLAG*pol* IP; Lane 6, FLAG*pol*A_6 IP.

presence and absence of benzonase. Due to the detection limit of MS analysis, we utilized Western Blot analysis in order to determine whether ICP8 could associate with HSV-1 Pol in infected cells. We were able to confirm the presence of HSV-1 Pol in all input fractions, which was specifically precipitated with αFLAG antibody from both FLAG*pol*- and FLAG*pol*A_6- infected cell lysates (Figure 4.5A and B). ICP8 did not appear to coprecipitate with WT Pol or PolA_6 in the presence and absence of benzonase (Figure 4.5A and B). Ultimately, we were unable to identify a protein whose association appeared to be disrupted as a result of the substitution mutation in PolA_6.

Discussion

We have used two complementary approaches in an attempt to identify a protein-protein interaction mediated by either the conserved motif at residues 44-49 of HSV-1 Pol that was responsible for efficient viral DNA synthesis during infection. While it appears that we have identified potential viral binding partners of HSV-1 Pol, we have yet to identify an interaction that has been abolished as a result of the deletion of the extreme N-terminal 51 residues or the substitution mutation(s) of the conserved motif. This raises interesting possibilities for the location and timing of specific HSV-1 Pol interactions. The implications of such findings are discussed below.

Associations between HSV-1 Pol and viral and cellular proteins *in vitro*.

UL8/ H/P complex. We have confirmed that the extreme N-terminal 51 residues of HSV-1 Pol are not required for interactions with either the UL8 or H/P complex. Our SPR analyses revealed a relatively high-affinity interaction between HSV-1 Pol and the H/P complex. We were unable to determine the binding kinetics for the HSV-1 Pol-UL8 complex, as this association did not produce a robust signal in this assay. The UL8 subunit when complexed with UL5 and UL52 may adopt a conformation that increases the affinity of binding for HSV-1 Pol. Interestingly, an interaction between HSV-1 Pol the UL5 subunit has recently been identified (S. Weller, personal communication). Thus, it is possible that the detected association between HSV-1 Pol and the H/P complex was mediated by an interaction with UL8 or the UL5 subunit, or both. Further analysis in regards to the stoichiometry of the HSV-1 Pol−H/P complex interaction may provide insight into the coordination of the viral replisome components during DNA replication.

ICP8. Our SPR analyses with purified proteins did not detect an interaction between ICP8 and HSV-1 Pol. Additionally, ICP8 did not coprecipitate with WT Pol from infected cell lysates in the presence and absence of benzonase. These results contradict previous reports that identified ICP8 in complex with HSV-1 in infected cell lysates via Western blot analysis (Strick et al., 1997; Vaughan et al., 1984). We were somewhat surprised that we failed to detect ICP8 in samples that were precipitated in the presence of nucleic acids, given that that both HSV-1 Pol and ICP8 are DNA binding proteins. It is possible that our immunoprecipitation conditions were too stringent to allow for the retention of potentially weak or transient interactions. An interaction between HSV-1 Pol and ICP8 may be more easily detected by immunoprecipitation analyses using purified proteins *in vitro*. Stimulation of HSV-1 5'-3' polymerase activity in the presence of ICP8, which is characteristic of SSBs and polymerase interactions in other systems, would suggest a functional interaction (reviewed in Chase and Williams, 1986; Hernandez and Lehman, 1990; Ruyechan et al., 1984). However, the E. coli SSB protein can also stimulate HSV-1 Pol, which would indicate that this effect is not species specific and potentially reflects DNA stabilization by SSB proteins rather than a specific interaction (O'Donnell et al., 1987).

UL2. HSV-1 Pol has been shown to interact directly with UL2 in the presence and absence of UL42 (Bogani et al., 2010), which led us to speculate that the binding interface lies at the extreme N-terminus of HSV-1 Pol. Additionally, the HCMV-encoded uracil DNA glycosylase binds an N-terminal 100kDa fragment of the viral DNA polymerase catalytic subunit UL54 (Strang and Coen, 2010). Our experiments with the full length and mutant UL2 proteins clearly demonstrated that the extreme N-terminal 51

residues of HSV-1 Pol were not required for binding. Therefore, the UL2 binding site could be mapped to any region within residues 51-1219 that comprise the majority of the HSV-1 Pol enzyme. Further investigation is needed in order to determine the timing, location, and significance of the HSV-1 Pol-UL2 interaction.

HSV-1 Pol interactions during cellular infection. A previous study demonstrated via Western blot analysis that viral replication proteins UL42, the H/P complex, and ICP8 coprecipitate with HSV-1 Pol from infected cell lysate (Strick et al., 1997). Unfortunately the general lack of available specific antisera for HSV-1 replication proteins limited our ability to determine whether or not our results are in accordance with this previous report. Although MS analysis was a useful technique in this global approach, it requires an abundant amount of protein for visual detection and isolation that greatly reduced the sensitivity and identification of precipitated proteins. Outside of UL42, we were unable to detect the presence of the H/P complex and ICP8 via MS analysis. However, we were able to identify potential associations between HSV-1 Pol and viral proteins UL39 and ICP27 that were not dependent upon the presence of nucleic acids. The large subunit of the viral ribonucleotide reductase (UL39) was the only viral DNA synthesis protein that was identified in our MS analysis other than UL42. There is no precedent for a ribonucleotide reductase−DNA polymerase interaction, and we cannot determine based on the presented data whether this observed association occurs naturally during viral infection or if it is induced upon cell lysis. The viral ribonucleotide reductase is important for viral DNA synthesis in resting cell cultures (Goldstein and Weller, 1988b; Jacobson et al., 1989). Thus, ribonucleotide reductase would be an interesting candidate for further analysis given that the *pol*A_6 viral DNA synthesis defect is

enhanced in serum starved cells (Chapter 2 – Terrell and Coen, 2012; Chapter 3). Quantitative analyses are required in order to determine whether UL39 is a bonafide HSV-1 Pol binding partner and if its binding affinity is altered as a result of the substitution mutation.

An interaction between HSV-1 Pol and ICP27 has not been previously reported in the literature. ICP27 coprecipitates with ICP8 from infected cell lysates and the two proteins were shown to interact directly *in vitro* (Olesky et al., 2005; Taylor and Knipe, 2004). Unlike HSV-1 Pol, ICP8 has a demonstrated role in stimulating late viral gene expression that is independent of viral DNA synthesis, which is further supported by reported interactions with ICP27-RNA polymerase II complexes (Chen and Knipe, 1996; Gao and Knipe, 1991; Zhou and Knipe, 2002). Further investigation is necessary in order to determine whether ICP27 can interact directly with HSV-1 Pol and if this would represent coupling of late viral gene expression and viral DNA synthesis.

Role of HSV-1 Pol interactions during viral replication. HSV-1 Pol is a multi-functional enzyme that can associate with a number of different viral and cellular proteins (reviewed in Lehman and Boehmer, 1997). Just recently, an interaction was identified between HSV-1 Pol and host cell factor 1 (HCF-1), which has been proposed to facilitate the recruitment of histone chaperone Asf1b and thereby promote efficient viral DNA synthesis during infection (Peng et al., 2009). The deletion and substitution mutations in HSV-1 Pol do not appear to disrupt this association (T. Kristie, personal communication). The variety of potential binding partners for HSV-1 Pol underscores the importance of appropriately timing specific interactions to allow for the efficient and faithful replication of the viral genome. The main job of HSV-1 Pol is to synthesize viral DNA, which is

supported by the essential interaction between the catalytic and processivity subunits of the viral DNA polymerase holoenzyme. HSV-1 Pol also encodes 3'-5' exonuclease activity, RNase H and apurinic/apyrimidinic and 5'- deoxyribose phosphate lyase activities that must be executed with specific substrates at the appropriate time (Bogani and Boehmer, 2008; Crute and Lehman, 1989; Knopf and Weisshart, 1988; O'Donnell et al., 1987). One would anticipate that the accessory HSV-1 Pol interactions would be of a lower affinity to allow for dissociation and reassociation of specific binding partners at any given stage during viral DNA synthesis. Therefore, our ability to investigate the exact mechanisms by which these processes occur is limited given that the replisome interactions are dynamic and dependent upon specific protein-protein, protein-DNA interactions and various conditions that are difficult to recapitulate *in vitro*. Single molecule studies have allowed for a more detailed analysis of replisome dynamics during DNA replication (van Oijen and Loparo, 2010). Although this has proven to be a less than straightforward process, identification of a protein interaction that is mediated by the conserved motif of HSV-1 Pol would provide further insight into the viral DNA synthesis processes and may very well represent a conserved mechanism for herpesvirus replication.

Acknowledgements

We would like to thank Ross Tomiano at the Taplin Facility at Harvard Medical School for help with MS analysis, Ping Bai in the laboratory of Sandy Weller at the University of Connecticut Health Center for conducting the SPR analyses with our WT and mutant polymerase proteins, Yaralid Sotomayor-Castro in the laboratory of Paul Boehmer at the University of Arizona College of Medicine-Pheonix for kindly providing purified UL2 proteins, and Jody Vogel in the laboratory of Thomas Kristie at the National Institutes of Allergy and Infectious Disease for examining the association of our mutant polymerases with HCF-1.

Chapter 5

Discussion

Summary of Results

HSV-1 Pol has been extensively studied for therapeutic purposes and has served as an effective drug target due to its importance for viral replication. A wealth of literature highlights the ability of HSV-1 Pol to perform various activities during the process of viral DNA synthesis and justifies further investigation into the significance of HSV-1 Pol function during infection. The conserved structural core of the enzyme that mediates 5'-3' polymerase and 3'-5' exonuclease activities is well defined, while the N-terminal half of the protein remains largely uncharacterized. This dissertation addresses the importance of the pre-NH_2-terminal domain of HSV-1 Pol for viral replication in cell culture and animal models of infection.

Our primary goal for the studies outlined in Chapter 2 was to identify a role for the pre-NH_2-terminal domain that was distinct from 5'-3' polymerase activity. Thus, we engineered and purified Pol mutant proteins for analysis of enzymatic activity and found that the extreme N-terminal 51 residues were not required for WT-like 5'-3' polymerase activity *in vitro*. Viral genetic analyses of *pol* mutant viruses revealed that the extreme N-terminal 42 residues of HSV-1 Pol were dispensable for viral replication despite conservation in HSV-1 and -2. Our studies identified a role for the conserved motif at residues 44-49 during viral DNA synthesis in the context of the infected cell.

In Chapter 3, we evaluated the importance of these conserved pre-NH_2-terminal elements for viral replication in a mouse ocular model of infection. The extreme N-terminal 42 residues of HSV-1 Pol were not required for viral replication, latency establishment, and reactivation in mice. In contrast, we found that the conserved motif FYNPYL was of even greater importance for acute ganglionic replication in mice.

Latency establishment and reactivation efficiencies were also perturbed in the absence of the conserved motif. Additionally, the viral replication defect exhibited by mutant virus *pol*A_6 in dividing cells was enhanced during infection of resting cell cultures.

We sought to determine whether the conserved motif mediated a protein-protein interaction that would enhance viral DNA synthesis during infection, which was addressed in Chapter 4. We employed a candidate-based approach with previously identified HSV-1 Pol binding partners, and an unbiased approach by analyzing protein interactions in infected cell lysates. As of yet, we have been unable to identify a protein whose interaction is disrupted as a result of the mutation.

These studies demonstrate that the pre-NH_2-terminal domain of HSV-1 Pol includes a function that is not important for 5'-3' polymerase activity, yet is required for efficient viral DNA synthesis in cell culture and is especially important for acute ganglionic replication in mice. Further investigation is necessary in order to elucidate the exact mechanism by which the conserved motif FYNPYL promotes efficient viral DNA synthesis, which may represent a conserved replication mechanism among herpesviruses. These studies have generated a plethora of questions that need to be addressed in order to better understand the role of the pre-NH_2-terminal domain for viral replication.

Hypotheses and Future Directions

What is the role of the extreme N-terminal 42 residues of HSV-1 Pol? We have demonstrated that the extreme N-terminal 42 residues of HSV-1 Pol are dispensable for viral replication both in cell culture and a mouse model of infection (Chapter 2 – Terrell and Coen, 2012, Chapter 3). However, we would not exclude the possibility of a potential role for these residues during viral infection. Conservation of the extreme N-terminal 42 residues is restricted to a subset of simplexviruses within the alphaherpesvirus subfamily that infect humans and monkeys (McGeoch et al., 2000). Viral DNA polymerases from Cercopithecine herpesvirus 1 (CeHV-1; B virus), CeHV-2 (simian agent 8; SA8), CeHV-16 (herpesvirus papio 2; HVP2), and Saimiriine herpesvirus 1 (SaHV-1) exhibit 31-50% protein sequence identity with the extreme N-terminal 42 residues of HSV-1 Pol (DiTommaso et al., 2011; Notredame et al., 2000). Additionally, pathogenesis of HSV infection in humans is very similar to that of B virus in its natural host, which is closely related to both HVP2 and SA8 (Elmore and Eberle, 2008). Therefore, we would suggest that this conserved element may confer an advantage during viral replication that is specific to the biology of simplexviruses.

Our data indicates that the extreme N-terminal 42 residues may play a role in the regulation of HSV-1 Pol expression during infection, as we observed that mutant virus *pol*ΔN43 exhibits a two-fold increase in viral DNA polymerase expression as compared to WT virus (Appendix). HSV-1 Pol proteins are less abundant as compared to other viral early gene products, which is presumably due to post-transcriptional mechanisms that prevent efficient translation (Yager et al., 1990). One possibility is that the 5' end of the pol coding sequence may be required for the formation of an RNA structure that impairs

protein translation. Deletion of an identified RNA inhibitory element upstream of the *pol* start site results in a two-fold increase in HSV-1 Pol expression and a six-fold defect in multicycle cycle replication kinetics as compared to WT virus (Bryant and Coen, 2008). Alternatively, the extreme N-terminal 42 residues may be required for *pol* mRNA binding and autoregulation of HSV-1 Pol expression during infection. While the possibility of autogenous regulation has yet to be demonstrated for HSV-1, both RNA structure and sequence are important for autoregulation of the Polα homologue T4 bacteriophage DNA polymerase (Pavlov and Karam, 2000). Thus, it is possible that sequence-dependent mechanisms may be employed in order to prevent the accumulation of HSV-1 Pol protein at late times post infection, which may be important for efficient viral replication. Further investigation is necessary in order to determine whether the 5' end of the *pol* coding sequence forms an RNA structure that directly impairs protein translation or functions in an autoregulatory fashion.

Does the conserved motif mediate a protein-protein interaction? The conserved motif is a cluster of hydrophobic and aromatic residues that resides within a flexible segment at the extreme N-terminus of HSV-1 Pol. Given that protein interactions can be accommodated at protein termini, we hypothesize that the conserved motif may represent a potential binding site. The strong conservation of this motif within the human herpesvirus Pol family would suggest that the binding partner is a cellular protein. One possibility is that the presumed protein-protein interaction has been abolished as the result of the substitution mutation, and that this interaction is more important during viral replication in resting cells and mice. Cellular polymerase Polδ was reported to exhibit

stimulated activity when bound to the werner helicase interacting protein 1 (WHNIP1) in the presence and absence of PCNA (Tsurimoto et al., 2005). Additionally, the Polδ−PCNA complex synthesized larger DNA products in the presence of WHNIP1, which led authors to suggest that WHNIP1 promoted reinitiation of DNA synthesis. Therefore, there is a possibility that HSV-1 Pol could associate with a protein that modulates its function during viral DNA synthesis in infected cells. Alternatively, the conserved motif may be part of a larger binding interface and loss of the motif would diminish the affinity of the interaction rather than abolishing it entirely. In this scenario, the formation of a stable complex would be imperative in resting cells and neurons due to limited expression of the binding partner. Thus, it would be imperative that we undertake a more quantitative approach to our future analyses regarding protein-binding assays. We are currently pursuing a collaboration with a lab that utilizes a high performance mass spectrometer that would provide more sensitive and quantitative analyses. In doing so, we hope to better execute our global approach in the identification of cellular and viral proteins that associate with HSV-1 Pol in infected cell lysates.

Can a cellular protein compensate for the polA_6 defect? The inability of the PolA_6 protein to efficiently synthesize viral DNA in dividing cells became more detrimental during infection of resting cells and especially in the ganglia. These characteristics are similar to those viruses in which the activity of nonessential viral DNA synthesis proteins was abolished. Specifically, viral TK and RR functions can be executed by cognate cellular enzymes in dividing cells, but not in resting cells wherein expression of these cellular proteins is repressed (Field and Wildy, 1978; Goldstein and Weller, 1988a;

Jameison et al., 1974). Thus, we hypothesize that a cellular protein could partially compensate for the $PolA_6$ impairment during infection of dividing cells. Following this line of reasoning, we could suggest that the cellular Polα or Polδ enzyme is the compensatory protein, as they are both structural homologs of HSV-1 Pol (Knopf and Weisshart, 1988; Liu et al., 2006; Wang et al., 1989). Evidence for a potential role for Polα in HSV-1 infection includes an interaction with the HSV-1 origin binding protein UL9 and a demonstrated ability of the enzyme to utilize RNA primers synthesized by the viral helicase-primase complex (Cavanaugh and Kuchta 2008; Lee et al., 1995). While Polα is necessary for the initiation of DNA replication, Polδ is responsible for processive DNA synthesis (Hubscher et al., 2002). Like HSV-1 Pol, Polδ exhibits 5'-3' polymerase and 3'-5' exonuclease activities and directly interacts with a processivity factor (e.g. PCNA). Additionally, Pol α and δ expression levels and activities are enhanced in actively dividing cells as compared to resting cells, which appears to correlate with the onset of DNA replication (Hao et al., 1992, Wahl et al., 1988; Wong et al., 1988; Yang et al., 1991). Several distinct cellular DNA polymerases have been identified as having a role during DNA replication (Hubscher et al., 2002). Whether or not the virus has the ability to actively engage cellular polymerases in viral DNA synthesis processes remains unclear. Studies utilizing cellular polymerase inhibitors are difficult to interpret given that these compounds also negatively impact viral polymerase activity. Perturbation of cellular polymerase expression and activity may result in undesired global alterations in cellular protein expression that could potentially complicate our investigations. Until we can recapitulate viral DNA synthesis *in vitro* or use single molecule studies to analyze

this process within the context of the infected cell, it will be very difficult to properly evaluate the importance of cellular polymerases for viral replication.

Does the conserved motif modulate HSV-1 Pol enzymatic activities? Our work has demonstrated that substitution of the conserved motif does not negatively impact intrinsic 5'-3' polymerase activity of HSV-1 Pol *in vitro*; however, the use of a non-physiological substrate in these assays may not accurately reflect the process of viral DNA synthesis within the infected cell. Therefore, it may be worthwhile to analyze PolA_6 activity on a circular template in the presence and absence of viral replication factors. Additionally, we have yet to test whether the PolA_6 enzyme is competent for other functions such as 3'-5' exonuclease, RNase H, and lyase activities. A viral mutant lacking detectable 3'-5' exonuclease activity exhibits a 50-fold decrease in viral yield with only a 3-fold decrease in viral DNA production, which largely reflects an alteration in replication fidelity rather than viral DNA synthesis (Tian et al., 2009). Based on the tight correlation between the observed defects in viral yield and viral DNA synthesis (Chapter 2 – Terrell and Coen, 2012), we do not anticipate that the substitution mutation would compromise 3'-5' exonuclease activity of HSV-1 Pol. The active sites for RNase H and lyase activities have yet to be mapped to HSV-1 Pol and the effect of disrupting these functions is unknown. Rigorous characterization of enzymatic activities on well-defined templates that reflect DNA replication substrates may provide a better understanding towards the mechanistic aspects of the *pol*A_6 defect.

Is the polA_6 replication defect at the periphery responsible for decreased latency establishment? Some viruses are able to establish latency as well as WT virus despite defective acute corneal and ganglionic replication in mice (Perng et al., 1996; Thompson and Sawtell, 2009). Both *pol*A_6 and TK^- viruses exhibit viral DNA synthesis defects in cell culture, acute replication defects in the mouse eye and ganglia in addition to decreases in latency establishment (Chapter 2 – Terrell and Coen, 2012; Thompson and Sawtell, 2000). Our results support the hypothesis that the level of viral DNA synthesis rather than infectious virus production at the peripheral site of infection is important for latency establishment. This possibility is supported by a report that demonstrated that latency can be established in a subset of neurons within the TG at the onset of acute ganglionic replication (Margolis et al., 1992). We would like to know whether the *pol*A_6 corneal replication defect directly impacts the number of viral genomes that gain access to the TG during acute replication. Comparison of *pol*A_6 with a mutant that exhibits corneal replication defects with WT-like latency establishment efficiencies (eg. VP16 mutant *in1814*; Thompson and Sawtell, 2009) may provide some insight as to whether viral replication or viral DNA synthesis is important latency establishment.

Is the conserved motif required for reactivation in vivo? We found it rather interesting that the *pol*A_6 virus was able to reactivate from explanted TG fairly efficiently despite the severe impairment in acute ganglionic replication. Interestingly, a VP16 mutant that displayed WT reactivation efficiencies from *in vitro* explant reactivation assays was unable to produce detectable infectious virus and lytic viral proteins in TG following hyperthermic stress *in vivo* (Thompson and Sawtell, 2009). The authors of this

study suggested that the excision of TG from mice might induce cellular changes that are not representative of what occurs *in vivo*. Additionally, reactivation of a UL2^{-} virus, as measured by recovery of infectious virus, was restricted *in vivo* in comparison to *in vitro* cocultivation assays (Pyles and Thompson, 1994). Thus, we would anticipate that the acute replication defect exhibited by *pol*A_6 might be more evident in a similar *in vivo* reactivation assay and that reactivation efficiencies could be further diminished. Ultimately, an *in vivo* reactivation assay would more accurately reflect the importance of the conserved motif in the context of reactivation.

Is the conserved motif important for replication of other herpesviruses? In addition to the human herpesvirus Pol family, the motif FYNPYL is well conserved among mammalian and avian herpesviruses (DiTommaso et al., 2011; Notredame et al., 2000). Conservation of the motif among the alpha, beta and gamma herpesvirus subfamilies would suggest that it mediates a function that is necessary for efficient viral replication in a variety of cell types. Our studies raise the possibility that the conserved motif may be especially important for replication in neurons and non-dividing cells. We are currently investigating whether the observed acute ganglionic replication defect represents a cell specific block for *pol*A_6 replication in neurons. Additionally, we plan to examine the molecular basis by which *pol*A_6 replication is further restricted in serum starved cultures and if this effect is specific for infection of non-dividing cells. The appropriate viral genetic analyses with lymphotropic herpesviruses such as EBV and KSHV may provide insight regarding the requirement of the conserved motif for viral replication in additional cell types. If the loss of this motif were to confer similar

replication defects in cell culture and animal models of infection for other herpesviruses, it could represent a conserved mechanism for herpesvirus replication in humans and potentially other mammalian and avian hosts.

Interestingly, the conserved motif is not present in herpesviruses that are specific for oysters (malacoherpesviridae), fish and amphibians (alloherpesviridae; DiTommaso et al., 2011; Notredame et al., 2000). Alloherpesvirus DNA polymerases contain a catalytic domain for 5'-3' polymerase activity that is conserved among Family B polymerases (Marchler-Bauer et al., 2011). Outside of the palm and fingers domains, alloherpesvirus DNA polymerase sequences exhibit little similarity with HSV-1 Pol (DiTommaso et al., 2011; Notredame et al., 2000), which would indicate that specific enzymatic functions are not shared among all herpesvirus DNA polymerases. Thus far, only the helicase, primase, and DNA polymerase have been identified as conserved viral DNA replication proteins within the alloherpesvirus family (Aoki et al., 2007; Hanson et al., 2011). More than likely, the evolutionary divergence of these animal species with respect to mammals would be reflected in distinct requirements that are necessary for efficient viral replication in its natural host. Additional information regarding replisome-mediated viral DNA synthesis with respect to alloherpesviruses would allow for comparative analyses that may provide further insight into the importance of the conserved motif for viral DNA replication.

Appendix

Protein Expression of Pre-NH_2-Terminal *pol* Mutant Viruses

Introduction

Herpes simplex virus 1 (HSV-1) infection is characterized by a regulated cascade of protein expression that correlates with defined stages of viral replication. Immediate early gene products prime the cell for infection, early viral proteins execute viral DNA synthesis, and late gene products are necessary for the assembly and egress of infectious viral particles. Thus, the virus must enable mechanisms that allow for temporal expression and the eventual downregulation of viral proteins at the appropriate times during infection. The expression and activity of the HSV-1 DNA polymerase catalytic subunit (HSV-1 Pol) is required for viral DNA synthesis and production of infectious virus (Aron et al., 1975; McGeoch et al., 1988; Wu et al., 1988). HSV-1 Pol expression is temporally regulated with peak protein synthesis at 4 hours post infection (hpi), with notable downregulation by 5 hpi despite the abundance of *pol* mRNA (Yager et al., 1990). Previously, we engineered two HSV-1 Pol mutants that retained WT-like 5'-3' polymerase activity *in vitro* yet exhibited decreased levels of viral DNA synthesis during cellular infection: deletion mutant *pol*ΔN52, in which the first 51 residues were deleted, and substitution mutant *pol*A_6 in which residues 44-49 were substituted with alanines (Chapter 2 – Terrell and Coen, 2012). The extreme N-terminal 42 residues were dispensable for viral replication in cell culture, while removal of the extreme N-terminal 140 residues resulted in a catalytically inactive HSV-1 Pol enzyme and replication incompetent mutant virus. We evaluated viral protein expression and observed differential expression of HSV-1 Pol during *pol* mutant virus infection as compared to WT virus. We sought to quantify these changes in HSV-1 Pol expression for specific *pol* mutant viruses in infected cell lysates.

Materials and Methods

Cells and viruses. Vero cells (American Catalog of Cell Culture) were maintained as previously described (Chapter 2 − Terrell and Coen, 2012). Wild type (WT), *pol*ΔN43, *pol*ΔN52, *pol*ΔN52R, *pol*A_6, and *pol*A_6R viruses were constructed as previously described (Chapter 2 − Terrell and Coen, 2012).

Western blot analysis. Vero cells (6×10^5) were either mock infected or infected at an MOI of 20 with the indicated viruses. At 8 hours post infection (hpi), monolayers were washed with DPBS and harvested with 330μl Laemmli buffer. Protein lysates were boiled at 90°C for 7 min and resolved by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were blocked in 5% milk/TBST and probed with PP5 α-Pol (1:2500), α-thymidine kinase (TK; Bill Summers (Yale University); 1:1000), αICP8 antibody (3-83; Knipe et al., 1987; 1:10,000), and α-β-actin (Abcam; 1:10000). Blots were exposed to secondary antibodies goat α-rabbit-HRP and goat α-mouse-HRP (Southern Biotech) and detected with chemiluminescence solution (Pierce).

Quantitation of protein expression. A dilution series of WT infected cell lysate was analyzed alongside mutant virus lysates for HSV-1 Pol and ICP8 expression via Western blot as described above. Relative quantitation Pol expression was achieved by analyzing the band intensity of scanned films with Quantity One software (Bio-Rad). Pol expression for each mutation was interpolated from a linear regression curve of standard band intensities generated from a dilution series of infected WT cell lysate and normalized to the level of ICP8 expression for each virus.

Results and Discussion

Previously, we generated *pol* mutant viruses using bacterial artificial chromosome technology in order to evaluate the importance of the pre-NH_2-terminal domain of HSV-1 Pol for viral replication. The extreme N-terminal 42, 51, and 140 residues of HSV-1 Pol are deleted in truncation mutants *pol*ΔN43, *pol*ΔN52, and *pol*ΔN141, respectively. A conserved motif at HSV-1 Pol residues 44-49 was replaced with six alanines in substitution mutant *pol*A_6. We sought to evaluate HSV-1 Pol protein expression during *pol* mutant virus infection by Western Blot analysis. Vero cells were infected at an MOI of 20 and cell lysates were harvested at 8 hpi. We found that deletion mutants *pol*ΔN43, *pol*ΔN52, and *pol*ΔN141 produced HSV-1 Pol polypeptides that exhibited a mobility shift that corresponded to the respective deletion (Figure A1). Interestingly, HSV-1 Pol expression was slightly elevated in mutants *pol*ΔN43, *pol*ΔN52, and *pol*A_6 as compared to WT virus (Figure A1). Moreover, HSV-1 Pol expression appeared to be restored to WT-like levels in rescued derivative viruses *pol*ΔN52R and *pol*A_6R (Figure A1). HSV-1 Pol expression during *pol*ΔN141 infection did not appear to be altered (Figure A1). The expression levels of ICP8 and TK appeared to be comparable among all viruses, which suggested that this observation was specific for mutant HSV-1 Pol expression (Figure A1). In order to quantify the changes in HSV-1 Pol expression, we measured HSV-1 Pol expression for each mutant with respect to ICP8 expression as described in the Materials and Methods (Figure A2). Mutants *pol*ΔN43, *pol*ΔN52, and *pol*A_6 each exhibited a ~2-fold increase in HSV-1 Pol expression compared to WT.

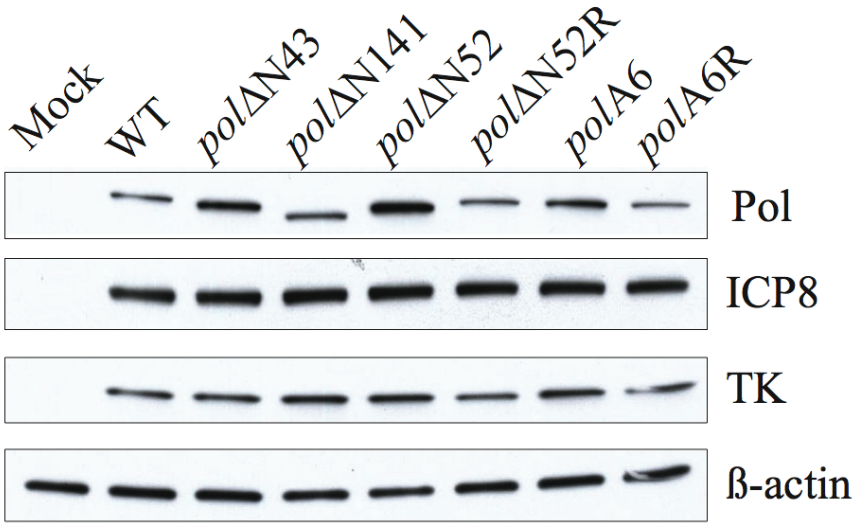

Figure A1. Viral protein expression during *pol* mutant virus infection. Vero cells were infected at an MOI of 20 and lysates were harvested at 8 hpi. Lysates were resolved via SDS-PAGE and membranes were probed specific antibodies targeting each of the indicated proteins.

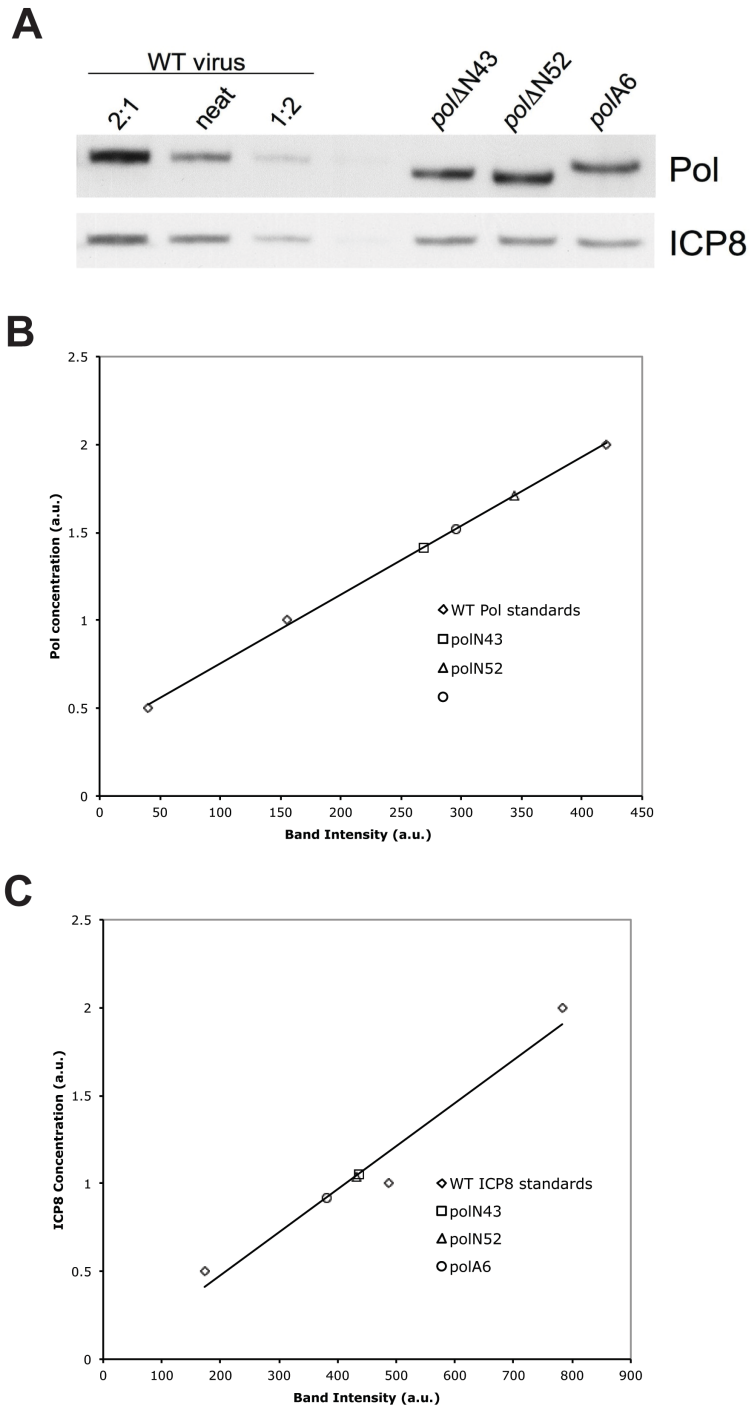

Figure A2. Quantitation of HSV-1 Pol expression during *pol* mutant virus infection. Dilutions of WT-infected cell lysates along with mutant virus samples were analyzed by SDS-PAGE and membranes were probed with HSV-1 Pol and ICP8 antibodies (A). Pol expression for each mutant was interpolated from a linear regression curve of standard band intensities generated from a dilution series of WT-infected cell lysate and normalized to the level of ICP8 expression for each virus (B to C).

Although *pol* mRNA levels are comparable to that of thymidine kinase transcripts, HSV-1 Pol protein is less abundant in infected cells due to inefficient translation (Yager et al., 1990). Authors speculate that a post-transcriptional mechanism may be responsible for preventing the accumulation of HSV-1 Pol proteins at late times post infection, as this may be detrimental to viral replication. Deletion of an identified RNA inhibitory element upstream of the *pol* start site resulted in a two-fold increase in HSV-1 Pol expression and a two-fold defect in single cycle replication kinetics as compared to WT virus (Bryant and Coen, 2008). However, we would not conclude that the modest overexpression of HSV-1 Pol in mutants *pol*ΔN52 and *pol*A_6 was responsible for the observed replication defect, given that deletion mutant *pol*ΔN43 replicated as efficiently as WT virus (Chapter 2 – Terrell and Coen, 2012). Due to the restoration of WT levels of HSV-1 Pol expression in rescued derivative viruses, we would suggest that the 147 nucleotides at the 5' end of the *pol* coding sequence aid in the regulation of HSV-1 Pol expression during infection. Such regulation may be dependent on protein activity and viral DNA synthesis, as we did not observe altered HSV-1 Pol expression for *pol*ΔN141. One possibility is that the 5' end of *pol* mRNA could function as an additional inhibitory RNA element that would prevent translation initiation due to the presence of secondary structure. Alternatively, these sequences may be important for autogenous regulation of DNA polymerase expression, which has been observed in bacteriophage T4 (Rodriguez et al., 2000; Wang et al., 1996). Although HSV-1 Pol has not been shown to autoregulate its own expression, there is evidence to support this possibility. For example, the NH_2-terminal domain of HSV-1 Pol contains an RNA binding motif that could function in this regard by binding *pol* mRNA. Additionally, autogenous regulation

may also contribute to the dramatic decline in HSV-1 Pol synthesis at early times post infection despite the relatively high abundance of *pol* transcripts (Yager et al., 1990). The possibility exists that several independent mechanisms may be responsible for the observed downregulation in HSV-1 Pol protein synthesis. Further investigation is necessary in order to determine how the 5' end of the *pol* coding sequence contributes to the regulation of HSV-1 Pol expression during infection.

References

Abraham, R.T. (2004). PI 3-kinase related kinases: 'big' players in stress-induced signaling pathways. DNA repair *3*, 883-887.

Alani, E., Thresher, R., Griffith, J.D., and Kolodner, R.D. (1992). Characterization of DNA-binding and strand-exchange stimulation properties of y-RPA, a yeast single-strand-DNA-binding protein. Journal of molecular biology *227*, 54-71.

Aoki, T., Hirono, I., Kurokawa, K., Fukuda, H., Nahary, R., Eldar, A., Davison, A.J., Waltzek, T.B., Bercovier, H., and Hedrick, R.P. (2007). Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. Journal of virology *81*, 5058-5065.

Aron, G.M., Purifoy, D.J., and Schaffer, P.A. (1975). DNA synthesis and DNA polymerase activity of herpes simplex virus type 1 temperature-sensitive mutants. Journal of virology *16*, 498-507.

Bacchetti, S., Evelegh, M.J., and Muirhead, B. (1986). Identification and separation of the two subunits of the herpes simplex virus ribonucleotide reductase. Journal of virology *57*, 1177-1181.

Balasubramanian, N., Bai, P., Buchek, G., Korza, G., and Weller, S.K. (2010). Physical interaction between the herpes simplex virus type 1 exonuclease, UL12, and the DNA double-strand break-sensing MRN complex. Journal of virology *84*, 12504-12514.

Balliet, J.W., Kushnir, A.S., and Schaffer, P.A. (2007). Construction and characterization of a herpes simplex virus type I recombinant expressing green fluorescent protein: acute phase replication and reactivation in mice. Virology *361*, 372-383.

Barnard, E.C., Brown, G., and Stow, N.D. (1997). Deletion mutants of the herpes simplex virus type 1 UL8 protein: effect on DNA synthesis and ability to interact with and influence the intracellular localization of the UL5 and UL52 proteins. Virology *237*, 97-106.

Batterson, W., Furlong, D., and Roizman, B. (1983). Molecular genetics of herpes simplex virus. VIII. further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. Journal of virology *45*, 397-407.

Birney, E., Kumar, S., and Krainer, A.R. (1993). Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. Nucleic acids research *21*, 5803-5816.

Blumel, J., Graper, S., and Matz, B. (2000). Structure of simian virus 40 DNA replicated by herpes simplex virus type 1. Virology *276*, 445-454.

Boehmer, P.E., Dodson, M.S., and Lehman, I.R. (1993). The herpes simplex virus type-1 origin binding protein. DNA helicase activity. The Journal of biological chemistry *268*, 1220-1225.

Boehmer, P.E., and Lehman, I.R. (1993). Physical interaction between the herpes simplex virus 1 origin-binding protein and single-stranded DNA-binding protein ICP8. Proceedings of the National Academy of Sciences of the United States of America *90*, 8444-8448.

Boehmer, P.E., and Lehman, I.R. (1997). Herpes simplex virus DNA replication. Annual review of biochemistry *66*, 347-384.

Bogani, F., and Boehmer, P.E. (2008). The replicative DNA polymerase of herpes simplex virus 1 exhibits apurinic/apyrimidinic and 5'-deoxyribose phosphate lyase activities. Proceedings of the National Academy of Sciences of the United States of America *105*, 11709-11714.

Bogani, F., Chua, C.N., and Boehmer, P.E. (2009). Reconstitution of uracil DNA glycosylase-initiated base excision repair in herpes simplex virus-1. The Journal of biological chemistry *284*, 16784-16790.

Bogani, F., Corredeira, I., Fernandez, V., Sattler, U., Rutvisuttinunt, W., Defais, M., and Boehmer, P.E. (2010). Association between the herpes simplex virus-1 DNA polymerase and uracil DNA glycosylase. The Journal of biological chemistry *285*, 27664-27672.

Bolovan, C.A., Sawtell, N.M., and Thompson, R.L. (1994). ICP34.5 mutants of herpes simplex virus type 1 strain 17syn+ are attenuated for neurovirulence in mice and for replication in confluent primary mouse embryo cell cultures. Journal of virology *68*, 48-55.

Brown, D.G., Visse, R., Sandhu, G., Davies, A., Rizkallah, P.J., Melitz, C., Summers, W.C., and Sanderson, M.R. (1995). Crystal structures of the thymidine kinase from herpes simplex virus type-1 in complex with deoxythymidine and ganciclovir. Nature structural biology *2*, 876-881.

Brown, S.M., Harland, J., MacLean, A.R., Podlech, J., and Clements, J.B. (1994). Cell type and cell state determine differential in vitro growth of non-neurovirulent ICP34.5-negative herpes simplex virus types 1 and 2. The Journal of general virology *75 (Pt 9)*, 2367-2377.

Bryant, K.F., and Coen, D.M. (2008). Inhibition of translation by a short element in the 5' leader of the herpes simplex virus 1 DNA polymerase transcript. Journal of virology *82*, 77-85.

Burd, C.G., and Dreyfuss, G. (1994). Conserved structures and diversity of functions of RNA-binding proteins. Science *265*, 615-621.

Bush, M., Yager, D.R., Gao, M., Weisshart, K., Marcy, A.I., Coen, D.M., and Knipe, D.M. (1991). Correct intranuclear localization of herpes simplex virus DNA polymerase requires the viral ICP8 DNA-binding protein. J Virol *65*, 1082-1089.

Calder, J.M., and Stow, N.D. (1990). Herpes simplex virus helicase-primase: the UL8 protein is not required for DNA-dependent ATPase and DNA helicase activities. Nucleic acids research *18*, 3573-3578.

Campadelli-Fiume, G., Cocchi, F., Menotti, L., and Lopez, M. (2000). The novel receptors that mediate the entry of herpes simplex viruses and animal alphaherpesviruses into cells. Reviews in medical virology *10*, 305-319.

Caradonna, S.J., and Cheng, Y.C. (1981). Induction of uracil-DNA glycosylase and dUTP nucleotidohydrolase activity in herpes simplex virus-infected human cells. The Journal of biological chemistry *256*, 9834-9837.

Carbone, R., Pearson, M., Minucci, S., and Pelicci, P.G. (2002). PML NBs associate with the hMre11 complex and p53 at sites of irradiation induced DNA damage. Oncogene *21*, 1633-1640.

Carrington-Lawrence, S.D., and Weller, S.K. (2003). Recruitment of polymerase to herpes simplex virus type 1 replication foci in cells expressing mutant primase (UL52) proteins. Journal of virology *77*, 4237-4247.

Cassady, K.A., Gross, M., and Roizman, B. (1998). The second-site mutation in the herpes simplex virus recombinants lacking the gamma134.5 genes precludes shutoff of protein synthesis by blocking the phosphorylation of eIF-2alpha. Journal of virology *72*, 7005-7011.

Cavanaugh, N.A., and Kuchta, R.D. (2009). Initiation of new DNA strands by the herpes simplex virus-1 primase-helicase complex and either herpes DNA polymerase or human DNA polymerase alpha. The Journal of biological chemistry *284*, 1523-1532.

Ceska, T.A., and Sayers, J.R. (1998). Structure-specific DNA cleavage by 5' nucleases. Trends in biochemical sciences *23*, 331-336.

Chase, J.W., and Williams, K.R. (1986). Single-stranded DNA binding proteins required for DNA replication. Annual review of biochemistry *55*, 103-136.

Chen, M.S., Summers, W.P., Walker, J., Summers, W.C., and Prusoff, W.H. (1979). Characterization of pyrimidine deoxyribonucleoside kinase (thymidine kinase) and thymidylate kinase as a multifunctional enzyme in cells transformed by herpes simplex virus type 1 and in cells infected with mutant strains of herpes simplex virus. Journal of virology *30*, 942-945.

Chen, S.H., Pearson, A., Coen, D.M., and Chen, S.H. (2004). Failure of thymidine kinase-negative herpes simplex virus to reactivate from latency following efficient establishment. Journal of virology *78*, 520-523.

Chen, Y.M., and Knipe, D.M. (1996). A dominant mutant form of the herpes simplex virus ICP8 protein decreases viral late gene transcription. Virology *221*, 281-290.

Chibo, D., Mijch, A., Doherty, R., and Birch, C. (2002). Novel mutations in the thymidine kinase and DNA polymerase genes of acyclovir and foscarnet resistant herpes simplex viruses infecting an immunocompromised patient. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology *25*, 165-170.

Chiou, H.C., Weller, S.K., and Coen, D.M. (1985). Mutations in the herpes simplex virus major DNA-binding protein gene leading to altered sensitivity to DNA polymerase inhibitors. Virology *145*, 213-226.

Chou, S., Lurain, N.S., Weinberg, A., Cai, G.Y., Sharma, P.L., and Crumpacker, C.S. (1999). Interstrain variation in the human cytomegalovirus DNA polymerase sequence and its effect on genotypic diagnosis of antiviral drug resistance. Adult AIDS Clinical Trials Group CMV Laboratories. Antimicrobial agents and chemotherapy *43*, 1500-1502.

Ciccia, A., and Elledge, S.J. (2010). The DNA damage response: making it safe to play with knives. Molecular cell *40*, 179-204.

Cimprich, K.A., and Cortez, D. (2008). ATR: an essential regulator of genome integrity. Nature reviews Molecular cell biology *9*, 616-627.

Coen, D.M., Kosz-Vnenchak, M., Jacobson, J.G., Leib, D.A., Bogard, C.L., Schaffer, P.A., Tyler, K.L., and Knipe, D.M. (1989). Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. Proceedings of the National Academy of Sciences of the United States of America *86*, 4736-4740.

Coen, D.M., and Schaffer, P.A. (2003). Antiherpesvirus drugs: a promising spectrum of new drugs and drug targets. Nature reviews Drug discovery *2*, 278-288.

Courcelle, C.T., Courcelle, J., Prichard, M.N., and Mocarski, E.S. (2001). Requirement for uracil-DNA glycosylase during the transition to late-phase cytomegalovirus DNA replication. Journal of virology *75*, 7592-7601.

Crute, J.J., and Lehman, I.R. (1989). Herpes simplex-1 DNA polymerase. Identification of an intrinsic 5'----3' exonuclease with ribonuclease H activity. The Journal of biological chemistry *264*, 19266-19270.

Crute, J.J., and Lehman, I.R. (1991). Herpes simplex virus-1 helicase-primase. Physical and catalytic properties. The Journal of biological chemistry *266*, 4484-4488.

Crute, J.J., Mocarski, E.S., and Lehman, I.R. (1988). A DNA helicase induced by herpes simplex virus type 1. Nucleic acids research *16*, 6585-6596.

Crute, J.J., Tsurumi, T., Zhu, L.A., Weller, S.K., Olivo, P.D., Challberg, M.D., Mocarski, E.S., and Lehman, I.R. (1989). Herpes simplex virus 1 helicase-primase: a complex of three herpes-encoded gene products. Proceedings of the National Academy of Sciences of the United States of America *86*, 2186-2189.

Darby, G., Churcher, M.J., and Larder, B.A. (1984). Cooperative effects between two acyclovir resistance loci in herpes simplex virus. Journal of virology *50*, 838-846.
Davison, A.J., and Scott, J.E. (1986). The complete DNA sequence of varicella-zoster virus. J Gen Virol *67*, 1759-1816.

Day, S.P., Lausch, R.N., and Oakes, J.E. (1988). Evidence that the gene for herpes simplex virus type 1 DNA polymerase accounts for the capacity of an intertypic recombinant to spread from eye to central nervous system. Virology *163*, 166-173.

de Bruyn Kops, A., and Knipe, D.M. (1988). Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. Cell *55*, 857-868.

de Jesus, O., Smith, P.R., Spender, L.C., Elgueta Karstegl, C., Niller, H.H., Huang, D., and Farrell, P.J. (2003). Updated Epstein-Barr virus (EBV) DNA sequence and analysis of a promoter for the BART (CST, BARF0) RNAs of EBV. J Gen Virol *84*, 1443-1450.

Delius, H., and Clements, J.B. (1976). A partial denaturation map of herpes simplex virus type 1 DNA: evidence for inversions of the unique DNA regions. The Journal of general virology *33*, 125-133.

Derse, D., Bastow, K.F., and Cheng, Y. (1982). Characterization of the DNA polymerases induced by a group of herpes simplex virus type I variants selected for growth in the presence of phosphonoformic acid. The Journal of biological chemistry *257*, 10251-10260.

Derse, D., and Cheng, Y.C. (1981). Herpes simplex virus type I DNA polymerase. Kinetic properties of the associated 3'-5' exonuclease activity and its role in araAMP incorporation. The Journal of biological chemistry *256*, 8525-8530.

Di Tommaso, P., Moretti, S., Xenarios, I., Orobitg, M., Montanyola, A., Chang, J.M., Taly, J.F., and Notredame, C. (2011). T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. Nucleic Acids Res *39*, W13-17.

Digard, P., Bebrin, W.R., Weisshart, K., and Coen, D.M. (1993a). The extreme C terminus of herpes simplex virus DNA polymerase is crucial for functional interaction with processivity factor UL42 and for viral replication. Journal of virology *67*, 398-406.

Digard, P., Chow, C.S., Pirrit, L., and Coen, D.M. (1993b). Functional analysis of the herpes simplex virus UL42 protein. Journal of virology *67*, 1159-1168.

Dodson, M.S., and Lehman, I.R. (1991). Association of DNA helicase and primase activities with a subassembly of the herpes simplex virus 1 helicase-primase composed of the UL5 and UL52 gene products. Proceedings of the National Academy of Sciences of the United States of America *88*, 1105-1109.

Dorsky, D.I., and Crumpacker, C.S. (1988). Expression of herpes simplex virus type 1 DNA polymerase gene by in vitro translation and effects of gene deletions on activity. J Virol *62*, 3224-3232.

Dracheva, S., Koonin, E.V., and Crute, J.J. (1995). Identification of the primase active site of the herpes simplex virus type 1 helicase-primase. The Journal of biological chemistry *270*, 14148-14153.

Efstathiou, S., Minson, A.C., Field, H.J., Anderson, J.R., and Wildy, P. (1986). Detection of herpes simplex virus-specific DNA sequences in latently infected mice and in humans. Journal of virology *57*, 446-455.

Efstathiou, S., and Preston, C.M. (2005). Towards an understanding of the molecular basis of herpes simplex virus latency. Virus research *111*, 108-119.

Elias, P., Gustafsson, C.M., and Hammarsten, O. (1990). The origin binding protein of herpes simplex virus 1 binds cooperatively to the viral origin of replication oris. The Journal of biological chemistry *265*, 17167-17173.

Elias, P., and Lehman, I.R. (1988). Interaction of origin binding protein with an origin of replication of herpes simplex virus 1. Proceedings of the National Academy of Sciences of the United States of America *85*, 2959-2963.

Elias, P., O'Donnell, M.E., Mocarski, E.S., and Lehman, I.R. (1986). A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. Proceedings of the National Academy of Sciences of the United States of America *83*, 6322-6326.

Elmore, D., and Eberle, R. (2008). Monkey B virus (Cercopithecine herpesvirus 1). Comparative medicine *58*, 11-21.

Falkenberg, M., Bushnell, D.A., Elias, P., and Lehman, I.R. (1997). The UL8 subunit of the heterotrimeric herpes simplex virus type 1 helicase-primase is required for the unwinding of single strand DNA-binding protein (ICP8)-coated DNA substrates. The Journal of biological chemistry *272*, 22766-22770.

Field, H.J., and Coen, D.M. (1986). Pathogenicity of herpes simplex virus mutants containing drug resistance mutations in the viral DNA polymerase gene. Journal of virology *60*, 286-289.

Field, H.J., and Wildy, P. (1978). The pathogenicity of thymidine kinase-deficient mutants of herpes simplex virus in mice. The Journal of hygiene *81*, 267-277.

Fierer, D.S., and Challberg, M.D. (1992). Purification and characterization of UL9, the herpes simplex virus type 1 origin-binding protein. Journal of virology *66*, 3986-3995.
Franklin, M.C., Wang, J., and Steitz, T.A. (2001). Structure of the replicating complex of a pol alpha family DNA polymerase. Cell *105*, 657-667.

Gallo, M.L., Dorsky, D.I., Crumpacker, C.S., and Parris, D.S. (1989). The essential 65-kilodalton DNA-binding protein of herpes simplex virus stimulates the virus-encoded DNA polymerase. Journal of virology *63*, 5023-5029.

Gallo, M.L., Jackwood, D.H., Murphy, M., Marsden, H.S., and Parris, D.S. (1988). Purification of the herpes simplex virus type 1 65-kilodalton DNA-binding protein: properties of the protein and evidence of its association with the virus-encoded DNA polymerase. Journal of virology *62*, 2874-2883.

Gao, M., and Knipe, D.M. (1991). Potential role for herpes simplex virus ICP8 DNA replication protein in stimulation of late gene expression. Journal of virology *65*, 2666-2675.

Garber, D.A., Beverley, S.M., and Coen, D.M. (1993). Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. Virology *197*, 459-462.

Gibbs, J.S., Chiou, H.C., Bastow, K.F., Cheng, Y.C., and Coen, D.M. (1988). Identification of amino acids in herpes simplex virus DNA polymerase involved in substrate and drug recognition. Proceedings of the National Academy of Sciences of the United States of America *85*, 6672-6676.

Gibbs, J.S., Chiou, H.C., Hall, J.D., Mount, D.W., Retondo, M.J., Weller, S.K., and Coen, D.M. (1985). Sequence and mapping analyses of the herpes simplex virus DNA polymerase gene predict a C-terminal substrate binding domain. Proc Natl Acad Sci U S A *82*, 7969-7973.

Goldstein, D.J., and Weller, S.K. (1988a). Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: characterization of an ICP6 deletion mutant. Virology *166*, 41-51.

Goldstein, D.J., and Weller, S.K. (1988b). Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis:

isolation and characterization of an ICP6 lacZ insertion mutant. Journal of virology *62*, 196-205.

Gottlieb, J., Marcy, A.I., Coen, D.M., and Challberg, M.D. (1990). The herpes simplex virus type 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. Journal of virology *64*, 5976-5987.

Hall, J.D., Orth, K.L., and Claus-Walker, D. (1996). Evidence that the nuclease activities associated with the herpes simplex type 1 DNA polymerase are due to the 3'-5' exonuclease. Journal of virology *70*, 4816-4818.

Hall, J.D., Orth, K.L., Sander, K.L., Swihart, B.M., and Senese, R.A. (1995). Mutations within conserved motifs in the 3'-5' exonuclease domain of herpes simplex virus DNA polymerase. The Journal of general virology *76 (Pt 12)*, 2999-3008.

Hamatake, R.K., Bifano, M., Hurlburt, W.W., and Tenney, D.J. (1997). A functional interaction of ICP8, the herpes simplex virus single-stranded DNA-binding protein, and the helicase-primase complex that is dependent on the presence of the UL8 subunit. The Journal of general virology *78 (Pt 4)*, 857-865.

Hanson, L., Dishon, A., and Kotler, M. (2011). Herpesviruses that infect fish. Viruses *3*, 2160-2191.

Hao, H., Jiang, Y., Zhang, S.J., Zhang, P., Zeng, R.X., and Lee, M.Y. (1992). Structural and functional relationships of human DNA polymerases. Chromosoma *102*, S121-127.
Hardy, W.R., and Sandri-Goldin, R.M. (1994). Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. Journal of virology *68*, 7790-7799.

Hayward, G.S., Jacob, R.J., Wadsworth, S.C., and Roizman, B. (1975). Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. Proceedings of the National Academy of Sciences of the United States of America *72*, 4243-4247.

He, X., and Lehman, I.R. (2001). An initial ATP-independent step in the unwinding of a herpes simplex virus type I origin of replication by a complex of the viral origin-binding protein and single-strand DNA-binding protein. Proceedings of the National Academy of Sciences of the United States of America *98*, 3024-3028.

Hernandez, T.R., and Lehman, I.R. (1990). Functional interaction between the herpes simplex-1 DNA polymerase and UL42 protein. The Journal of biological chemistry *265*, 11227-11232.

Honess, R.W., and Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. Journal of virology *14*, 8-19.

Hubscher, U., Maga, G., and Spadari, S. (2002). Eukaryotic DNA polymerases. Annu Rev Biochem *71*, 133-163.

Huszar, D., and Bacchetti, S. (1981). Partial purification and characterization of the ribonucleotide reductase induced by herpes simplex virus infection of mammalian cells. Journal of virology *37*, 580-588.

Hwang, Y.T., and Hwang, C.B. (2003). Exonuclease-deficient polymerase mutant of herpes simplex virus type 1 induces altered spectra of mutations. J Virol *77*, 2946-2955.

Hwang, Y.T., Liu, B.Y., Coen, D.M., and Hwang, C.B. (1997). Effects of mutations in the Exo III motif of the herpes simplex virus DNA polymerase gene on enzyme activities, viral replication, and replication fidelity. J Virol *71*, 7791-7798.

Ingemarson, R., and Lankinen, H. (1987). The herpes simplex virus type 1 ribonucleotide reductase is a tight complex of the type alpha 2 beta 2 composed of 40K and 140K proteins, of which the latter shows multiple forms due to proteolysis. Virology *156*, 417-422.

Isegawa, Y., Hara, J., Amo, K., Osugi, Y., Takemoto, M., Yamanishi, K., Fukunaga, R., Shibata, M., Ohshima, A., Horiguchi, Y*., et al.* (2009). Human herpesvirus 6 ganciclovir-resistant strain with amino acid substitutions associated with the death of an allogeneic stem cell transplant recipient. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology *44*, 15-19.

Jacob, R.J., Morse, L.S., and Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. Journal of virology *29*, 448-457.

Jacobson, J.G., Leib, D.A., Goldstein, D.J., Bogard, C.L., Schaffer, P.A., Weller, S.K., and Coen, D.M. (1989). A herpes simplex virus ribonucleotide reductase deletion mutant is defective for productive acute and reactivatable latent infections of mice and for replication in mouse cells. Virology *173*, 276-283.

Jamieson, A.T., Gentry, G.A., and Subak-Sharpe, J.H. (1974). Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. The Journal of general virology *24*, 465-480.

Jarvis, T.C., Paul, L.S., Hockensmith, J.W., and von Hippel, P.H. (1989). Structural and enzymatic studies of the T4 DNA replication system. II. ATPase properties of the polymerase accessory protein complex. The Journal of biological chemistry *264*, 12717-12729.

Katz, J.P., Bodin, E.T., and Coen, D.M. (1990). Quantitative polymerase chain reaction analysis of herpes simplex virus DNA in ganglia of mice infected with replication-incompetent mutants. Journal of virology *64*, 4288-4295.

Kieff, E.D., Bachenheimer, S.L., and Roizman, B. (1971). Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. Journal of virology *8*, 125-132.

Klinedinst, D.K., and Challberg, M.D. (1994). Helicase-primase complex of herpes simplex virus type 1: a mutation in the UL52 subunit abolishes primase activity. Journal of virology *68*, 3693-3701.

Knipe, D.M., Senechek, D., Rice, S.A., and Smith, J.L. (1987). Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. Journal of virology *61*, 276-284.

Knopf, C.W., and Weisshart, K. (1988). The herpes simplex virus DNA polymerase: analysis of the functional domains. Biochimica et biophysica acta *951*, 298-314.

Knopf, C.W., and Weisshart, K. (1990). Comparison of exonucleolytic activities of herpes simplex virus type-1 DNA polymerase and DNase. European journal of biochemistry / FEBS *191*, 263-273.

Knopf, K.W. (1979). Properties of herpes simplex virus DNA polymerase and characterization of its associated exonuclease activity. Eur J Biochem *98*, 231-244.

Koff, A., and Tegtmeyer, P. (1988). Characterization of major recognition sequences for a herpes simplex virus type 1 origin-binding protein. Journal of virology *62*, 4096-4103.

Komazin-Meredith, G., Santos, W.L., Filman, D.J., Hogle, J.M., Verdine, G.L., and Coen, D.M. (2008). The positively charged surface of herpes simplex virus UL42 mediates DNA binding. J Biol Chem *283*, 6154-6161.

Kong, X.P., Onrust, R., O'Donnell, M., and Kuriyan, J. (1992). Three-dimensional structure of the beta subunit of E. coli DNA polymerase III holoenzyme: a sliding DNA clamp. Cell *69*, 425-437.

Kramer, M.F., Jurak, I., Pesola, J.M., Boissel, S., Knipe, D.M., and Coen, D.M. (2011). Herpes simplex virus 1 microRNAs expressed abundantly during latent infection are not essential for latency in mouse trigeminal ganglia. Virology *417*, 239-247.

Krishna, T.S., Kong, X.P., Gary, S., Burgers, P.M., and Kuriyan, J. (1994). Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. Cell *79*, 1233-1243.

Kristie, T.M., Liang, Y., and Vogel, J.L. (2010). Control of alpha-herpesvirus IE gene expression by HCF-1 coupled chromatin modification activities. Biochimica et biophysica acta *1799*, 257-265.

Kuhn, F.J., and Knopf, C.W. (1996). Herpes simplex virus type 1 DNA polymerase. Mutational analysis of the 3'-5'-exonuclease domain. J Biol Chem *271*, 29245-29254.
LaBoissiere, S., and O'Hare, P. (2000). Analysis of HCF, the cellular cofactor of VP16, in herpes simplex virus-infected cells. Journal of virology *74*, 99-109.

Lafferty, W.E., Downey, L., Celum, C., and Wald, A. (2000). Herpes simplex virus type 1 as a cause of genital herpes: impact on surveillance and prevention. The Journal of infectious diseases *181*, 1454-1457.

Larder, B.A., and Darby, G. (1985). Selection and characterisation of acyclovir-resistant herpes simplex virus type 1 mutants inducing altered DNA polymerase activities. Virology *146*, 262-271.

Lausch, R.N., Yeung, K.C., Miller, J.Z., and Oakes, J.E. (1990). Nucleotide sequences responsible for the inability of a herpes simplex virus type 2 strain to grow in human lymphocytes are identical to those responsible for its inability to grow in mouse tissues following ocular infection. Virology *176*, 319-328.

Lee, C.K., and Knipe, D.M. (1985). An immunoassay for the study of DNA-binding activities of herpes simplex virus protein ICP8. Journal of virology *54*, 731-738.

Lee, S.S., Dong, Q., Wang, T.S., and Lehman, I.R. (1995). Interaction of herpes simplex virus 1 origin-binding protein with DNA polymerase alpha. Proceedings of the National Academy of Sciences of the United States of America *92*, 7882-7886.

Lehman, I.R., and Kaguni, L.S. (1989). DNA polymerase alpha. J Biol Chem *264*, 4265-4268.

Leib, D.A., Nadeau, K.C., Rundle, S.A., and Schaffer, P.A. (1991). The promoter of the latency-associated transcripts of herpes simplex virus type 1 contains a functional cAMP-response element: role of the latency-associated transcripts and cAMP in reactivation of viral latency. Proceedings of the National Academy of Sciences of the United States of America *88*, 48-52.

Lilley, C.E., Carson, C.T., Muotri, A.R., Gage, F.H., and Weitzman, M.D. (2005). DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proceedings of the National Academy of Sciences of the United States of America *102*, 5844-5849.

Linke, S.P., Sengupta, S., Khabie, N., Jeffries, B.A., Buchhop, S., Miska, S., Henning, W., Pedeux, R., Wang, X.W., Hofseth, L.J.*, et al.* (2003). p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination. Cancer research *63*, 2596-2605.

Liptak, L.M., Uprichard, S.L., and Knipe, D.M. (1996). Functional order of assembly of herpes simplex virus DNA replication proteins into prereplicative site structures. Journal of virology *70*, 1759-1767.

Liu, S., Knafels, J.D., Chang, J.S., Waszak, G.A., Baldwin, E.T., Deibel, M.R., Jr., Thomsen, D.R., Homa, F.L., Wells, P.A., Tory, M.C.*, et al.* (2006). Crystal structure of the herpes simplex virus 1 DNA polymerase. J Biol Chem *281*, 18193-18200.

Lycke, E., Kristensson, K., Svennerholm, B., Vahlne, A., and Ziegler, R. (1984). Uptake and transport of herpes simplex virus in neurites of rat dorsal root ganglia cells in culture. The Journal of general virology *65 (Pt 1)*, 55-64.

Makhov, A.M., Boehmer, P.E., Lehman, I.R., and Griffith, J.D. (1996). The herpes simplex virus type 1 origin-binding protein carries out origin specific DNA unwinding and forms stem-loop structures. The EMBO journal *15*, 1742-1750.

Malik, A.K., Martinez, R., Muncy, L., Carmichael, E.P., and Weller, S.K. (1992). Genetic analysis of the herpes simplex virus type 1 UL9 gene: isolation of a LacZ insertion mutant and expression in eukaryotic cells. Virology *190*, 702-715.

Marchler-Bauer, A., Lu, S., Anderson, J.B., Chitsaz, F., Derbyshire, M.K., DeWeese-Scott, C., Fong, J.H., Geer, L.Y., Geer, R.C., Gonzales, N.R.*, et al.* (2011). CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic acids research *39*, D225-229.

Marcy, A.I., Olivo, P.D., Challberg, M.D., and Coen, D.M. (1990). Enzymatic activities of overexpressed herpes simplex virus DNA polymerase purified from recombinant baculovirus-infected insect cells. Nucleic Acids Res *18*, 1207-1215.

Margolis, T.P., Sedarati, F., Dobson, A.T., Feldman, L.T., and Stevens, J.G. (1992). Pathways of viral gene expression during acute neuronal infection with HSV-1. Virology *189*, 150-160.

Marsden, H.S., Cross, A.M., Francis, G.J., Patel, A.H., MacEachran, K., Murphy, M., McVey, G., Haydon, D., Abbotts, A., and Stow, N.D. (1996). The herpes simplex virus type 1 UL8 protein influences the intracellular localization of the UL52 but not the ICP8 or POL replication proteins in virus-infected cells. The Journal of general virology *77 (Pt 9)*, 2241-2249.

Marsden, H.S., McLean, G.W., Barnard, E.C., Francis, G.J., MacEachran, K., Murphy, M., McVey, G., Cross, A., Abbotts, A.P., and Stow, N.D. (1997). The catalytic subunit of the DNA polymerase of herpes simplex virus type 1 interacts specifically with the C terminus of the UL8 component of the viral helicase-primase complex. J Virol *71*, 6390-6397.

Martinez, R., Shao, L., Bronstein, J.C., Weber, P.C., and Weller, S.K. (1996). The product of a 1.9-kb mRNA which overlaps the HSV-1 alkaline nuclease gene (UL12) cannot relieve the growth defects of a null mutant. Virology *215*, 152-164.

Martinez, R., Shao, L., and Weller, S.K. (1992). The conserved helicase motifs of the herpes simplex virus type 1 origin-binding protein UL9 are important for function. Journal of virology *66*, 6735-6746.

Maul, G.G., Ishov, A.M., and Everett, R.D. (1996). Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. Virology *217*, 67-75.

McGeoch, D.J., Dalrymple, M.A., Dolan, A., McNab, D., Perry, L.J., Taylor, P., and Challberg, M.D. (1988). Structures of herpes simplex virus type 1 genes required for replication of virus DNA. Journal of virology *62*, 444-453.

McGeoch, D.J., Dolan, A., and Ralph, A.C. (2000). Toward a comprehensive phylogeny for mammalian and avian herpesviruses. Journal of virology *74*, 10401-10406.

McLean, G.W., Abbotts, A.P., Parry, M.E., Marsden, H.S., and Stow, N.D. (1994). The herpes simplex virus type 1 origin-binding protein interacts specifically with the viral UL8 protein. The Journal of general virology *75 (Pt 10)*, 2699-2706.

McLennan, J.L., and Darby, G. (1980). Herpes simplex virus latency: the cellular location of virus in dorsal root ganglia and the fate of the infected cell following virus activation. The Journal of general virology *51*, 233-243.

Megaw, A.G., Rapaport, D., Avidor, B., Frenkel, N., and Davison, A.J. (1998). The DNA sequence of the RK strain of human herpesvirus 7. Virology *244*, 119-132.
Miyamoto, K., and Morgan, C. (1971). Structure and development of viruses as observed in the electron microscope. XI. Entry and uncoating of herpes simplex virus. Journal of virology *8*, 910-918.

Mohni, K.N., Dee, A.R., Smith, S., Schumacher, A.J., and Weller, S.K. (2012). Efficient Herpes Simplex Virus type 1 replication requires cellular ATR pathway proteins. Journal of virology.

Mohni, K.N., Livingston, C.M., Cortez, D., and Weller, S.K. (2010). ATR and ATRIP are recruited to herpes simplex virus type 1 replication compartments even though ATR signaling is disabled. Journal of virology *84*, 12152-12164.

Morgan, C., Rose, H.M., and Mednis, B. (1968). Electron microscopy of herpes simplex virus. I. Entry. Journal of virology *2*, 507-516.

Moshkin, Y.M., Armstrong, J.A., Maeda, R.K., Tamkun, J.W., Verrijzer, P., Kennison, J.A., and Karch, F. (2002). Histone chaperone ASF1 cooperates with the Brahma chromatin-remodelling machinery. Genes & development *16*, 2621-2626.

Mullaney, J., Moss, H.W., and McGeoch, D.J. (1989). Gene UL2 of herpes simplex virus type 1 encodes a uracil-DNA glycosylase. The Journal of general virology *70 (Pt 2)*, 449-454.

Munakata, T., Adachi, N., Yokoyama, N., Kuzuhara, T., and Horikoshi, M. (2000). A human homologue of yeast anti-silencing factor has histone chaperone activity. Genes to cells : devoted to molecular & cellular mechanisms *5*, 221-233.

Negorev, D., and Maul, G.G. (2001). Cellular proteins localized at and interacting within ND10/PML nuclear bodies/PODs suggest functions of a nuclear depot. Oncogene *20*, 7234-7242.

Neipel, F., Albrecht, J.C., and Fleckenstein, B. (1997). Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? J Virol *71*, 4187-4192.

Notredame, C., Higgins, D.G., and Heringa, J. (2000). T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol *302*, 205-217.

Nutter, L.M., Grill, S.P., and Cheng, Y.C. (1985). Can ribonucleotide reductase be considered as an effective target for developing antiherpes simplex virus type II (HSV-2) compounds? Biochemical pharmacology *34*, 777-780.

O'Donnell, M.E., Elias, P., Funnell, B.E., and Lehman, I.R. (1987). Interaction between the DNA polymerase and single-stranded DNA-binding protein (infected cell protein 8) of herpes simplex virus 1. The Journal of biological chemistry *262*, 4260-4266.
Olesky, M., McNamee, E.E., Zhou, C., Taylor, T.J., and Knipe, D.M. (2005). Evidence for a direct interaction between HSV-1 ICP27 and ICP8 proteins. Virology *331*, 94-105.

Olivo, P.D., Nelson, N.J., and Challberg, M.D. (1988). Herpes simplex virus DNA replication: the UL9 gene encodes an origin-binding protein. Proceedings of the National Academy of Sciences of the United States of America *85*, 5414-5418.

Parkinson, J., Lees-Miller, S.P., and Everett, R.D. (1999). Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. Journal of virology *73*, 650-657.

Parry, M.E., Stow, N.D., and Marsden, H.S. (1993). Purification and properties of the herpes simplex virus type 1 UL8 protein. The Journal of general virology *74 (Pt 4)*, 607-612.

Pavlov, A.R., and Karam, J.D. (1994). Binding specificity of T4 DNA polymerase to RNA. The Journal of biological chemistry *269*, 12968-12972.

Pellet, P.E., and Roizman, B. (2006). The Family Herpesviridae: A Brief Introduction. In Fields Virology, D.M. Knipe, and P.M. Howley, eds. (Philadelphia, PA: Lippincott, Williams, & Wilkins), pp. 2479-2495.

Pelosi, E., Mulamba, G.B., and Coen, D.M. (1998). Penciclovir and pathogenesis phenotypes of drug-resistant Herpes simplex virus mutants. Antiviral research *37*, 17-28.

Penfold, M.E., Armati, P., and Cunningham, A.L. (1994). Axonal transport of herpes simplex virions to epidermal cells: evidence for a specialized mode of virus transport and assembly. Proceedings of the National Academy of Sciences of the United States of America *91*, 6529-6533.

Peng, H., Nogueira, M.L., Vogel, J.L., and Kristie, T.M. (2010). Transcriptional coactivator HCF-1 couples the histone chaperone Asf1b to HSV-1 DNA replication components. Proc Natl Acad Sci U S A *107*, 2461-2466.

Perng, G.C., Ghiasi, H., Slanina, S.M., Nesburn, A.B., and Wechsler, S.L. (1996). High-dose ocular infection with a herpes simplex virus type 1 ICP34.5 deletion mutant produces no corneal disease or neurovirulence yet results in wild-type levels of spontaneous reactivation. Journal of virology *70*, 2883-2893.

Pesola, J.M., Zhu, J., Knipe, D.M., and Coen, D.M. (2005). Herpes simplex virus 1 immediate-early and early gene expression during reactivation from latency under conditions that prevent infectious virus production. Journal of virology *79*, 14516-14525.

Pilger, B.D., Cui, C., and Coen, D.M. (2004). Identification of a small molecule that inhibits herpes simplex virus DNA Polymerase subunit interactions and viral replication. Chemistry & biology *11*, 647-654.

Poffenberger, K.L., and Roizman, B. (1985). A noninverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. Journal of virology *53*, 587-595.

Ponce de Leon, M., Eisenberg, R.J., and Cohen, G.H. (1977). Ribonucleotide reductase from herpes simplex virus (types 1 and 2) infected and uninfected KB cells: properties of the partially purified enzymes. The Journal of general virology *36*, 163-173.

Powell, K.L., and Purifoy, D.J. (1977). Nonstructural proteins of herpes simplex virus. I. Purification of the induced DNA polymerase. Journal of virology *24*, 618-626.

Preston, V.G., Darling, A.J., and McDougall, I.M. (1988). The herpes simplex virus type 1 temperature-sensitive mutant ts1222 has a single base pair deletion in the small subunit of ribonucleotide reductase. Virology *167*, 458-467.

Prichard, M.N., Duke, G.M., and Mocarski, E.S. (1996). Human cytomegalovirus uracil DNA glycosylase is required for the normal temporal regulation of both DNA synthesis and viral replication. Journal of virology *70*, 3018-3025.

Pyles, R.B., and Thompson, R.L. (1994). Evidence that the herpes simplex virus type 1 uracil DNA glycosylase is required for efficient viral replication and latency in the murine nervous system. Journal of virology *68*, 4963-4972.

Quinlan, M.P., Chen, L.B., and Knipe, D.M. (1984). The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell *36*, 857-868.

Randell, J.C., Komazin, G., Jiang, C., Hwang, C.B., and Coen, D.M. (2005). Effects of substitutions of arginine residues on the basic surface of herpes simplex virus UL42 support a role for DNA binding in processive DNA synthesis. Journal of virology *79*, 12025-12034.

Reuven, N.B., Staire, A.E., Myers, R.S., and Weller, S.K. (2003). The herpes simplex virus type 1 alkaline nuclease and single-stranded DNA binding protein mediate strand exchange in vitro. Journal of virology *77*, 7425-7433.

Reuven, N.B., and Weller, S.K. (2005). Herpes simplex virus type 1 single-strand DNA binding protein ICP8 enhances the nuclease activity of the UL12 alkaline nuclease by increasing its processivity. Journal of virology *79*, 9356-9358.

Roberts, C.M., Pfister, J.R., and Spear, S.J. (2003). Increasing proportion of herpes simplex virus type 1 as a cause of genital herpes infection in college students. Sexually transmitted diseases *30*, 797-800.

Rock, D.L., and Fraser, N.W. (1983). Detection of HSV-1 genome in central nervous system of latently infected mice. Nature *302*, 523-525.
Rodriguez, A.C., Park, H.W., Mao, C., and Beese, L.S. (2000). Crystal structure of a pol alpha family DNA polymerase from the hyperthermophilic archaeon Thermococcus sp. 9 degrees N-7. J Mol Biol *299*, 447-462.

Roizman, B., Knipe, D.M., and Whitley, R. (2006). Herpes Simplex Viruses. In Fields Virology, D.M. Knipe, and P.M. Howley, eds. (Philadelphia, PA: Lippincott, Williams & Wilkins), pp. 2501-2601.

Romanova, L.Y., Willers, H., Blagosklonny, M.V., and Powell, S.N. (2004). The interaction of p53 with replication protein A mediates suppression of homologous recombination. Oncogene *23*, 9025-9033.

Rothwell, P.J., and Waksman, G. (2005). Structure and mechanism of DNA polymerases. Advances in protein chemistry *71*, 401-440.

Ruyechan, W.T. (1983). The major herpes simplex virus DNA-binding protein holds single-stranded DNA in an extended configuration. Journal of virology *46*, 661-666.

Ruyechan, W.T., and Weir, A.C. (1984). Interaction with nucleic acids and stimulation of the viral DNA polymerase by the herpes simplex virus type 1 major DNA-binding protein. Journal of virology *52*, 727-733.

Schulz, L.L., and Tyler, J.K. (2006). The histone chaperone ASF1 localizes to active DNA replication forks to mediate efficient DNA replication. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *20*, 488-490.

Schumacher, A.J., Mohni, K.N., Kan, Y., Hendrickson, E.A., Stark, J.M., and Weller, S.K. (2012). The HSV-1 exonuclease, UL12, stimulates recombination by a single strand annealing mechanism. PLoS pathogens *8*, e1002862.

Severini, A., Scraba, D.G., and Tyrrell, D.L. (1996). Branched structures in the intracellular DNA of herpes simplex virus type 1. Journal of virology *70*, 3169-3175.

Shao, L., Rapp, L.M., and Weller, S.K. (1993). Herpes simplex virus 1 alkaline nuclease is required for efficient egress of capsids from the nucleus. Virology *196*, 146-162.

Sherman, G., Gottlieb, J., and Challberg, M.D. (1992). The UL8 subunit of the herpes simplex virus helicase-primase complex is required for efficient primer utilization. Journal of virology *66*, 4884-4892.

Shirata, N., Kudoh, A., Daikoku, T., Tatsumi, Y., Fujita, M., Kiyono, T., Sugaya, Y., Isomura, H., Ishizaki, K., and Tsurumi, T. (2005). Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. The Journal of biological chemistry *280*, 30336-30341.

Sirbu, B.M., Lachmayer, S.J., Wulfing, V., Marten, L.M., Clarkson, K.E., Lee, L.W., Gheorghiu, L., Zou, L., Powell, S.N., Dahm-Daphi, J.*, et al.* (2011). ATR-p53 restricts homologous recombination in response to replicative stress but does not limit DNA interstrand crosslink repair in lung cancer cells. PloS one *6*, e23053.

Spear, M.A., Schuback, D., Miyata, K., Grandi, P., Sun, F., Yoo, L., Nguyen, A., Brandt, C.R., and Breakefield, X.O. (2003). HSV-1 amplicon peptide display vector. Journal of virological methods *107*, 71-79.

Spivack, J.G., and Fraser, N.W. (1988). Expression of herpes simplex virus type 1 latency-associated transcripts in the trigeminal ganglia of mice during acute infection and reactivation of latent infection. Journal of virology *62*, 1479-1485.

Steitz, T.A., Smerdon, S.J., Jager, J., and Joyce, C.M. (1994). A unified polymerase mechanism for nonhomologous DNA and RNA polymerases. Science *266*, 2022-2025.

Stevens, J.G., and Cook, M.L. (1971). Latent herpes simplex virus in spinal ganglia of mice. Science *173*, 843-845.

Stevens, J.G., Wagner, E.K., Devi-Rao, G.B., Cook, M.L., and Feldman, L.T. (1987). RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science *235*, 1056-1059.

Stow, N.D. (1982). Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. The EMBO journal *1*, 863-867.

Strang, B.L., and Coen, D.M. (2010). Interaction of the human cytomegalovirus uracil DNA glycosylase UL114 with the viral DNA polymerase catalytic subunit UL54. The Journal of general virology *91*, 2029-2033.

Strang, B.L., and Stow, N.D. (2005). Circularization of the herpes simplex virus type 1 genome upon lytic infection. Journal of virology *79*, 12487-12494.

Strick, R., Hansen, J., Bracht, R., Komitowski, D., and Knopf, C.W. (1997). Epitope mapping and functional characterization of monoclonal antibodies specific for herpes simplex virus type I DNA polymerase. Intervirology *40*, 41-49.

Strom, T., and Frenkel, N. (1987). Effects of herpes simplex virus on mRNA stability. Journal of virology *61*, 2198-2207.

Sturzbecher, H.W., Donzelmann, B., Henning, W., Knippschild, U., and Buchhop, S. (1996). p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction. The EMBO journal *15*, 1992-2002.

Sung, P. (1994). Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. Science *265*, 1241-1243.

Swan, M.K., Johnson, R.E., Prakash, L., Prakash, S., and Aggarwal, A.K. (2009). Structural basis of high-fidelity DNA synthesis by yeast DNA polymerase delta. Nature structural & molecular biology *16*, 979-986.

Tanguy Le Gac, N., Villani, G., Hoffmann, J.S., and Boehmer, P.E. (1996). The UL8 subunit of the herpes simplex virus type-1 DNA helicase-primase optimizes utilization of DNA templates covered by the homologous single-strand DNA-binding protein ICP8. The Journal of biological chemistry *271*, 21645-21651.

Taylor, T.J., and Knipe, D.M. (2004). Proteomics of herpes simplex virus replication compartments: association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. Journal of virology *78*, 5856-5866.

Tenney, D.J., Hurlburt, W.W., Micheletti, P.A., Bifano, M., and Hamatake, R.K. (1994). The UL8 component of the herpes simplex virus helicase-primase complex stimulates primer synthesis by a subassembly of the UL5 and UL52 components. The Journal of biological chemistry *269*, 5030-5035.

Tenney, D.J., Sheaffer, A.K., Hurlburt, W.W., Bifano, M., and Hamatake, R.K. (1995). Sequence-dependent primer synthesis by the herpes simplex virus helicase-primase complex. The Journal of biological chemistry *270*, 9129-9136.

Tenser, R.B., Hay, K.A., and Edris, W.A. (1989). Latency-associated transcript but not reactivatable virus is present in sensory ganglion neurons after inoculation of thymidine kinase-negative mutants of herpes simplex virus type 1. Journal of virology *63*, 2861-2865.

Terrell, S.L., and Coen, D.M. (2012). The Pre-NH2-Terminal Domain of the Herpes Simplex Virus 1 DNA Polymerase Catalytic Subunit Is Required for Efficient Viral Replication. Journal of virology *86*, 11057-11065.

Thompson, R.L., Preston, C.M., and Sawtell, N.M. (2009). De novo synthesis of VP16 coordinates the exit from HSV latency in vivo. PLoS pathogens *5*, e1000352.

Thompson, R.L., and Sawtell, N.M. (2000). Replication of herpes simplex virus type 1 within trigeminal ganglia is required for high frequency but not high viral genome copy number latency. Journal of virology *74*, 965-974.

Tian, W., Hwang, Y.T., Lu, Q., and Hwang, C.B. (2009). Finger domain mutation affects enzyme activity, DNA replication efficiency, and fidelity of an exonuclease-deficient DNA polymerase of herpes simplex virus type 1. J Virol *83*, 7194-7201.

Tischer, B.K., Smith, G.A., and Osterrieder, N. (2010). En passant mutagenesis: a two step markerless red recombination system. Methods Mol Biol *634*, 421-430.

Tischer, B.K., von Einem, J., Kaufer, B., and Osterrieder, N. (2006). Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. BioTechniques *40*, 191-197.

Tsurimoto, T., Shinozaki, A., Yano, M., Seki, M., and Enomoto, T. (2005). Human Werner helicase interacting protein 1 (WRNIP1) functions as a novel modulator for DNA polymerase delta. Genes to cells : devoted to molecular & cellular mechanisms *10*, 13-22.

Tyler, J.K., Adams, C.R., Chen, S.R., Kobayashi, R., Kamakaka, R.T., and Kadonaga, J.T. (1999). The RCAF complex mediates chromatin assembly during DNA replication and repair. Nature *402*, 555-560.

van Oijen, A.M., and Loparo, J.J. (2010). Single-molecule studies of the replisome. Annual review of biophysics *39*, 429-448.

Vaughan, P.J., Banks, L.M., Purifoy, D.J., and Powell, K.L. (1984). Interactions between herpes simplex virus DNA-binding proteins. The Journal of general virology *65 (Pt 11)*, 2033-2041.

Vaughan, P.J., Purifoy, D.J., and Powell, K.L. (1985). DNA-binding protein associated with herpes simplex virus DNA polymerase. Journal of virology *53*, 501-508.

Wadsworth, S., Hayward, G.S., and Roizman, B. (1976). Anatomy of herpes simplex virus DNA. V. Terminally repetitive sequences. Journal of virology *17*, 503-512.

Wagner, E.K., and Bloom, D.C. (1997). Experimental investigation of herpes simplex virus latency. Clinical microbiology reviews *10*, 419-443.

Wahl, A.F., Geis, A.M., Spain, B.H., Wong, S.W., Korn, D., and Wang, T.S. (1988). Gene expression of human DNA polymerase alpha during cell proliferation and the cell cycle. Molecular and cellular biology *8*, 5016-5025.

Wang, J., Sattar, A.K., Wang, C.C., Karam, J.D., Konigsberg, W.H., and Steitz, T.A. (1997). Crystal structure of a pol alpha family replication DNA polymerase from bacteriophage RB69. Cell *89*, 1087-1099.

Wang, J., Yu, P., Lin, T.C., Konigsberg, W.H., and Steitz, T.A. (1996). Crystal structures of an NH2-terminal fragment of T4 DNA polymerase and its complexes with single-stranded DNA and with divalent metal ions. Biochemistry *35*, 8110-8119.

Wang, T.S., Wong, S.W., and Korn, D. (1989). Human DNA polymerase alpha: predicted functional domains and relationships with viral DNA polymerases. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *3*, 14-21.

Weissbach, A., Hong, S.C., Aucker, J., and Muller, R. (1973). Characterization of herpes simplex virus-induced deoxyribonucleic acid polymerase. The Journal of biological chemistry *248*, 6270-6277.

Weisshart, K., and Knopf, C.W. (1988). The herpes simplex virus type I DNA polymerase. Polypeptide structure and antigenic domains. European journal of biochemistry / FEBS *174*, 707-716.

Weisshart, K., Kuo, A.A., Hwang, C.B., Kumura, K., and Coen, D.M. (1994). Structural and functional organization of herpes simplex virus DNA polymerase investigated by limited proteolysis. The Journal of biological chemistry *269*, 22788-22796.

Weller, S.K., Spadaro, A., Schaffer, J.E., Murray, A.W., Maxam, A.M., and Schaffer, P.A. (1985). Cloning, sequencing, and functional analysis of oriL, a herpes simplex virus type 1 origin of DNA synthesis. Molecular and cellular biology *5*, 930-942.

Wilcock, D., and Lane, D.P. (1991). Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. Nature *349*, 429-431.

Wilkinson, D.E., and Weller, S.K. (2003). The role of DNA recombination in herpes simplex virus DNA replication. IUBMB life *55*, 451-458.

Wilkinson, D.E., and Weller, S.K. (2004). Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. Journal of virology *78*, 4783-4796.

Wilkinson, D.E., and Weller, S.K. (2005). Inhibition of the herpes simplex virus type 1 DNA polymerase induces hyperphosphorylation of replication protein A and its accumulation at S-phase-specific sites of DNA damage during infection. Journal of virology *79*, 7162-7171.

Wilkinson, D.E., and Weller, S.K. (2006). Herpes simplex virus type I disrupts the ATR-dependent DNA-damage response during lytic infection. Journal of cell science *119*, 2695-2703.

Wong, S.W., Wahl, A.F., Yuan, P.M., Arai, N., Pearson, B.E., Arai, K., Korn, D., Hunkapiller, M.W., and Wang, T.S. (1988). Human DNA polymerase alpha gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerases. The EMBO journal *7*, 37-47.
Wu, C.A., Nelson, N.J., McGeoch, D.J., and Challberg, M.D. (1988). Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. Journal of virology *62*, 435-443.

Yager, D.R., Marcy, A.I., and Coen, D.M. (1990). Translational regulation of herpes simplex virus DNA polymerase. Journal of virology *64*, 2217-2225.

Yang, C.L., Zhang, S.J., Toomey, N.L., Palmer, T.N., and Lee, M.Y. (1991). Induction of DNA polymerase activities in the regenerating rat liver. Biochemistry *30*, 7534-7541.

York, I.A., Roop, C., Andrews, D.W., Riddell, S.R., Graham, F.L., and Johnson, D.C. (1994). A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell *77*, 525-535.

Zhou, C., and Knipe, D.M. (2002). Association of herpes simplex virus type 1 ICP8 and ICP27 proteins with cellular RNA polymerase II holoenzyme. Journal of virology *76*, 5893-5904.

Zhu, L.A., and Weller, S.K. (1992a). The six conserved helicase motifs of the UL5 gene product, a component of the herpes simplex virus type 1 helicase-primase, are essential for its function. Journal of virology *66*, 469-479.

Zhu, L.A., and Weller, S.K. (1992b). The UL5 gene of herpes simplex virus type 1: isolation of a lacZ insertion mutant and association of the UL5 gene product with other members of the helicase-primase complex. Journal of virology *66*, 458-468.

Zuccola, H.J., Filman, D.J., Coen, D.M., and Hogle, J.M. (2000). The crystal structure of an unusual processivity factor, herpes simplex virus UL42, bound to the C terminus of its cognate polymerase. Molecular cell *5*, 267-278.