Epigenetic Regulation of Lytic and Latent Herpes Simplex Virus 1 Infection

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Epigenetic Regulation of Lytic and Latent Herpes Simplex Virus 1 Infection

A dissertation presented

by

Jennifer Sohn Lee

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Virology

Harvard University
Cambridge, Massachusetts

April 2015
Epigenetic Regulation of Lytic and Latent Herpes Simplex Virus 1 Infection

Abstract

Epigenetic regulation plays a major role in whether the herpes simplex virus 1 (HSV-1) will initiate viral gene expression and lytic infection or instead suppress its gene expression and establish a latent infection. Prior to this study, it was known that cells respond to naked DNA by assembling chromatin to silence foreign genetic material. However, during lytic infection of epithelial cells, viral proteins VP16 and ICP0 have been implicated in limiting chromatin association and promoting euchromatic histone modifications on the HSV-1 genome. We hypothesized that the viral genome would also be subject to silencing by heterochromatin modification during lytic infection. To test this we examined the association of chromatin and heterochromatic modifications during lytic infection with WT viruses and ICP0-null mutant viruses. We found that heterochromatin modifications H3K9me3 and H3K27me3 associate initially with all viruses, but were removed rapidly during infection with WT HSV-1. ICP0-null viruses were not able to remove histones or heterochromatin, indicating a role for ICP0 in reversing epigenetic silencing.

In latent infection, HSV-1 undergoes epigenetic silencing as a means to suppress gene expression and persist in neurons. Surprisingly, in this study, we find that ICP0-null viruses accumulate less heterochromatin on lytic gene promoters relative to WT
viruses. This suggests that ICP0 may function to promote infection of neurons, or assist in the establishment or maintenance of latent infection.

Additionally, during latency the viral genome maintains active expression from the latency-associated transcript (LAT) region, and this region retains markers of euchromatin that are excluded from the lytic viral genes. The insulator protein, CTCF, binds to a site downstream of this region between the LAT and ICP0 promoters. We find that during latent infection, deletion of this site promoted accumulation of H3K27me3 at the LAT promoter and reduced reactivation competence of the virus, but surprisingly enhanced LAT expression. This suggests that CTCF balances epigenetic repression to promote latency and maintain reactivation competence. In summary, this dissertation suggests that during lytic infection HSV reverses cell-mediated epigenetic repression and promotes viral gene expression, while during latency, the virus co-opts epigenetic mechanisms to maintain a silenced but poised genome.
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Chapter 3

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Chapter 1. Introduction
1.1. Herpes Simplex Viruses

1.1.1. Herpesvirus Classification

Herpesviruses are large, enveloped DNA viruses that are widely distributed and collectively able to infect a broad host range. The *Herpesvirales* order includes three families: *Malacoherpesviridae*, the herpesviruses of bivalves, *Alloherpesviridae*, the herpesviruses of fish and amphibians, and *Herpesviridae*, the herpesviruses of birds, reptiles, and mammals (Davison et al., 2009; Pellett et al., 2011). The *Herpesviridae* family can be further sorted into three subfamilies: alpha-, beta-, and gamma- *Herpesvirinae* (Roizman, 1982). Individually, however, herpesviruses are generally restricted to infecting a single host species. Nine herpesviruses have thus far been identified as infecting primarily humans, with members in each of the subfamilies: the alphaherpesviruses include the genera *Simplexvirus* (HSV-1 and HSV-2) and *Varicellovirus* (VZV), betahepesviruses include the genera *Cytomegalovirus* (HCMV) and *Roseolovirus* (HHV-6 and HHV-7), and gammaherpesviruses include the genera *Lymphocryptovirus* (EBV) and *Rhadinovirus* (KSHV) (Pellett and Roizman, 2013). The focus of this dissertation will be on herpes simplex virus 1 (HSV-1).

1.1.2. Herpesvirus Epidemiology and Pathology

Herpes simplex viruses are distributed globally and are readily transmitted through close contact, resulting in a lifelong infection throughout which infected
individuals are capable of shedding transmissible virus even in the absence of clinical symptoms. Within the United States, in 2005-2010, among 14-49 year olds the average seroprevalence for HSV-1 was 59.3% and 15.7% for HSV-2 infection; although, within specific demographic groups prevalence is much higher (Bradley et al., 2013). Rates of HSV-1 and HSV-2 are even higher in the developing world, with some studies finding nearly 100% seroprevalence for herpes simplex viruses (Smith and Robinson, 2002). This is of particular concern in areas with a high prevalence of endemic human immunodeficiency virus (HIV), because genital herpes infection can cause a 2-4 fold increase in the likelihood of HIV transmission and infection (Freeman et al., 2006; Wald and Link, 2002).

Clinically, HSV-1 is generally associated with orofacial lesions, known as cold sores, while HSV-2 is generally associated with genital lesions; however, both can infect either mucosal surface (Roizman et al., 2013). Primary HSV-1 infection is often clinically asymptomatic, but is followed by transmission of the virus from the site of primary infection at the mucosa to the innervating sensory ganglia. Here the virus can establish a latent infection within neural ganglia where it persists for the lifetime of the host. During latent infection the virus is relatively dormant and does not produce infectious progeny virus. However, neuronal stress can induce periodic reactivation of the virus, resulting in production of infectious progeny that can undergo anterograde transport to return to the site of primary infection and cause recurrent vesicular lesions. In addition to vesicular lesions, HSV is also responsible for a number of rare but clinically severe disease pathologies. Infection of neonates or immunocompromised
individuals can lead to disseminated viral infection and encephalitis, which results in significant morbidity and mortality despite antiviral drug treatments that are currently available (Kimberlin, 2004). Additionally, infection of the eye by HSV can result in recurrent corneal infection and keratoconjunctivitis, which is a leading cause of infectious blindness in the developed world (Farooq and Shukla, 2012).

1.1.3. The Herpesvirus Virion

The HSV-1 virion has an average diameter of 186 nm and is surrounded by a viral envelope (Grünewald et al., 2003). The lipid bilayer envelope of HSV-1 is derived and modified from host cellular membranes and contains spikes of embedded viral glycoproteins (Roizman et al., 2013). HSV-1 virions carry as many as 13 distinct viral glycoproteins to facilitate attachment and entry of the virus. Underneath the envelope, lies the tegument. The tegument is a non-structured, asymmetrical protein layer that contains at least 18 viral proteins, including VP16, the viral protein transactivator, and the virion host shutoff (VHS) protein, as well as a number of other proteins that assist in productive infection of permissive cells (Honess and Roizman, 1973). Within the tegument, the structured viral capsid is approximately 100 nm in diameter and contains 162 capsomeres, with an icosahedral T=16 symmetry (Wildy and Watson, 1962; Zhou et al., 1994). The capsid surrounds the HSV-1 genomic core that consists of a single molecule of approximately 150 kbp of double stranded (ds) DNA. Additionally, unlike other DNA viruses, such as the polyomaviruses or papillomaviruses, herpesvirus genomes are not associated with nucleosome proteins within the virion (Cohen et al.,
1980; Oh and Fraser, 2008; Pignatti and Cassai, 1980). Instead, HSV-1 balances the negative charge of the DNA phosphate backbone within the virion through association with the anionic polyamine spermine (Kutluay and Triezenberg, 2009b; Lu and Triezenberg, 2009).

1.1.4. HSV Genome Organization

The HSV-1 genome is organized into two covalently linked regions called the unique long (UL) and unique short (US) segments, each of which are flanked by inverted repeat long (RL) and repeat short (RS) regions, respectively, and joined with a variable number of repeated a sequences (Wadsworth et al., 1975) (Figure 1.1). Some genes, including ICP0, ICP34.5, and the LAT, are located within the repeat regions and therefore maintained as two copies within the genome. Interestingly, the HSV-1 genome has an ~68% GC content, with many potential CpG motifs for DNA methylation; however, methylation of the viral genome has not been detected and likely does not influence viral infection (Dressler et al., 1987; Kieff et al., 1971; Kubat et al., 2004b).
Figure 1.1: Map of the LAT transcriptional unit. (A) The HSV-1 genome with the unique long (L) and short (S) components of the genome denoted as $U_L$ and $U_S$, respectively. (B) An expanded view of the repeat L ($R_L$) and repeat S ($R_S$) junction shows regulatory elements within the LAT transcriptional unit, including the LAT promoter (LAP), long-term enhancer element (LTE), CCCTC-binding factor (CTCF) binding site (CTRL2), and ICP0 Promoter (ICP0 P). (C) Locations and orientations of transcripts including the 8.3 kbp LAT primary transcript, the 2.0 kbp stable LAT intron, ICP0, and ICP34.5. (D) MicroRNAs encoded by the LAT region: miR-H1, miR-H2, miR-H3, miR-H4, miR-H5, miR-H6, miR-H7, miR-H8, miR-H14, miR-H15, miR-H27.
1.2. Lytic Infection and Replication

1.2.1. Viral Entry

Infection with HSV-1 begins at the mucosal epithelium or through a break in in the skin wherein a productive infection initiates within epithelial cells (Roizman et al., 2013). To achieve lytic replication the herpesvirus virion glycoproteins attach to cell surface receptors and fuse the viral envelope with a cellular membrane to release the tegument and core-containing capsid into the cytoplasm (Gianni et al., 2010; Nicola and Straus, 2004). The viral nucleocapsid is then transported to a nuclear pore where it docks to release the naked viral genome into the nucleus (Sodeik et al., 1997). The linear viral genome circularizes rapidly upon nuclear entry (Garber et al., 1993; Poffenberger and Roizman, 1985).

1.2.2. Viral Gene Expression

Viral gene transcription is dependent on the host cell RNA polymerase II (RNAPII) and viral factors are required to regulate the ordered cascade of viral gene product accumulation (Alwine et al., 1974; Costanzo et al., 1977). The more than 80 viral gene products produced during productive infection can be grouped into at least three kinetic classes of expression referred to as α- or immediate-early (IE) genes, β- or early (E) genes, and γ- or late (L) genes (Honess and Roizman, 1975; Honess and Roizman, 1974). The IE genes are defined by their ability to be expressed and
transcribed prior to *de novo* viral protein synthesis; however, transcriptional activation of the IE genes is facilitated by the viral VP16 protein which enters in the tegument (Ace et al., 1989). VP16 forms an activator complex with host cell factor (HCF) and octamer-binding protein 1 (OCT1) that binds target viral sequences within IE gene promoters to recruit additional transcription factors and stimulate IE gene expression (Wysocka and Herr, 2003). The IE genes encode proteins ICP0, ICP4, ICP22, ICP27, and ICP47, and the expression of IE proteins, particularly ICP4, is required to activate E gene expression, and for progression through the viral lytic infection cycle (Carrozza and DeLuca, 1996; Smith et al., 1993). The E genes include products that are necessary for viral DNA replication, including ICP8, the viral ssDNA binding protein (Chen and Knipe, 1996; Gao and Knipe, 1991). Initiation of viral DNA synthesis stimulates transcription of the L genes, and these L genes include products necessary for assembly of infectious progeny virions (Roizman et al., 2013).

### 1.2.3. Viral Replication

Viral DNA synthesis requires seven viral proteins including ICP8 (UL29), the viral DNA polymerase (UL30), and a viral helicase-primase complex (Challberg, 1986). Additional viral proteins, such as thymidine kinase (UL23) (Kit and Dubbs, 1965) and ribonucleotide reductase (UL39/UL40) (Bacchetti et al., 1986), are not considered non-essential, but are required for replication in some tissue types. Viral DNA synthesis occurs near nuclear domain 10 (ND10) sites (Ishov and Maul, 1996), and progresses to the formation of replication compartments that eventually fill the nucleus and
marginalize cellular chromatin (Quinlan et al., 1984). The circularized genome initiates DNA replication at one of three origins of replication, beginning with a theta replication mechanism, but subsequently converting to a rolling circle mechanism to generate head-to-tail concatemers (Jacob et al., 1979).

1.2.4. Viral Assembly and Egress

Viral capsids accumulate and assemble in the infected cell nucleus and viral DNA is then inserted in an energy-dependent process (Conway and Homa, 2011; Dasgupta and Wilson, 1999; Vlazny et al., 1982). Egress of the mature nucleocapsid requires crossing cellular membranes to acquire the viral envelope in a process that is still not fully defined, however, resulting virus eventually egresses the infected cell and enters the extracellular space for subsequent rounds of lytic infection or progression to latent infection (Granzow et al., 2001; Johnson and Baines, 2011; Skepper et al., 2001; Wild et al., 2005).

1.3. Latent Infection and Reactivation

1.3.1. Viral Transcription in Latent Infection

In latent infection, the only transcripts that accumulate abundantly are microRNAs and the latency-associated transcripts (LATs) (Stevens et al., 1987; Umbach et al., 2008). LAT expression has not been detected in all latently infected
neurons, and \textit{in situ} hybridization experiments estimate that only a third of all latently infected cells accumulate LATs (Chen et al., 2002b; Maggioncalda et al., 1996; Mehta et al., 1995; Ramakrishnan et al., 1994). Differences in viral genome copy number in neuronal subpopulations suggest that viral genomes at high copy number localize near centromeric chromatin and express LATs, while neurons containing low-copy numbers of viral genomes are silenced within PML nuclear bodies, and do not express LAT (Catez et al., 2012). However, a recent single cell analysis of latent infection detected LAT expression in nearly all latently infected neurons after \textit{in situ} qRT-PCR amplification, and may reflect differences in latent infection or experimental sensitivity (Ma et al., 2014).

The LAT transcripts originate from a neuron specific promoter and include the primary \~8.3 kbp LAT, from which is spliced a stable 2.0 kbp intron and a smaller 1.5 kbp intron (Batchelor and O'Hare, 1990; Farrell et al., 1991; Stevens et al., 1987; Zwaagstra et al., 1990) (Figure 1.1B-C). Only low levels of the full-length primary LAT RNA are detected in neurons (Wagner et al., 1988); however, the spliced introns possess an unusually stable lariat structure that facilitates their abundant nuclear accumulation (Farrell et al., 1991). Efficient expression of LAT transcriptional unit requires the upstream regulatory promoter sequences that span the approximately 800 base-pairs (bp) upstream of the transcriptional start site (Dobson et al., 1989), as well as a downstream enhancer element that ensures long-term promoter activity during latency (Lokensgard et al., 1997; Lokensgard et al., 1994). Currently, no strong
evidence exists for protein expression from the LAT, so functions are attributed to forms of the LAT transcript itself (Drolet et al., 1998).

The actual function of the LATs has been a source of much speculation and debate. Many attempts have been made to resolve their role in the establishment, maintenance, or reactivation from latent infection. Infection with viral LAT-mutants resulted in increased neuronal death, more severe trigeminal tissue pathology, and ultimately increased mortality (Leib et al., 1989a; Thompson and Sawtell, 2001; Wang et al., 2005). LAT expression has, therefore, been associated with preventing neuronal cell death (Bloom, 2004), potentially by inhibiting apoptosis (Ahmed et al., 2002; Perng et al., 2000a; Thompson and Sawtell, 2000), protecting from CD8+ T-cell killing (Jiang et al., 2011), or inhibiting dendritic cell maturation (Chentoufi et al., 2012), to ultimately promote survival and enhance long-term latency (Perng et al., 2000b).

Many studies have suggested that the primary function of LATs is to mediate suppression of viral lytic genes so as to establish a latent gene expression profile and evade immune detection. Viral LAT-mutants exhibit elevated lytic gene expression in neurons during acute (Garber et al., 1997) and latent infection of mice (Chen et al., 1997), and LAT expression in trans in cultured cells reduced IE gene expression (Mador et al., 1998). However, it should be noted that some studies have observed LAT mediated enhancement of lytic gene expression (Giordani et al., 2008). Additionally, expression of LATs during latent infection promotes the assembly of repressive heterochromatin on viral lytic gene promoters, further supporting the role of LATs in suppression of viral transcription (Cliffe et al., 2009; Wang et al., 2005).
The overlap of the LAT with important lytic genes including *ICP0, ICP34.5*, and *ICP4*, has suggested an antisense-mediated mechanism of repression. Recently, as many as 27 HSV-1 microRNA (miRNA) species have been identified, many of which are encoded within the LAT sequences (Du et al., 2015; Jurak et al., 2010; Umbach et al., 2008; Umbach et al., 2009) (Figure 1.1D). Primary LAT, or other transcripts originating from the LAT region could be the priRNA precursor to regulatory miRNAs that suppress lytic infection by virtue of their perfect complementarity to essential lytic genes (Jurak et al., 2011). Indeed several of these miRNAs have been shown to be capable of binding RISC, targeting viral mRNAs, and downregulating lytic gene expression (Flores et al., 2013; Umbach et al., 2009). Additionally, it has been hypothesized that viral miRNAs could target cellular gene expression to facilitate latent infection; however, specific targets have not been identified (Flores et al., 2013; Grey, 2014; Umbach et al., 2009).

1.3.2. Establishment of Latent Infection

After primary infection at the mucosal epithelium, the virus can spread to innervating sensory neurons through fusion at the axonal termini and retrograde transport of the nucleocapsid to the neuronal cell body within a ganglion (Lycke et al., 1984; Penfold et al., 1994). Within the neural tissue of animal models an initial acute phase of viral lytic gene expression accompanied by a transient accumulation of viral IE and E gene transcripts occurs during the first 24-72 hpi (Garber et al., 1997; Kosz-Vnenchak et al., 1990; Kramer et al., 1998; Valyi-Nagy et al., 1991). However expression of viral lytic genes is limited and becomes progressively reduced in the days
and weeks following infection. Transcription from the LAT region also begins during the acute phase of infection in the trigeminal ganglia (Kramer et al., 1998). However, unlike lytic genes, LAT expression continues and accumulates during the establishment of latent infection (Stevens et al., 1987).

The cells within trigeminal ganglia are a diverse population including neuronal support cells, as well as dozens of distinct neuronal populations that have been characterized and distinguished based on their surface markers and receptor expression. Herpes simplex viruses appear to show preference for the establishment of latency within specific neuronal subtypes (Yang et al., 2000). A subset of neurons that bind to a monoclonal antibody specific to the A5 receptor act as a primary latent reservoir for HSV-1 during in vivo mouse infection (Imai et al., 2009; Yang et al., 2000). During in vitro infection A5-positive neurons are relatively non-permissive to HSV-1 but are supportive of productive HSV-2 infection (Bertke et al., 2011). Interestingly, HSV-2 preferentially establishes latency in a different neuronal subset that is KH10-positive, and non-permissive to HSV-2 infection in vitro but permissive to HSV-1 (Bertke et al., 2012; Bertke et al., 2013; Margolis et al., 2007). Neuronal subtype selection has been associated with cis-acting elements within the LAT region supporting the concept that the LAT sequences regulate the establishment of latency (Bertke et al., 2012; Bertke et al., 2013).

The mechanism underlying the progression from active lytic gene expression to silencing during latency has yet to be clearly defined. Previous hypotheses have suggested neurons could lack a necessary transcription factor to bind IE gene
promoters and stimulate initiation of the lytic gene cascade (Hagmann et al., 1995; Valyi-Nagy et al., 1991), or could express a transcriptional repressor that would block access to viral gene promoters (Akhova et al., 2005; Kemp et al., 1990; Lillycrop et al., 1991). Other hypotheses have suggested that HCF localization in neurons could be cytoplasmic rather than nuclear (Kristie et al., 1999), making it unable to transactivate viral gene expression and unable to facilitate the transport the viral transactivator VP16 to the nucleus (Boissière et al., 1999; Sears et al., 1991). Finally, the kinetics and features of LAT expression has focused speculation on these transcripts as likely candidates responsible for the progression towards latency (Garber et al., 1997). However, LAT expression is not absolutely required for the establishment of latent infection (Javier et al., 1988; Leib et al., 1989a; Steiner et al., 1989). Additionally, while some studies with LAT-negative mutant viruses have detected reduced numbers of neurons able to establish latent infection (Sawtell and Thompson, 1992a; Thompson and Sawtell, 1997), others have not (Javier et al., 1988; Leib et al., 1989a; Steiner et al., 1989). Finally, epigenetic regulation is also believed to play an integral role in latent infection, and its role in the suppression of viral lytic gene expression will be discussed further in subsequent sections.

1.3.3. Maintenance of Latent infection

Within the sensory ganglia, the virus establishes a persistent latent infection that is maintained for the lifetime of the host. Latent infection is classically defined by the lack of infectious virus detectable in the ganglionic tissue, accompanied by the ability of
infectious virus production upon explant and co-cultivation with permissive cells (Leist et al., 1989). Latency can be established in neurons with or without prior lytic replication in the ganglia (Leib et al., 1989a; Stevens et al., 1987). During latency, the viral genome exists as a circular episome that is associated with a regular nucleosomal array (Deshmane and Fraser, 1989). Among latently infected neurons, the viral genome load per neuron is highly variable. While most neurons contain 10-100 copies of the HSV-1 genome, a small population of neurons can contain over 1000 viral genomes (Sawtell, 1997). Lytic gene expression is repressed, and the virus does not engage in active replication. Previously, it has been hypothesized that the initial expression of lytic viral genes or expression of LATs occurs within mutually exclusive neuronal cell populations, leading to the suggestion that neurons expressing lytic genes die and are cleared, while the population expressing LATs remain and establish latent infection (Margolis et al., 1992; Speck and Simmons, 1991). However, low levels of lytic transcripts can be detected in latent infection (Kramer and Coen, 1995; Pesola et al., 2005; Speck and Simmons, 1991). These transcripts have been attributed to rare neurons with high levels of ongoing lytic transcription (Feldman et al., 2002), or to low levels of lytic transcription preceding but failing to surpass the threshold for reactivation (Kramer and Coen, 1995). Interestingly, more recent advances to techniques detecting transcripts at the single-cell level suggest that almost all infected neurons initially undergo some lytic gene transcription (Proença et al., 2008), and that lytic transcription is ongoing in a majority of latently infected LAT-expressing neurons (Ma et al., 2014). Surprisingly, even transcripts of the potent viral lytic gene transactivator, ICP0, can be detected during
latency at low levels independently of spontaneous reactivation events (Chen et al., 2002a; Maillet et al., 2006). The incomplete silencing of lytic genes suggests ongoing active and dynamic regulation to prevent lytic transcripts from initiating progression to productive infection during inappropriate circumstances while permitting reactivation when advantageous.

1.3.4. Reactivation from Latent Infection

Periodic viral reactivation is triggered by various stimuli such as stress or neuronal damage and can occur for the lifetime of the host. Some studies have detected reduced explant reactivation from neurons infected with LAT-negative viruses, and suggest that LATs facilitate reactivation (Bloom et al., 1996; Hill et al., 1990; Leib et al., 1989a; Perng et al., 1994). However, the role of LATs in reactivation is unclear. During reactivation, the levels of LATs and viral miRNAs decrease while lytic gene transcripts simultaneously accumulate, eventually leading to viral replication and production of viral progeny (Du et al., 2011; Kosz-Vnenchak et al., 1993; Spivack and Fraser, 1988). Subsequently, virions or virion components are carried by anterograde transport down neuronal axons, and infectious virus is released at the axonal termini (Antinone et al., 2010; Wisner et al., 2011). From here the virus gains access to permissive peripheral tissue, generally near the site of primary infection and can resume lytic replication. Reactivation is of clinical significance because it can result in painful recurrent lesions, blindness in the case of ocular reactivation, and transmission to new susceptible hosts (Roizman et al., 2013). Shedding of reactivated virus can occur even in the absence of
clinical symptoms, increasing the difficulty of prevention, as infected individuals may not know they are infected and able to spread disease (Wald et al., 2000).

1.3.5. Experimental Models of Latent infection

Many model systems have been and are currently used in the field of HSV latent infection. While lytic infection is commonly studied using cell culture models, the quiescent infection that predominates at the neuronal site of HSV infection is difficult to mimic in vitro. Cell culture models of latency generally require the use of HSV inhibitors or replication-defective viral mutants to inhibit productive infection (Arthur et al., 2001; Wilcox and Johnson, 1988). These artificial manipulations can result in quiescent infection that is not necessarily representative of true in vivo latency. Additionally, in vitro tissue culture infections generally lack neuronal subset diversity, ganglia architecture, and immune response effectors such as macrophages and CD8+ T cells factors that may influence latent infection (Liu et al., 2000; Shimeld et al., 1995; Yang et al., 2000). Therefore, the use of small animal systems to model latent infection has been essential to our understanding of latency, despite that these animals are not naturally hosts for HSV infection.

There are currently three main animal models used to study latent HSV infection. The guinea pig model is useful for studying HSV-2 genital infection for its ability to closely simulate the pathology associated with acute infection as well as recurrent disease (Stanberry et al., 1982). To study HSV-1, rabbit and mouse models are used most commonly. Ocular infection results in primary lytic replication that peaks within 48
hours followed by decline, while transport of HSV-1 to the trigeminal ganglia is detectable one day after infection and proceeds to the establishment of latent infection (Knotts et al., 1974). The rabbit model of infection is able to mimic spontaneous reactivation of HSV-1 that is characteristic of human infection; however, their large size, prohibitive cost, and the limited availability of transgenic strains make the rabbit a less practical model (Webre et al., 2012). The advantages of the mouse system include their reduced expense, the extensive characterization and availability of inbred and transgenic strains, and the diversity of useful molecular and immunological reagents (Kollias et al., 2014; Webre et al., 2012). However they do not exhibit spontaneous reactivation; therefore, reactivation studies rely on induction through ex vivo explant (Stevens and Cook, 1971), or in vivo trauma (Hill et al., 1978), pharmacological manipulation (Willey et al., 1984), exposure to UV irradiation (Laycock et al., 1991), or hyperthermic stress (Sawtell and Thompson, 1992b).

1.4. Epigenetic Regulation and Herpes Simplex Virus

1.4.1. Limiting the Host Chromatin Response in Lytic Infection

Nucleosome assembly on foreign DNA serves as an intrinsic host defense mechanism to silence incoming foreign genetic material, and this consequently occurs rapidly after the introduction of naked viral DNA or transfected plasmid DNA into the nucleus (Cereghini and Yaniv, 1984). The HSV-1 capsid contains the viral genome but
lacks histones; therefore, the viral genome enters the cell as naked DNA (Homa and Brown, 1997; Pignatti and Cassai, 1980). Upon entry into the nucleus, chromatin assembly on the viral episome begins rapidly and can be detected within the first few hours of infection (Cliffe and Knipe, 2008; Oh and Fraser, 2008). Nuclease digestion studies of the viral genome during lytic infection do not detect abundant nucleosome association or the nucleosomal laddering characteristic of an ordered array of regularly spaced nucleosomes (Kent et al., 2004; Leinbach and Summers, 1980; Lentine and Bachenheimer, 1990). Therefore, it appears that cellular response is never able to fully chromatinize the viral genome during lytic infection. However, some studies suggest that chromatin association may be less stable, and nucleosomes may be only loosely associated and difficult to detect (Lacasse and Schang, 2010). Post-translational covalent modifications to histone tails also contribute to transcriptional regulation (Jenuwein and Allis, 2001). Studies using chromatin immunoprecipitation (ChIP) techniques have revealed that as productive infection progresses, histone H3 accumulation at lytic viral promoters is reduced (Cliffe and Knipe, 2008; Herrera and Triezenberg, 2004), and remaining histones are enriched for markers of active euchromatin, such as histone H3 lysine 4 trimethylation (H3K4me3) (Huang et al., 2006; Kent et al., 2004) or histone H3 lysine 9 or 14 acetylation (H3K9ac or H3K14ac) (Cliffe and Knipe, 2008; Herrera and Triezenberg, 2004; Kent et al., 2004).

Many host cellular factors function in recruitment and regulation of chromatin on to the HSV genome. In addition to covalent modification of histone tails, the positioning of nucleosomes, and the incorporation of histone variants provide an additional layer of
regulatory control. The chromatin remodeler SNF2H is required for expression of IE genes including ICP0 (Bryant et al., 2011). Conversely, in HeLa cells, depletion of the chromatin remodeler CHD3 increases viral IE gene expression, implicating CHD3 in repression and silencing (Arbuckle and Kristie, 2014). Repressive effects in early infection of HeLa cells have been demonstrated by the histone chaperone anti-silencing factor1a (Asf1a) (Oh et al., 2012), while the Asf1b chaperone promotes viral DNA synthesis (Peng et al., 2008). Additionally, the selective incorporation of histone variants has been demonstrated in HSV-1 infection, and viral IE gene promoters can exchange histone H3 for the histone variant H3.3 to facilitate transcription (Placek et al., 2009). The effects of these factors in both promoting and suppressing viral gene transcription during lytic infection demonstrate the highly specific chromatin environment that HSV-1 must maintain.

Mechanistically, control of the cellular chromatin assembly response and prevention of viral gene silencing during lytic infection have thus far largely been attributed to the HSV-1 viral proteins VP16 and ICP0. VP16 (U_{48}) enters with the virion as part of the tegument and is therefore present throughout the entire course of infection. Once released from the tegument, VP16 forms a transactivator complex with cellular proteins HCF-1 and Oct-1 that binds to response elements in IE genes (Gerster and Roeder, 1988; Kristie et al., 1989). The VP16-HCF-1-Oct1 complex activates IE gene expression by recruiting transcription factors to promote the formation of the RNAPII pre-initiation complex, and by regulating the epigenetic environment at the promoters of IE genes (Kristie, 2007). VP16 recruits Set1 and MLL1 histone
methyltransferases to promote euchromatic H3K4me3 modifications (Narayanan et al., 2007), while also recruiting the LSD1 H3K9me1/2 demethylase (Liang et al., 2009) and the JMJD H3K9me3 demethylase (Liang et al., 2013b) to reduce heterochromatic modifications. (Liang et al., 2013a). VP16 also recruits histone acetyl transferases (HATs) such as p300 and cAMP response element binding (CREB)-binding protein (CBP), as well as nucleosome remodelers such as BRG1 and BRM, the human orthologs of the yeast SWI/SNF complex, to viral IE promoters (Herrera and Triezenberg, 2004). Consequently, viruses lacking the transactivation domain of VP16 accumulate histones at IE gene promoters, and demonstrate reduced levels of acetylated histones associated with E gene promoters (Herrera and Triezenberg, 2004; Kutluay and Triezenberg, 2009b). Furthermore, VP16 is involved in intranuclear positioning of HSV-1 genomes, and targeting of HSV-1 to the nuclear periphery through association with nuclear lamin A reduces heterochromatin formation on viral genes and increased IE gene expression (Silva et al., 2008; Silva et al., 2012).

ICP0, an IE viral protein, is an E3 ubiquitin ligase and promiscuous transactivator with multiple roles in promoting gene expression and limiting host antiviral defense. While not essential, infections of most cell types require ICP0 for efficient gene expression when infected at low multiplicities of infection (Chen and Silverstein, 1992; Sacks and Schaffer, 1987; Stow and Stow, 1986). A number of mechanisms have been reported to contribute to the regulation of viral chromatin by ICP0. Defects caused by the loss of ICP0 can be partially restored by treatment with histone deacetylase (HDAC) inhibitors trichostatin A (Hobbs and DeLuca, 1999) and sodium butyrate (Poon et al.,
2003), suggesting a role for ICP0 in opposing HDACs to counter the cellular epigenetic silencing response. To prevent silencing and facilitate gene expression through acetylation modifications, ICP0 can bind to and displace components of the cellular repressor complex comprised of HDAC1/2, lysine-dependent demethylase 1 (LSD1), RE1 silencing transcription factor (REST), and co-repressor to REST (CoREST) (Gu et al., 2005; Zhou et al., 2013; Zhou et al., 2011). Replacement of ICP0 with a dominant negative CoREST that can bind to REST but not HDAC1 enhanced viral yield in a low PFU/cell infection (Gu and Roizman, 2007). Additionally, a mutant virus encoding an ICP0 that cannot bind to CoREST demonstrated reduced ability to reactivate HSV-1 from quiescent infection (Ferenczy et al., 2011). Furthermore, transfected ICP0 can form a complex with class II histone deacetylases, HDAC5, HDAC6, and HDAC7, to limit their activity (Lomonte et al., 2004). These studies support the hypothesis that ICP0 is functionally significant as an HDAC inhibitor to counter host-mediated repression.

Studies of quiescent infection also indicate that ICP0 can prevent accumulation of H3K9me3 and HP1\(^\gamma\) heterochromatin (Ferenczy and DeLuca, 2009) and reverse association with these heterochromatin markers when provided in trans (Ferenczy and DeLuca, 2011). Several regions of ICP0, including the RING-finger domain, which exhibits E3 ubiquitin ligase activity (Boutell et al., 2002; Everett, 2000), and the C-terminal domain, which is involved in the disruption of the HDAC1/2/LSD-1/REST/CoREST complex (Gu and Roizman, 2007), contribute to the efficient removal of heterochromatin (Ferenczy et al., 2011). However, the mechanism of this activity has not yet been defined.
As well as countering the host epigenetic silencing response, ICP0 can also directly promote active gene expression. Chromatin immunoprecipitation experiments demonstrate that the introduction of ICP0 increases histone acetylation at viral gene promoters during quiescent infection (Coleman et al., 2008), and that ICP0 reduces total histone H3 accumulation and promotes histone acetylation during the progression of lytic infection of HeLa cells (Cliffe and Knipe, 2008). To increase accumulation of euchromatin, ICP0 can bind to BMAL1 (Kawaguchi et al., 2001). BMAL1, in turn, binds and stabilizes the CLOCK histone H3/H4 acetyltransferases (HATs) to promote accumulation of active acetylation modifications to histones on the viral genome (Kalamvoki and Roizman, 2010; Kalamvoki and Roizman, 2011).

Furthermore, rather than directly interacting with histone modifying proteins, as an E3 ubiquitin ligase, ICP0 can target cellular proteins for ubiquitination and subsequent degradation by the proteasome. Incoming HSV DNA is targeted by host protein promyelocytic leukemia (PML), which forms repressive nuclear bodies, also called nuclear domain 10 (ND10), in association with other proteins such as Sp100 and hDaxx that are associated with epigenetic silencing (Everett and Murray, 2005; Kim et al., 2011; Shalginskikh et al., 2013). However, ICP0 degrades PML and Sp100 and disrupts the formation of ND10 to counter host repression and facilitate the transition from IE to E gene expression (Chelbi-Alix, 1999; Everett et al., 2006). This mechanism also appears particularly effective at countering the intrinsic antiviral epigenetic silencing response. In HFF cells, the cellular DNA-sensor IFI16 promotes the accumulation of the heterochromatin modifications H3K9me3 on any incoming unchromatinized foreign
DNA, including incoming viral HSV genomes; however, it is degraded by ICP0 (Orzalli et al., 2013). This serves as an indirect mechanism of ICP0-dependent regulation of histone methylation.

Finally, several other viral proteins have been implicated in manipulating the viral epigenetic landscape during lytic infection. The Us3 protein is a serine-threonine protein kinase that may prevent silencing through its ability to phosphorylate HDAC1, HDAC2, and COREST to inactivate them (Everett, 1984; Gelman and Silverstein, 1985). Gene expression from the baculovirus is facilitated by the ability of Us3 to prevent histone deacetylation (Poon et al., 2006). Additionally, ICP8 can recruit chromatin-remodeling complexes to replication compartments and on to progeny DNA (Taylor and Knipe, 2004), which may facilitate the stimulation of L gene expression by ICP8 (Gao and Knipe, 1991). The variety and redundancy of viral proteins that target cellular epigenetic factors support the importance of chromatin regulation during lytic infection.

1.4.2. Chromatin in Latent HSV-1 Infection

Unlike lytic infection, which is characterized by sparse or loosely associated nucleosomes on the viral genome, latent infection of HSV-1 is known for the accumulation of ordered nucleosomes forming a regular nucleosomal array (Deshmane and Fraser, 1989). The nucleosomes associated with viral DNA are modified with silencing heterochromatic markers such as H3K9me2, H3K9me3, and H3K27me3 at viral lytic gene promoters, and consequently lytic gene expression is highly repressed (Cliffe et al., 2009; Kubat et al., 2004b; Kwiatkowski et al., 2009; Wang et al., 2005).
Interestingly, despite the high GC content of HSV-1, current evidence indicates that DNA methylation does not occur on the viral genome and does not play a role in silencing lytic gene expression (Dressler et al., 1987; Kubat et al., 2004b). The contrasting chromatin profile of viral genomes during lytic and latent infection has led to models in which a “chromatin switch” is a major determinant of the transition between lytic and latent infection (Knipe and Cliffe, 2008). HSV-1 is therefore believed to exploit cellular chromatin repression of viral lytic genes as a means to evade immune detection and persist for the lifetime of the host, while also maintaining the flexibility to restore active gene transcription during situations advantageous to viral spread.

The only transcripts expressed abundantly during latency originate from the LAT region (Stevens et al., 1987), and the LAT promoter is the only region identified that accumulates markers of active euchromatin, such as histone acetylation, in addition to the heterochromatin markers that are seen in other regions of the viral genomes (Kubat et al., 2004a). While expression of the LAT transcript is not necessary for establishment of latency, it has been demonstrated to promote H3K27me3 modifications on viral chromatin (Cliffe et al., 2009; Wang et al., 2005). No strong evidence for protein encoded by the LATs has been identified; however, long non-coding RNAs are known to mediate heterochromatin assembly and maintenance (Drolet et al., 1998).

In lytic infection, histone accumulation begins rapidly post entry and decreases throughout the progression of infection (Cliffe and Knipe, 2008). Kinetic experiments describing the establishment phase of latency demonstrate very different kinetics of chromatin association. Viral genomes within the trigeminal ganglia (TGs) of mice show
histone H3 accumulation by 7 dpi (Cliffe et al., 2013; Wang et al., 2005), but accumulation of the repressive H3K9me2 (Wang et al., 2005) and H3K27me3 (Cliffe et al., 2013) modifications occurred later in the period of 7 to 14 dpi. Additionally, Suz12, a component of the PRC2 complex also increased on lytic promoters by 14 dpi (Cliffe et al., 2013). Suz12 recruitment was surprisingly independent of LAT expression, indicating that perhaps additional viral factors are involved in maintaining heterochromatin modifications. Additionally, the PRC1 component Bmi1 was enriched less than 2-fold relative to cellular control in the LAT region, suggesting that maintenance of heterochromatin modifications may not be maintained by PRC1 (Cliffe et al., 2009; Kwiatkowski et al., 2009).

Explant reactivation studies in vitro have provided further support for the epigenetic switch model. After induction of reactivation, the levels of LAT transcript and miRNAs are reduced (Du et al., 2011; Kosz-Vnenchak et al., 1993; Spivack and Fraser, 1988) and the histones associated with the LAT region become deacetylated (Amelio et al., 2006a). Conversely, lytic gene transcripts accumulate (Devi-Rao et al., 1994; Kosz-Vnenchak et al., 1993), and histones associated with lytic gene regions become progressively associated with acetylated histones (Amelio et al., 2006a). These studies suggest an opposing relationship between histone modifications and transcript levels from the LAT and lytic gene elements may drive the transition from latent to productive infection. However, it remains to be determined whether changes to this epigenetic code precede and facilitate transcriptional changes, or whether transcription of viral genes is required to initiate histone modification.
Pharmacological manipulation of latent infection has also provided support for the model of epigenetic control of the lytic to latent transition of HSV. Treatment of mice with an HDAC inhibitor, butyrate, results in acetylation of histones on lytic genes and reactivation of the virus (Neumann et al., 2007). Inhibition of the LSD1 histone demethylases of H3K9me/me2 increased repressive histone modifications at IE gene promoters, reduced IE gene and protein expression, and inhibited explant reactivation (Liang et al., 2009). Inhibition of the JMJD2 H3K9me3 demethylase also reduced viral yield in explant reactivation and inhibited IE gene expression with concurrent increases in H3K9me3 accumulation (Liang et al., 2013b). Similar results upon inhibition of H3K27me3 demethylases JMJD3 and UTX suggest that all three types of histone modifications are important for suppression of viral gene expression during latent infection (Hill et al., 2014). Significantly, in studies using various animal models, increasing epigenetic suppression through inhibition of LSD1 also reduced in vivo recurrence and viral shedding during long term latency, even surpassing the efficacy of current antiviral treatments (Hill et al., 2014). Overall, these studies highlight the importance of understanding the mechanism of epigenetic suppression and the maintenance of latent infection as a means to control recurrent infection, and prevent spread of the disease.
1.5. The CCCTC-binding Factor and HSV-1

1.5.1. The CCCTC-binding factor (CTCF)

CTCF is an 11-zinc-finger DNA-binding cellular protein that is essential, multifunctional, ubiquitously expressed, and highly conserved among species ranging from Drosophila to humans (Banahmad et al., 1990; Lobanenkov et al., 1990). Genome-wide mapping of CTCF binding sites have identified as many as 65,000 sites in mammalian genomes, at both inter- and intra-genic sites, and with unique cell- and tissue-type specific distribution patterns (Barski et al., 2007; Chen et al., 2012a; Cuddapah et al., 2009; Kim et al., 2007).

CTCF, also initially called negative protein 1 (NeP1), was initially classified as a transcriptional repressor from two independent lines of investigation at MYC promoters and the chicken lysozyme silencer (Banahmad et al., 1990; Köhne et al., 1993; Lobanenkov and Gudvin, 1988; Lobanenkov et al., 1990). Isolation and characterization proved that CTCF and NeP1 were identical, despite their highly divergent DNA binding motifs (Burcin et al., 1997). Subsequently, CTCF was also independently identified binding to sites within the amyloid precursor protein (APP) gene promoter and the HS4 region of the chicken β-globin locus (Quitschke et al., 1996; Walters et al., 1999). CTCF consequently emerged as a highly multifunctional transcription factor, capable of binding to diverse yet specific target sequences using different combinations of its zinc-fingers, to act variously as a transcriptional repressor, activator, enhancer-blocker, or insulator (Bell et al., 1999; Filippova et al., 1996; Klenova et al., 1993).
Importantly among these functions, CTCF is the only identified vertebrate insulator protein (Felsenfeld et al., 2004). Insulators are boundary elements that maintain distinct regulatory control of adjacent genetic elements. CTCF-bound insulators can prevent the linear spread of heterochromatin modifications, particularly H3K27me3, from regions of silenced chromatin to neighboring active regions, and can restrict the polarity of bidirectional enhancer elements, to prevent inappropriate activation of nearby promoters (Bell et al., 1999; Chung et al., 1993; Hark et al., 2000; Kim et al., 2007; Schwartz et al., 2012). This allows regions of active transcription and euchromatin modification to exist in close proximity to regions of silenced transcription and heterochromatin, and also contributes to X chromosome inactivation, gene imprinting, and monoallelic gene expression (Fedoriw et al., 2004; Filippova et al., 2005; Lee, 2003; Ling et al., 2006).

The ability to bind diverse yet specific targets also underlies the mechanism by which CTCF mediates multiple functions. In addition to recognizing a variety of sequence motifs, CTCF also recruits and binds to many proteins, and therefore acts as the bridge between genetic elements and cellular effectors. CTCF can directly regulate transcription through RNA polymerase II recruitment and pausing to affect RNA splicing and intron selection (Shukla et al., 2011). CTCF can also affect transcription through epigenetic regulation by recruiting self-PARyalted PARP1 to inhibit CpG methylation (Michele et al., 2012). CTCF also has multiple mechanisms to regulate nucleosomes, such as recruiting histone-modifying proteins including the SNF2-like chromodomain helicase protein (CHD8), the co-repressor of histone deacetylases, Sin3A, or the PRC2 complex component, Suz12 (Ishihara et al., 2006; Li et al., 2008; Lutz et al., 2000).
CTCF can also act as an anchor point for histone binding to regulate the specific positioning and downstream phasing of nucleosomes (Fu et al., 2008). Using this mechanism, CTCF can mediate which downstream sequences are exposed in the space between nucleosomes and therefore available for interaction with transcription factors and other host proteins. Additionally, by positioning nucleosomes, CTCF can create gaps in the chain of regularly spaced nucleosomes across which histone-modifying proteins cannot traverse, thereby disrupting the linear spread of heterochromatin. Increasingly, it has emerged that a main function of CTCF is to regulate the topology of genomes (Handoko et al., 2011; Ong and Corces, 2014; Splinter et al., 2006). CTCF can interact with structural elements such as cohesins and lamin A/C, and can also homodimerize to form loops between two CTCF bound sites on the genome. These interactions can establish physical barriers by creating isolated chromatin loop domains, mediate long-range inter- or intra-chromosome interactions, and organize chromatin positioning within the three dimensional nuclear space.

1.5.2. CTCF and Herpesviruses

In addition to vertebrate genomes, CTCF binding sites have also been identified within herpesviral genomes. CTCF association with gammaherpesvirus Epstein-Barr virus (EBV) occurs at several regulatory regions including upstream of the \( C_p \), \( W_p \), \( EBERs \), and \( Q_p \) gene promoters, and also forms a loop between the \( OriP \) and the control region for the latency membrane proteins LMP1 and LMP2A (Arvey et al., 2012; Chau et al., 2006; Day et al., 2007; Holdorf et al., 2011; Tempera et al., 2010). CTCF
accumulation at the \( Cp \) region has been associated with repression of EBNA2 transcription and establishment of restricted latency types (Chau et al., 2006; Hughes et al., 2012; Salamon et al., 2009). The \( Qp \) promoter is essential in type I latency for maintenance of EBNA1 transcription and viral genomes; thus, disruption of the \( Qp \) CTCF sites results in silencing at \( Qp \) with H3K9 methylation and CpG DNA methylation (Tempera et al., 2010). Disruption of the CTCF binding site at the intron of \( LMP2A \) and 3' UTR of \( LMP1 \) leads to elevated episomal copy number, reduction of latent \( LMP1/LMP2A \) transcription, and a corresponding reduction in euchromatic H3K9ac and increase in heterochromatic H3K9me3 at the \( LMP2A \) promoter (Chen et al., 2014). In another gammaherpesvirus, KSHV, CTCF is involved in coordinating lytic and latent gene control, restricting lytic transcription and replication, and regulating latent transcription through histone modifications, nucleosome phasing, and RNA polymerase II recruitment and programming (Chen et al., 2012b; Kang et al., 2013; Kang et al., 2011; Li et al., 2014; Stedman et al., 2008). Interestingly, in the distantly related betaherpesvirus HCMV, infection with HCMV initially induced higher CTCF mRNA levels, and CTCF binding within the intron of the major immediate early gene (MIE) affected RNA polymerase II leading to repression of MIE expression and viral replication (Martinez et al., 2014). Cumulatively these studies suggest that herpesviruses have evolved to co-opt cellular CTCF functions to regulate epigenetic modifications and differential viral gene expression profiles associated with different phases of infection.

Among the alphaherpesviruses, at least seven CTCF binding regions have been identified in HSV-1 and are associated with CTCF during latent infection (Amelio et al.,
2006b; Chen et al., 2007). One such site, named *CTRL2*, is located in the repeat-long region of the HSV-1 genome within the 2.0 kbp *LAT* intron, positioned between the *LAT* and *ICP0* promoter regions. The other CTCF binding sites are positioned between each of the IE genes and the *LAT* promoter/5' exon, isolating each into a unique domain. In transgene assays, HSV-1 sequences that include the *CTRL2* site are capable of enhancer blocking, silencing, and preventing the spread of heterochromatin (Amelio et al., 2006b; Chen et al., 2007). Additionally, CTCF association with the *CTRL2* site, as well as the nearby CTCF binding sites upstream of the *ICP0* and *ICP4* promoters is disrupted after induction of reactivation (Ertel et al., 2012). These results suggest that in HSV-1, CTCF binding may also regulate transcriptional control of the *LAT* and IE genes to facilitate latent infection.
Contributions: I constructed the \( r212R, \Delta Prom, \) and \( PromR \) HSV-1 viral mutants. I performed subsequent infections, ChIP experiments, and data analyses in collaboration with Priya Raja.
2.1. ABSTRACT

HSV-1 is a double-stranded DNA virus that must contend with a cellular chromatinization response upon entry into a permissive cell nucleus. In this study, we demonstrated that unchromatinized HSV-1 genomes enter primary human foreskin fibroblasts and are rapidly subjected to assembly of nucleosomes and association with repressive heterochromatin modifications such as H3K9me3 and H3K27me3 during the first one to two hours post infection. Kinetic analysis of the association of histones and heterochromatin modifications over the course of lytic infection demonstrated a progressive removal that coincided with initiation of viral gene expression. We also evaluated the role of the viral IE protein, ICP0, in regulating nucleosome assembly or removal, and in the regulation of specific heterochromatin modifications localized to viral gene promoters. Using two HSV-1 ICP0-mutant virus strains, we demonstrated that ICP0 did not affect the initial assembly of nucleosomes or association with heterochromatin modifications, because the levels of accumulation were similar among WT and ICP0-mutant HSV-1 strains. However, both ICP0-mutant viruses were unable to remove histones and reduce heterochromatin modifications during the subsequent twelve hours post infection, coinciding with delayed and reduced accumulation of viral gene products and an absence of viral replication. Overall, these results indicated that the virus must overcome silencing by host-cell-mediated repressive chromatin assembly to allow the progression through the lytic infection cycle. HSV-1 has therefore evolved mechanisms to regulate viral chromatin to both counter the cellular antiviral response and direct the cascade of viral gene expression during lytic infection.
2.2. INTRODUCTION

The assembly of histones onto incoming naked DNA appears to have evolved in eukaryotic cells as an intrinsic response to silence foreign DNA through compaction into repressive heterochromatin (Cereghini and Yaniv, 1984). However, chromatin is a dynamic structure also integral to regulating effective gene expression. Chromatin remodelers can modulate the density and positioning of histones, while a variety of chromatin modifying factors can direct the addition or removal of specific covalent modifications to histone tails. Silenced heterochromatin is densely compacted with regularly spaced nucleosomes through association with heterochromatin protein 1 (HP1) and covalent modifications at histone tails such as histone 3 (H3) lysine 9-trimethylation (H3K9me3) and lysine 27-trimethylation (H3K27me3) (Greer and Shi, 2012; Lachner et al., 2001). Conversely, active euchromatin is associated with reduced histones that are enriched in modifications such as H3 lysine 9-aceylation (H3K9ac) or lysine 4-trimethylation (H3K4me3) (Strahl and Allis, 2000). Histone-mediated interactions also regulate many processes including transcriptional response to signaling (Badeaux and Shi, 2013; Hübner et al., 2013; Suganuma and Workman, 2013), mRNA splicing (Luco et al., 2011), DNA repair (Price and D’Andrea, 2013), and nuclear positioning of chromatin (Dion and Gasser, 2013).

The double-stranded DNA genome of HSV-1 enters the host cell as naked DNA from within the virion (Cohen et al., 1980; Knipe and Cliffe, 2008; Oh and Fraser, 2008; Pignatti and Cassai, 1980). Therefore, each round of lytic infection must contend with the cellular chromatinization response to prevent silencing, initiate the ordered cascade
of lytic gene expression, and facilitate production of infectious progeny. Previously, studies have demonstrated that as lytic infection of HSV-1 progresses viral promoters associate with unstable nucleosomes that contain histones with activating modifications (Cliffe and Knipe, 2008; Huang et al., 2006; Kent et al., 2004; Lacasse and Schang, 2012). The chromatin remodeler SNF2H is required for immediate early (IE) viral gene expression (Bryant et al., 2011), while the chromatin remodeler CHD3 has been implicated in initial repression of gene expression (Arbuckle and Kristie, 2014). Additional factors such as the histone chaperone human anti-silencing factor1a (Asf1a) and the exchange of histone variants have also been implicated in epigenetic regulation of HSV-1 (Oh et al., 2012; Placek et al., 2009). Epigenetic changes can occur as both cause and consequence of transcription, and their specific role and relative importance to viral infection has been widely debated (Henikoff and Shilatifard, 2011; Kutluay and Triezenberg, 2009b). However, the importance of chromatin regulation is supported by the presence of viral proteins, such as VP16 and ICP0, which are involved in manipulating chromatin.

The tegument protein, VP16, activates IE gene expression through interaction with cellular transcription factors HCF-1 and Oct-1, and the recruitment of chromatin methylation modifiers including SETD1A methyltransferase, LSD1 (an H3K9 di- or mono-demethylase), and JMJD2 (an H3K9me3 demethylase) (Liang et al., 2013b). Additionally, VP16 can recruit nucleosome remodelers such as BRG1 and BRM, as well as histone acetyl transferases (HATs) such as p300 and CBP to viral IE promoters (Herrera and Triezenberg, 2004; Kutluay and Triezenberg, 2009a). Accordingly, in the
absence of VP16, IE gene promoters are associated with increased histone accumulation and reduced histone acetylation at E gene promoters (Herrera and Triezenberg, 2004).

The IE viral protein, ICP0, is a multi-functional E3 ubiquitin ligase and potent gene transactivator, with the ability to modulate viral chromatin in a number of ways. Initially, ICP0 was found to promote the acetylation of viral chromatin, and reduce total histone accumulation on the viral genome during a lytic infection of HeLa cells (Cliffe and Knipe, 2008). The anti-silencing activity of ICP0 has been attributed to a variety of activities. ICP0 can prevent the removal of histone acetylations by binding to CoREST and disrupting HDAC1 binding to the HDAC1/CoREST/LSD1/REST (HCLR) repressor complex, ultimately causing relocalization of HCLR components to the cytoplasm later in infection (Gu et al., 2005; Gu and Roizman, 2007; Zhou et al., 2013; Zhou et al., 2011). ICP0 has also been implicated in promoting histone acetylation. During infection the stabilization and recruitment of CLOCK H3/H4 acetyltransferases facilitates expression of viral genes and CLOCK overexpression can compensate for defects in ICP0-deficient viruses (Kalamvoki and Roizman, 2010; Kalamvoki and Roizman, 2011). Additionally, ICP0 can indirectly affect histone methylation modifications that are associated with heterochromatin. Upon activation, the cellular DNA sensor, IFI16, can promote accumulation of silencing H3K9me3 modifications on the viral genome and other foreign unchromatinized DNA; however, IFI16 is degraded by ICP0 in infection with HSV-1 (Orzalli et al., 2013). These studies highlight the importance and diversity of targets of ICP0 in lytic infection associated with its ability to prevent chromatin-mediated silencing.
The dynamic balance between the cellular repressive chromatin response and subsequent viral ability to establish active chromatin led to the development of our hypothesis that during lytic infection early chromatin-mediated repression is followed by dynamic derepression of viral genes through the removal of histones and repressive histone tail methylations. Previous studies of HSV-1 chromatin have focused on the mainly on the accumulation of euchromatic acetylation modifications during lytic infection, while examining the role of heterochromatin in latent infection. However, several studies have identified H3K9me3 heterochromatin associated with viral genomes during initial acute infection (Liang et al., 2009; Narayanan et al., 2007), but excluded from replication compartments (Silva et al., 2008). Additionally, a reduction of H3K9me3 heterochromatin at the ICP0 promoter coincided with the expression of IE genes (Liang et al., 2009). In this study, we examined the kinetics of histone association and heterochromatin modifications H3K9me3 and H3K27me3 during lytic infection of primary human foreskin fibroblasts (HFFs). Additionally, we evaluated the role ICP0 protein by comparison to two ICP0-deficient viruses that fail to transcribe the ICP0 gene, or express only a truncated ICP0 protein. We found that during lytic infection, viral genomes were initially and rapidly repressed into compact nucleosome structures and associated with repressive heterochromatin modifications. The progression of infection correlated with derepression of viral genomes characterized by the reduction in histone association and reductions in histone methylation associated with silenced heterochromatin. The ICP0-null viruses were unable to effectively reverse host-
mediated epigenetic silencing, indicating a role for ICP0 in the removal of heterochromatin to facilitate viral gene expression.
2.3. MATERIALS AND METHODS

Cells and viruses

Primary HFF, U2OS, Vero, and HeLa cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). U2OS, Vero, and HeLa cells were maintained at 37°C in Dulbecco’s Modified Eagles Medium (DMEM; Gibco-BRL) supplemented with 5% (vol/vol) heat-inactivated bovine calf serum (BCS), 5% (vol/vol) heat-inactivated fetal bovine serum (FBS). HFF cells were maintained in 10% (vol/vol) FBS, 2 mM L-glutamine.

The HSV-1 ICP0 nonsense mutant virus, n212, contains a nonsense mutation at codon 212 of ICP0 and was constructed and described previously (Cai and Schaffer, 1989). For this study, we have constructed a corresponding wild-type (WT) rescued virus n212R by homologous recombination with a full-length ICP0 gene in a linearized plasmid with infectious viral DNA from n212 virus in U2OS cells. Progeny viruses were screened and plaque purified on Vero cells, and ICP0+ viruses were confirmed by diagnostic PCR, restriction endonuclease digestion, and western blotting for ICP0 protein.

An HSV-1 ICP0 promoter deletion mutant, ΔProm, and its corresponding WT rescued virus, PromR, were constructed by homologous recombination with the ICP0-null 7134 infectious viral DNA (Cai and Schaffer, 1989) and either an ICP0 promoter mutant plasmid or WT full-length ICP0 plasmid. The ICP0 promoter mutant plasmid, pICP0ΔProm, was constructed by digestion with Ncol and Stul restriction enzymes and subsequent ligation to create a 711 bp deletion of the ICP0 start site and upstream
promoter region. Virus isolates were screened using an agarose overlay containing X-gal to distinguish plaques that did not express β-galactosidase, and plaque-purified four times. Mutations were confirmed by diagnostic PCR and restriction endonuclease digestion, and confirmed with western blotting for ICP0 protein.

The WT HSV-1 KOS strain virus was propagated and titered on Vero cells by plaque assay. The n212, n212R, ΔProm, and PromR virus stocks were grown on U2OS cells and titered in parallel on U2OS cells by plaque assay. The expression of ICP0 mRNA was confirmed in infection with n212, n212R, and PromR viruses, but absent in ΔProm virus with qRT-PCR. The expression of LAT mRNA, which is absent in 7134 virus, was confirmed from n212, n212R, ΔProm, and PromR viruses by Northern Blotting and qRT-PCR. Finally, we verified that all rescued viruses, n212R and PromR followed similar infection kinetics to WT HSV-1 KOS strain. To test for viral replication HFF or Vero cells were infected at a multiplicity of infection (MOI) of 3 for 24 h. Cells were scraped in the medium and the entire volume was frozen, sonicated, and titrated on U2OS cells.

**Western blotting**

Cells were lysed in NuPage LDS Sample Buffer (Invitrogen), boiled, and proteins were resolved on NuPAGE 4-12% Bis-Tris Gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Perkin-Elmer Life Sciences). The membranes were blocked in a 5% skim milk solution in tris-buffered saline (TBS) with 0.1% Tween-20 (TBS-T). Membranes were incubated with antibodies specific for HSV
ICP27 (1:10,000) (Abcam), HSV ICP8 (1:10,000, 3-83) (Knipe and Smith, 1986), and GAPDH (1:10,000) (Abcam). Western blots were developed using chemiluminescence reagents to detect horseradish peroxidase signal (Luminate Forte Western HRP Substrate, Millipore) and X-ray film (Hyblot ES, Denville).

**Cell culture Infection for chromatin immunoprecipitation**

HFF cells were plated into 15 cm dishes to obtain 95% confluence on the following day, at which time they were infected at 3 PFU/cell with viruses diluted in cold phosphate-buffered saline (PBS) containing 0.1% glucose, and 1% heat-inactivated BCS. Virus was allowed to adsorb to cells for 1 h at 37°C after which cells were washed with 10 ml of PBS, then 5 ml of citric acid wash buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) (Highlander et al., 1987) to inactivate remaining bound virus, followed by three washes with 10 ml of DMEM containing 1% heat-inactivated BCS. Subsequently cells were overlaid with DMEM containing 1% heat inactivated BCS, and incubated at 37°C for the indicated length of time. For experiments testing the effects of the HSV replication inhibitor sodium phosphonoacetic acid (PAA) on infection, the viral inoculum and overlay media was prepared as above with the addition of 200 µg/ml of PAA and 10 mM HEPES throughout the adsorption and infection period as described previously (Cliffe and Knipe, 2008).
Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was conducted as described previously (Wang et al., 2005, Cliffe et al., 2008, Cliffe et al., 2009) with modifications. Cells in 15 cm plates were fixed for 15 min at 37°C in 20 ml serum-free DMEM containing 1% formaldehyde (Sigma, 36.5-38%). To quench the formaldehyde, 0.125 M glycine was added and incubated for 3 min at room temperature. Samples were subsequently rinsed three times with cold PBS. Cells were then scraped gently and collected by centrifugation in PBS containing 1 mM phenylmethanesulfonylfluoride (PMSF) at 2000 rpm for 5 min at 4°C. Cell pellets were then frozen at -80°C.

Cell pellets were resuspended in 1 ml of 1% SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1), incubated for 10 min on ice, and subsequently sonicated at 4°C in a Diagenode Biorupter for approximately 6 cycles of 5 minutes each (30 sec ON 30 sec OFF) at the high setting in 15 ml polystyrene tubes. Sonication was checked on a 1.2% agarose gel to confirm chromatin fragments of approximately 500 bp. Chromatin concentration was estimated using a nanodrop spectrometer, and 15 µg of chromatin was used per IP reaction.

IP reactions were performed in MaxRecovery tubes (Axygen) with equivalent amounts of chromatin diluted to one ml in ChIP dilution buffer (150 mM NaCl, 10 mM Na₂HPO₄, 2 mM EDTA, 1.1% Triton, 0.1% SDS) overnight at 4°C with 2.5 µg of antibody per IP reaction (H3, Abcam ab1791; H3K9me3, Abcam ab8898; H3K27m3, Active motif 39156). A negative control Rabbit IgG (Millipore, NG1893918) was used. To measure total chromatin input 10 µl (1%) and 40 µl (4%) from a 1 ml IP reaction was
retained from each sample. The IP reaction was incubated for 2 h with 20 \( \mu l \) of MagnaChIP magnetic beads at 4°C after which the beads were washed 3 times with cold low-salt buffer (150 mM NaCl, 20 mM Tris-HCL, pH 8.1, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1 mM PMSF) and 3 times with cold LiCl wash buffer (50mM HEPES, pH 7.5, 250 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% sodium deoxycholate, and 1 mM PMSF) and once with cold Tris-EDTA buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). The chromatin complexes were eluted twice and combined by adding 90 \( \mu l \) of elution buffer (1% SDS, 0.1 M NaHCO_3) and incubating at 65°C for 10 min rotation for 10 min at room temperature for a total of 180 \( \mu l \) elution volume.

To process immunoprecipitated samples and inputs for analysis, the protein-DNA crosslinks were reversed by adding 0.2M NaCl and incubating for 30 min at 95°C. To remove RNA and protein from samples, RNase digestion was carried out with 1 \( \mu l \) of RNase (1 mg/ml Ambion) for 1 h at 37°C, followed by treatment with 2 \( \mu l \) of Proteinase K (Roche) with Tris/EDTA at 45°C for 2 h. The DNA was purified using the QIAquick PCR purification kit (Qiagen) and eluted twice in 50 \( \mu l \) of elution buffer to yield 100 \( \mu l \) total eluate volume.

Quantitative PCR for DNA quantification

Real-time quantitative PCR (qPCR) was performed using the Power SYBR Green PCR Master Mix and a Prism 7300 realtime system (Applied Biosystems) according to the manufacturer’s instructions in a total reaction volume of 25 \( \mu l \) with 2.5 \( \mu l \) DNA and 100 nM of each primer as described previously (Cliffe and Knipe, 2008). Primer pair
specificity was confirmed by running a dissociation curve of the PCR products for each reaction. All DNA samples were run in duplicate, and relative copy numbers were determined by comparison with a standard curve generated from 10-fold serial dilutions of DNA isolated from HSV-infected HFF cells. The fraction of viral DNA immunoprecipitated relative to the input 1% sample was normalized to the amount of cellular GAPDH (glyceraldehyde 3-phosphate dehydrogenase) DNA immunoprecipitated relative to 1% input from the same reaction. A list of primer sequences used follows below in Table 2.1.
Table 2.1: Sequences of primers used for qPCR quantification of DNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP8 F</td>
<td>GAGACCGGGTGGGGGAATGAATC</td>
</tr>
<tr>
<td>ICP8 R</td>
<td>CCCCGGGGTTGTCTGTGAAGG</td>
</tr>
<tr>
<td>ICP27 F</td>
<td>ACCCAGCCACGTATCCACC</td>
</tr>
<tr>
<td>ICP27 R</td>
<td>ACACCATAAGTGTCGATGT</td>
</tr>
<tr>
<td>LAP F</td>
<td>CCCGGCCCGCACGAT</td>
</tr>
<tr>
<td>LAP R</td>
<td>CAACACCCCGCCGCTTT</td>
</tr>
<tr>
<td>LAT 5’ exon F</td>
<td>TTCGTTTCCCCGCTTT</td>
</tr>
<tr>
<td>LAT 5’ exon R</td>
<td>CAGACGGGTAAAGAACAGAAACC</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>CAGGCGCCCAATACGACCAAATC</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>TTCGACAGTCAGTCAGCCGCATCTTTTT</td>
</tr>
</tbody>
</table>
Viral and cellular analysis of RNA and DNA by quantitative RT-PCR

Total RNA from infected cells was extracted using the Qiagen RNeasy Kit according to the manufacturer’s directions. Samples were DNase treated using the DNA-free Kit (Ambion) and quantified by quantitative PCR (qPCR) using the Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems) with primers previously described and listed in Table 2.2 below (Cliffe et al., 2013; Cliffe et al., 2009; Orzalli et al., 2013). To distinguish the ICP0 mRNA transcripts from the overlapping LAT mRNA transcripts, we employed a strategy previously described in Cliffe et al., 2013, in which only the reverse primer was added for the RT step (30 min at 48°C) after which the reaction was paused to allow addition of the forward primer. In all other reactions both primers were added simultaneously at the start of the RT reaction. Mock samples without the addition of reverse transcriptase were included as negative controls. Viral RNA was normalized to a cellular 18S RNA control.

DNA was extracted from the same samples using the Qiagen Blood and Tissue DNeasy Kit as per the manufacturer’s instructions. Isolated DNA was then quantified by real-time PCR as described above with viral ICP8 and cellular GAPDH specific primers to measure viral genomes present within each sample.
Table 2.2: Sequences of primers used for qRT-PCR quantification of RNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP0 RNA F</td>
<td>TGCAACGCAAGCTGGTGTA</td>
</tr>
<tr>
<td>ICP0 RNA R</td>
<td>ACGATCGGGATGGTGCTGAA</td>
</tr>
<tr>
<td>LAT Intron F</td>
<td>TGTGTGGTGCCCGTGTTCTT</td>
</tr>
<tr>
<td>LAT Intron R</td>
<td>CCAGCCAAATCCGTTCGGG</td>
</tr>
<tr>
<td>ICP8 RNA F</td>
<td>GGAGGTGCACCGGATAACC</td>
</tr>
<tr>
<td>ICP8 RNA R</td>
<td>GGCTAAAATCCGGCATGAAC</td>
</tr>
<tr>
<td>18S RNA F</td>
<td>GCATTCGTATTGCGCCGCTA</td>
</tr>
<tr>
<td>18S RNA R</td>
<td>AGCTGCCGGCGCGGGTC</td>
</tr>
</tbody>
</table>
2.4. RESULTS

Kinetic Analysis of HSV-1 Chromatin Reveals Early Association with Histones and Heterochromatin Modifications During Lytic Infection

To examine the kinetics of chromatin association with the HSV-1 genome, we infected HFF cells at 3 PFU/cell with WT KOS strain HSV-1 virus. After an hour of adsorption, we treated cells with acidic buffer to remove surface-attached virions and synchronize infection as described previously (Cliffe and Knipe, 2008). At every hour post infection for 12 hours we formaldehyde-fixed the intact cellular monolayer and subsequently collected and froze cells for ChIP analysis. Cellular lysates were then sonicated and subjected to immunoprecipitation with antibodies specific for histone H3, H3K9me3, or H3K27me3. We measured the extent of association with histones and specific histone modifications using qPCR and primers specific for the viral ICP8 early gene promoter to quantify the proportion of viral DNA immunoprecipitated normalized to the proportion of cellular GAPDH DNA immunoprecipitated from the same reaction (Figure 2.1). Three independent ChIP experiments were performed on chromatin from 3 independent infections.

We observed that the ICP8 gene promoter accumulated the highest levels of histone H3 and repressive chromatin modifications H3K9me3 and H3K27me3 as early as 1-2 hpi. Over the subsequent 12 hours post infection (hpi) the levels of total histone and heterochromatin modifications were reduced on the viral ICP8 promoter while remaining constant at the cellular GAPDH promoter (Figure 2.1A-C). Relative to the initial peak accumulation, at the ICP8 promoter there was a 10-fold reduction in the
levels of total histone H3, and a 50- to 100-fold reduction in the H3K9me3 and H3K27me3 heterochromatin modifications. Additionally, while the greatest change in total H3 levels occurred during the first 4 hpi, H3K9me3 and H3K27me3 removal was most prominent between 3-7 hpi and 4-6 hpi respectively. These results indicated that kinetics of total histone removal and the reduction in heterochromatin modifications are regulated independently during the course of infection. Analysis of the proportion of H3K9me3 and H3K27me3 relative to total H3 suggested that while absolute levels of peak and rapidly drop during the first four hours, the relative proportion of H3 and heterochromatin modifications remains the same (Figure 2.2). However, at 4-6 hpi the proportion of histones with H3K9me3 or H3K27me3 modifications relative to total H3 was reduced. This analysis suggested that early in infection the virus may rely on chromatin remodelers to remove histones along with the repressive modifications they carry, while later in infection the virus may target specific modifications through recruitment of histone demethylases.
Figure 2.1: Kinetic analysis of chromatin on the viral ICP8 gene promoter during lytic infection with WT KOS strain HSV-1. Human foreskin fibroblasts were infected with WT KOS strain HSV-1 at 3 PFU/cell. ChIP was performed using antibodies specific for (A) Histone H3, (B) H3K9me3, and (C) H3K27me3. The proportion bound to specific antibodies was measured at the viral ICP8 promoter and normalized to a cellular GAPDH control. (D) Viral genomes were quantified from input samples normalized to GAPDH.
Figure 2.2: Kinetic analysis of heterochromatin markers H3K9me3 and H3K27me3 relative to total H3 at the ICP8 promoter during lytic HSV-1 Infection. ChIP analysis of the proportion of histones with heterochromatin modifications (A) H3K9me3 and (B) H3K27me3 expressed relative to total H3 at the viral ICP8 promoter. The proportion of modified histones at the cellular GAPDH control region is presented in parallel.
Construction and Analysis of HSV-1 ICP0-Mutant Viruses

We wanted to determine whether ICP0 was facilitating the dynamic reversal in chromatin association during lytic infection to reduce chromatin silencing and promote viral gene expression. We tested two independently constructed ICP0-mutant HSV-1 viruses, n212 and ΔProm (Figure 2.3). The n212 virus, previously described (Cai and Schaffer, 1989) contained a nonsense mutation in codon 212, to prevent synthesis of functional ICP0 protein while retaining ICP0 transcript accumulation and translation of a small fragment of ICP0. We constructed the rescued virus n212R as the ICP0+ control virus. The ΔProm virus contained a 711 bp deletion of the ICP0 transcription start site and upstream promoter region to abrogate transcription of the ICP0 gene. Construction of ΔProm and the cognate rescued virus PromR are described in Materials and Methods. Both ICP0-mutant viruses showed a reduction in accumulation of the viral ICP8 early protein and the ICP27 IE protein (Figure 2.4A). Expression of ICP8 transcripts was also delayed and reduced in both viral mutants (Figure 2.4B). Additionally, both ICP0-mutant viruses showed an approximately ~2-log replication defect in HFF and Vero cells compared to infection with their respective ICP0+ rescued viruses (Figure 2.4C), consistent with replication defects of other ICP0-mutant viruses.
Figure 2.3: Map of the ICP0 gene and mutations. The unique-short (U_s) and unique-long (U_l) regions of the HSV-1 genome are flanked by inverted repeats. A section of the repeat region is magnified to show the primary 8.3 kbp LAT and the spliced 2.0 kbp LAT intron transcripts which are antisense and overlapping to primary ICP0 and ICP34.5 transcripts. The n212 ICP0-mutant virus has a nonsense mutation in codon 212 in exon 2 of the ICP0 transcript and is indicated with an asterisk. The ΔProm ICP0-mutant has a 711 bp deletion of the ICP0 promoter and transcriptional start site between the Ncol (N) and Stul (S) restriction endonuclease sites depicted on the map.
Figure 2.4: Analysis of ICP0-mutant viruses. HFF cells were infected at 3 PFU/ml with n212, n212R, ΔProm, or PromR HSV-1 viruses. (A) Whole cell lysate was collected and analyzed by western blotting for ICP8, ICP27, and GAPDH protein expression at 2, 4, 6, 8, and 12 hpi. (B) Total RNA was extracted from infected cells hourly from 1-5 hpi and ICP8 RNA expression was quantified relative to cellular 18S rRNA. (C) HFF cells were infected at 1 PFU/ml with n212, n212R, ΔProm, or PromR HSV-1 viruses. At 24 hpi infected cells in their overlay medium were collected and titered on U2OS cells.
ICP0-Mutant HSV-1 Viruses Show Debilitated Removal of Nucleosomes and Heterochromatin

To study the kinetics and structure of viral chromatin in ICP0-mutant infected cells, we infected HFF cells with n212 or ΔProm virus, and their corresponding rescued viruses at 3 PFU/cell. Three independent infections with each virus, and independent ChIP experiments were carried out to assess the association of total H3, and the H3K9me3 and H3K27me3 histone modifications with the HSV-1 genome. These were measured by quantitative PCR as the proportion immunoprecipitated at the ICP8 gene promoter normalized to the proportion immunoprecipitated at the GAPDH promoter from the same reaction. We observed that the ICP0+ rescued viruses n212R and PromR demonstrated kinetics of initial histone and heterochromatin association and subsequent removal similar to those observed previously with the WT KOS strain virus (Figure 2.5A-C and Figure 2.6A-C). The n212 and ΔProm ICP0-mutant strain HSV-1 viruses initially accumulated histones and heterochromatin modifications at comparable levels to WT viral strains. Combined analysis of the first four hours of infection failed to demonstrate statistically significant differences between virus strains (Table 2.3). However, during 5 to 8 hpi the ICP0-mutant viruses were unable to effectively remove histones and repressive histone modifications resulting in significant differences compared to infection with WT viruses (Table 2.3). The trend was upheld at late times post infection, but not statistically significant in ΔProm and PromR infection, caused by high variability of the samples taken at 12 hpi, likely resulting from cell death. Viral replication and genome accumulation was quantified by measurement of ICP8 from reserved input
samples normalized to GAPDH. ICP0-mutant viruses demonstrated reduced and delayed viral replication (Figure 2.5D and Figure 2.6D). Overall, these results indicated that the presence of ICP0 promotes removal of histones and heterochromatin during the progression of lytic infection and facilitates viral replication.
Figure 2.5: Kinetics of chromatin on the *ICP8* gene promoter of an ICP0-mutant HSV-1 virus ΔProm relative to its WT rescued PromR during lytic infection. HFF cells were infected with ΔProm or PromR HSV-1 virus at 3 PFU/cell. ChIP analysis with antibodies specific for (A) histone H3, (B) H3K9me3, or (C) H3K27me3 are presented as the proportion bound measured at the *ICP8* promoter relative to normalization to the cellular GAPDH control. (D) Viral genomes were measured by quantification of *ICP8* sequences relative to GAPDH sequences from input IP samples.
Figure 2.6: Kinetics of chromatin on the *ICP8* gene promoter of an ICP0-mutant HSV-1 virus *n212* relative to its WT rescue *n212R* during lytic infection. HFF cells were infected with *n212* or *n212R* HSV-1 virus at 3 PFU/cell. ChIP analysis with antibodies specific for (A) histone H3, (B) H3K9me3, or (C) H3K27me3 are presented as the proportion bound measured at the *ICP8* promoter relative to normalization to the cellular GAPDH control. (D) Viral genomes were measured by quantification of *ICP8* sequences relative to *GAPDH* sequences from input IP samples.
Table 2.3: Statistical significance of infection in ICP0-mutant and WT HSV-1 using paired t-tests at three time-segments post infection

<table>
<thead>
<tr>
<th>IP Sample Conditions</th>
<th>ΔProm vs. PromR</th>
<th>n212 vs. n212R</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-4h</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>5-8h</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>9-12h</td>
<td>NS</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>K9me3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-4h</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>5-8h</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>9-12h</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>K27me3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-4h</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>5-8h</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>9-12h</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
Removal of Nucleosomes and H3K9me3 Heterochromatin Modifications Occurred Independently of Viral DNA Synthesis

The time period of most prominent heterochromatin removal relative to total histone H3 removal occurred after 4 hours post infection, coinciding with the initiation of viral genome replication. Therefore, we wanted to determine whether viral replication, and/or potentially dilution of histones and heterochromatin modification by newly synthesized genomes were responsible for their apparent reduction. We infected HFF cells at 3 PFU/cell with the WT n212R HSV-1 virus in the presence or absence of a viral DNA polymerase inhibitor sodium-phosphonoacetate (PAA). Control treated infections demonstrated a significant increase in viral genomes between 3 and 6 hpi (p < 0.05, paired t-test), while PAA treated cells showed no increased viral genome copy number (Figure 2.7A). We subsequently performed ChIP experiments from samples isolated at 3 and 6 hpi with antibodies specific for H3, H3K9me3, and H3K27me3 as above. In control cells significant decreases in total H3 (p < 0.01), H3K9me3 (p < 0.05) and H3K27me3 (p < 0.05) were detected between 3-6hpi. In PAA treated cells, the levels of H3 (p < 0.01) and H3K9me3 (p <0.01) decreased significantly; however, H3K27me3 levels did not change significantly between 3 and 6hpi (Figure 2.7B-D). These results suggested that removal of histones and H3K9me3 was not dependent on viral DNA synthesis. However, surprisingly, the H3K27me3 modification was not removed upon treatment with PAA, possibly indicating that removal of H3K27me3 is dependent on viral DNA synthesis or the participation of a viral L gene product.
Figure 2.7: Effect of viral DNA synthesis on removal of histones and heterochromatin modifications. HFF cells were treated with replication inhibitor sodium phosphonoacetate (PAA), and viral genome accumulation (A) from 3 to 6 hours was measured. ChIP analysis at 3 and 6 hpi with antibodies specific for (B) total histone H3, (C) H3K9me3, and (D) H3K27me3 heterochromatin levels were performed, and results are shown as the fraction bound compared to input chromatin at the \textit{ICP8} promoter.
2.5. DISCUSSION

Upon entering the host cell nucleus, the HSV-1 genome immediately encounters cellular silencing machinery that assembles nucleosomes and facilitates the deposition of heterochromatin modifications on foreign naked DNA. To progress through the cascade of gene expression characteristic of a successful lytic infection, HSV-1 must counteract host-mediated silencing using mechanisms to limit chromatin association entirely and/or regulate histones that remain associated. The HSV-1 genome therefore provides a model to address the role of nucleosome compaction and histone modifications in anti-viral silencing by host cells. Previous studies comparing early and late times post infection found that overall histone association with viral genomes declined while euchromatin modifications increased (Cliffe and Knipe, 2008; Oh and Fraser, 2008). This chromatin structure is established by VP16, which recruits chromatin remodelers to remove histones (Herrera and Triezenberg, 2004) and promotes the accumulation of H3K4me3 euchromatin on the remaining histones (Narayanan et al., 2007), and ICP0, which promotes acetylated euchromatin by disrupting the CoREST/HDAC1 complex (Gu et al., 2005) and recruiting the CLOCK histone acetyltransferases (Kalamvoki and Roizman, 2010).

Additionally, heterochromatin has been detected on the viral genome during initial infection (Liang et al., 2009; Narayanan et al., 2007), and accumulation of H3K9me3 heterochromatin at the ICP0 promoter was reduced by four hours post infection (Liang et al., 2009). Therefore, we hypothesized that in addition to promoting euchromatin, HSV-1 would also engage mechanisms to restrict or remove H3K9me3
and H3K27me3 heterochromatin. In this study we examined the kinetics of total nucleosome and heterochromatin association on HSV-1 genomes during the lytic cycle replication of primary human foreskin fibroblasts and found that histones and heterochromatin modifications H3K9me3 and H3K27me3 were rapidly associated but subsequently removed as infection progressed with WT HSV-1 virus strains (Figure 2.8). The most pronounced reduction of H3K9me3 and H3K27me3 heterochromatin relative to total histone H3 occurred four hours after infection, coinciding with the derepression of E viral gene transcription and initiation of viral DNA synthesis. This argues that chromatin reduction is an important stage in the progression of lytic infection. Furthermore, while the rate of histone removal peaked at 2-4 hpi and slowed thereafter, the rate of heterochromatin removal peaked after 4 hpi, suggesting that while early in infection histone and heterochromatin removal may be dependent on nucleosome remodeling, specific demethylases may be recruited later during infection.

Treatment of cells with the viral DNA polymerase inhibitor, PAA, demonstrated that histone removal and removal of the H3K9me3 modification are not dependent on viral replication, indicating that newly replicated and non-histone-associated viral genomes are not responsible for the apparent reduction in immunoprecipitation levels. Surprisingly, unlike H3K9me3, the removal of the H3K27me3 modification was affected by PAA treatment. The removal of H3K27me3 may be triggered by events after replication, or may actually represent dilution by viral genomes rather than removal from incoming genomes. Newly replicated viral genomes are potential targets for nucleosome binding and heterochromatin modification; however, genomes packaged within viral
cores lack associated histones. Therefore, a future challenge will be to determine whether newly synthesized genomes destined for progeny virions avoid histone association entirely or whether they must remove bound histones before packaging.

The differential dependence on DNA replication could also represent a more targeted effort by the virus to remove H3K9me3 than H3K27me3 heterochromatin. In eukaryotic cells, H3K9me3 is generally associated with constitutive heterochromatin and permanent repression, while H3K27me3 is associated with facultative heterochromatin that is characterized by repression with periodic activation, as seen in in developmental genes, and in bivalent domains in stem cells (Barski et al., 2007; Trojer and Reinberg, 2007). In HSV-1 latent infection, H3K27me3 is the major form of heterochromatin enriched on lytic gene promoters (Cliffe et al., 2009; Kwiatkowski et al., 2009), and is believed to suppress lytic gene expression through recruitment of PRC2, which can block RNAPII recruitment (Cliffe et al., 2013; Watson et al., 2013). Consequently, during in vitro reactivation, inhibition of the H3K27me3 demethylases, UTX and JMJD3, reduces transcription of all classes of lytic genes and lowers viral yield (Messer et al., 2014). While these studies indicate that H3K27me3 suppresses lytic gene, the detection of ongoing lytic transcription during latent infection (Kramer and Coen, 1995; Speck and Simmons, 1991) suggests that H3K27me3 heterochromatin may limit but not entirely block transcription. Therefore, in lytic infection removal of H3K27me3 may not be essential to lytic gene expression. Future studies examining additional epigenetic modifications, along with studies targeting specific modifications for inhibition or
enhancement, will be necessary to determine which aspects of epigenetic regulation are most essential.

An additional objective of this study was to assess the role of ICP0 in histone and heterochromatin removal. In addition to its other functions, VP16 can reduce methylated heterochromatin by recruiting demethylases of H3K9me2/1 (Liang et al., 2009) and H3K9me3 (Liang et al., 2013b) to promote viral gene transcription. We hypothesized that ICP0 might similarly restrict or remove methylated heterochromatin in lytic infection. Indeed, two independently constructed ICP0-mutant viruses demonstrated that ICP0 promoted removal of histones and heterochromatin from the viral genome.

ICP0 mRNA transcripts were present in cells infected with n212 but not ΔProm virus, but both showed similar defects in histone and heterochromatin removal. Therefore, we conclude that transcription of the ICP0 gene, and/or ICP0 RNA is not sufficient to remove histones and heterochromatin in lytic infection. The truncated 212 amino acid ICP0 protein expressed from the n212 virus is also insufficient for histone and heterochromatin removal, despite the inclusion of the RING finger domain that was previously shown to be essential for heterochromatin removal and IFI16 degradation (Ferenczy et al., 2011; Orzalli et al., 2012). Interestingly, it has recently been shown that the small ICP0 fragment encoded by n212 localizes to the nucleus despite the lack of a nuclear localization signal (NLS) domain, and can partially disperse PML from ND10 bodies; however, it is unable to mediate PML degradation despite having in vitro E3 ubiquitin ligase activity (Lanfranca et al., 2013). Our results agree with studies of the role of ICP0 in activation of gene expression and suggest that the RING finger domain
requires full-length ICP0 including the C-terminal region to promote the removal of histones and heterochromatin (Cai and Schaffer, 1992).

Additionally, the persistence of histones and heterochromatin on the ICP8 promoter of ICP0-mutant viruses coincided with a delay and reduction in ICP8 transcript accumulation. This supports our conclusion that removal of histones and reduction of H3K9me3 heterochromatin is essential for efficient lytic gene expression, and may be promoted by removal of H3K27me3. Thus, the removal of histones and heterochromatin may be an essential mechanism of ICP0-mediated transactivation.

Overall, these findings support the importance of chromatin regulation in viral gene expression during lytic infection. Incoming viral genomes are subjected to rapid nucleosome and heterochromatin repression. To our knowledge, this study is the first to characterize the kinetics of histone H3, H3K9me3, and H3K27me3 heterochromatin removal during lytic infection. Our results suggest several independent mechanisms contribute to chromatin modulation to relieve epigenetic suppression. Additionally we identify a function of the viral protein, ICP0, in removal of heterochromatin from the viral genome to facilitate viral gene expression during lytic infection.
Figure 2.8: Model of HSV-1 chromatin regulation during lytic infection. HSV-1 enters the nucleus and is subjected to host cell repression through the assembly of silencing chromatin during the first 2 hours post infection. After 2 hours, HSV-1 begins to remove histones to reduce total histone occupancy and heterochromatin levels while maintaining the proportion of histones bearing heterochromatin modifications. RNA polymerase II (RNAPII) is recruited and initiates transcription at early genes. ICP0 promotes histone and heterochromatin removal and promotes viral gene transcription. After 4 hours, specific removal of H3K9me3 and H3K27me3 modifications occurs, reducing the proportion of histones bearing heterochromatin markers. Removal of H3K27me3 is dependent on viral DNA synthesis. ICP0 promotes accumulation of histones bearing euchromatin modifications.
Chapter 3. HSV-1 ICP0 Regulates the Structure of Latent Viral Chromatin

Contributions: I constructed the n212R, ΔProm, and PromR HSV-1 viral mutants. I performed subsequent infections, animal studies, and ChIP experiments, and data analyses in collaboration with Priya Raja, except for ICP27 and gC transcript measurements, which were performed by Dongli Pan and Jean Pesola.
3.1. ABSTRACT

The ability of HSV-1 to transition from lytic to latent infection is thought to rely heavily on control of viral gene expression through epigenetic mechanisms. We previously showed that during lytic infection, HSV-1 genomes initially associated with repressive chromatin, but these modifications were rapidly removed in ICP0-expressing viruses. However, ICP0-mutant viruses were unable to remove silencing modifications. In neuronal infection, because association with repressive chromatin occurs gradually over the weeks following infection, ultimately resulting in stable heterochromatin associations with viral lytic promoters and quiescent infection. The latency-associated transcripts (LATs) are the only abundantly expressed viral transcripts in latent infection, and have been associated with regulating viral chromatin at lytic gene promoters and specifically promoting H3K27me3 modifications, as well as some effect on H3K9me3. The LATs and ICP0 transcripts are expressed antisense and overlapping to each other and have often been posited to have opposing functions to regulate latent and lytic infection. In this study we evaluated the chromatin profiles of ICP0-expressing and ICP0-null viruses to assess the role of ICP0 in latent infection. Paradoxically, in latent infection of sensory neurons, the genomes of ICP0-expressing viruses are enriched in histones associated with heterochromatin modifications relative to ICP0-mutant viruses. ICP0-expressing viruses are also able to establish higher latent genome loads and express higher total levels of LAT transcript accumulation. We propose that ICP0 is not a negative regulator of LAT but instead has a positive role in regulating viral chromatin structure to promote the establishment and/or maintenance of latent infection.
3.2. INTRODUCTION

To establish a latent infection, HSV-1 spreads from the site of primary lytic infection at the epithelium to the enervating sensory neurons. Here the virus can be maintained within neurons for the lifetime of the host. Unlike in lytic infection, during latency the virus does not produce progeny virions, and the ordered cascade of gene expression is replaced with very limited lytic gene transcription, and the only abundantly accumulating viral transcripts originate from the LAT region (Kramer and Coen, 1995; Ma et al., 2014; Pesola et al., 2005). The LAT region is located within the long repeat sequences of the viral genome and includes a neuron-specific LAT promoter (LAP) that directs transcription of an unstable primary 8.3 kbp LAT, from which an intron is spliced to generate the stable 2.0 kbp and 1.5 kbp forms (Farrell et al., 1991; Stevens et al., 1987).

To successfully establish lifelong latency, the virus must promote cell survival and contend with ongoing intrinsic, innate, and adaptive host immune responses. Expression of the LAT transcripts has been implicated in many activities including promoting cell survival (da Silva and Jones, 2013; Thompson and Sawtell, 2001), inhibiting apoptosis (Jones, 2013; Thompson and Sawtell, 2000), anti-sense repression of several lytic genes including ICP0 (Chen et al., 1997; Garber et al., 1997), and facilitating the accumulation of heterochromatin modifications at the viral genome (Cliffe et al., 2009; Wang et al., 2005). The suppression of the viral lytic genes through association with heterochromatin has been hypothesized to reduce the cytopathic
effects of infection and facilitate evasion of immune detection by limiting the accumulation of viral gene products within the cell (Knipe and Cliffe, 2008).

Repression of viral gene expression correlates with the accumulation of histones and heterochromatin modifications during the establishment of latency, to the extent that in established latent infection viral genomes are almost entirely compacted into an ordered nucleosomal array (Cliffe et al., 2013; Cliffe et al., 2009; Deshmane and Fraser, 1989; Kwiatkowski et al., 2009). Furthermore, while viral lytic promoters are silenced and associated with heterochromatin characterized by H3K9me2, H3K9me3, and H3K27me3, the LAT promoter is associated with bivalent chromatin accumulation characterized by association with both euchromatin and heterochromatin (Cliffe et al., 2009; Kubat et al., 2004a; Kwiatkowski et al., 2009; Wang et al., 2005). This has led to the hypothesis that an epigenetic switch initiates the transition of the viral genome from lytic to latent infection.

Latent chromatin is not established rapidly in the initial hours post infection as it is in lytic infection; rather, chromatin accumulation during latent infection occurs more gradually over the days and weeks following infection. Previous experiments in the mouse ocular infection model indicate that histone H3 association with the viral genome occurs by 7 days post infection (dpi) within trigeminal ganglia (TG), but accumulation of the H3K27me3 modification is not detectable until after 10-14 dpi (Cliffe et al., 2013). Enrichment of H3K27me3 corresponds to a significant accumulation of the PRC2 component Suz12 at lytic viral promoters by 14 dpi, but not the PRC1 component Bmi1 (Cliffe et al., 2013; Kwiatkowski et al., 2009). Interestingly, recruitment of Suz12 occurs
independently of LAT transcription, suggesting additional viral mechanisms to promote H3K27me3 accumulation (Cliffe et al., 2013). While heterochromatic modifications were previously believed to represent a terminally silenced genome, it has become increasingly clear that latent infection is not characterized by permanent and static repression of the viral genome, but represents an ongoing dynamic balance mediated by both virus and the infected cell (Hill et al., 2014).

During latent infection, HSV-1 must maintain its silenced genome in a poised and reactivation-competent state, while constantly surveying the environment of the cell for conditions advantageous to reactivation. Repressive modifications can spread linearly or can lead to the accumulation of increasingly repressive modifications, association with HP1, and eventually DNA methylation (Lachner et al., 2001; Wang et al., 2014). However, methylation of the HSV-1 genome has not been detected (Dressler et al., 1987; Kubat et al., 2004b). HSV-1 must therefore halt the progression of silencing, to limit the accumulation of modifications that would be difficult to remove and refractory to reactivation. Accordingly, treatment with histone demethylase inhibitors that target LSD1 or JMJD2 family members can increase accumulation of H3K9me3 at lytic gene promoters, reduce lytic gene expression, and ultimately inhibit viral reactivation (Hill et al., 2014; Liang et al., 2013b).

In this study we evaluated a role for ICP0 in modulating chromatin during latent infection. The viral IE gene ICP0 is transcribed antisense and overlapping to the primary LAT transcript, and can act as a potent transactivator to promote the cascade of lytic gene expression during productive infection. Many studies have focused on a role for
the LATs in latent infection, and have even proposed a central function of LATs in repression of *ICP0*. However, there have been limited studies on the function of ICP0 itself in latent infection, partially due to the belief that ICP0 expression would be negligible in latency (Kramer et al., 1998). Previously, studies have attempted to demonstrate a role for ICP0 in reactivation. ICP0-mutant viruses can establish and maintain latency; however, they reactivate less efficiently in *in vitro* explant reactivation assays (Cai et al., 1993; Cai and Schaffer, 1992; Halford and Schaffer, 2001; Leib et al., 1989b). Therefore, it was hypothesized that enhanced expression of ICP0 in latent infection could initiate aberrant and inappropriate reactivation. However, studies have also demonstrated that ICP0 is not critical for the initiation of reactivation from latency *in vivo* (Thompson and Sawtell, 2006). More recently, it has been shown that in latent infection ICP0 expression precedes the establishment of latency in approximately one third of latently infected neurons, and low levels of ICP0 transcripts are detected in latency in the absence of reactivation (Chen et al., 2002a; Maillet et al., 2006; Pesola et al., 2005; Proença et al., 2008; Speck and Simmons, 1991). These studies suggest that ICP0 may function early in neuronal infection to affect establishment of latency, or exhibit ongoing activity during maintenance of latent infection.

Previously we have shown that ICP0 reduces heterochromatin and histone association with viral lytic promoters during productive infection (see Chapter 2) (Cliffe and Knipe, 2008). We therefore hypothesized that ICP0 in latent infection would also function to limit heterochromatin associated with viral genomes during latent infection. Surprisingly, however, we found that the latent genome of ICP0-mutant viruses
exhibited reduced association with heterochromatin, suggesting that ICP0 expression promoted association with heterochromatin on the viral genome in latent infection. Interestingly, ICP0 also did not negatively regulate LAT expression, despite enhancing H3K27me3 accumulation at the LAT promoter. Ultimately, ICP0-expressing viruses were able to establish higher levels of viral genomes, suggesting that ICP0 expression and/or heterochromatin association facilitates establishment or maintenance of latency.
3.3. MATERIALS AND METHODS

Cells and Viruses

U2OS cells (ATCC; Manassas, VA) were maintained in DMEM supplemented with 5% FCS (v/v), 5% BCS (v/v), 2 mM L-glutamine, at 37°C. The n212 HSV-1 ICP0 nonsense mutant, which contains a nonsense mutation in codon 212 has been previously described (Cai and Schaffer, 1989). The WT rescued virus n212R was constructed as described previously, as were the ∆Prom ICP0-promoter deletion mutant virus, and PromR WT rescued viruses (see Chapter 2.3). Virus stocks were propagated and titered in U2OS cells in parallel with their corresponding WT rescued virus.

Mouse infections and isolation of trigeminal ganglia

Six-week-old CD-1 mice (Charles River Laboratories) were anesthetized in an isoflurane chamber followed by intraperitoneal injection of ketamine hydrochloride (3.7 mg/mouse) and zylazine hydrochloride (0.5 mg/mouse) and inoculated with 2 x 10^6 PFU/eye of ICP0-mutant virus (n212 or ∆Prom) or 2 x 10^4 PFU/eye of WT virus (n212R or PromR) as previously described (Tenser and Dunstan, 1979). Doses were adjusted for ICP0 mutant and WT ICP0 rescued viruses to achieve roughly equivalent replication in the eye for the first 2-3 days post infection.

To measure infection within the cornea, eyeswabs were collected from tear films using sterile polyester applicators (Puritan) for the first 5 days post infection, and titered on U2OS cells as previously described (Coen et al., 1989). Survival was measured for 28 days, and mice that succumbed to disease or were sacrificed due to severe...
neurological impairment were recorded. After approximately 28 days mice were sacrificed and trigeminal ganglia (TG) were dissected and immediately frozen in liquid nitrogen and stored at -80°C. Mice were housed in accordance with institutional and National Institute of Health guidelines for the care and use of animals in research, and all procedures were approved by the Institutional Animal Care and Use Committee of Harvard Medical School.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was performed on TGs as previously described, with modifications (Cliffe et al., 2009). Intact TGs were incubated in 20 ml of serum-free DMEM with 1% formaldehyde at 37°C for 15 min to cross-link the chromatin, and then washed 3 times in ice-cold PBS. TGs were then homogenized in a TissueLyser LT (Qiagen) with 5 mm stainless steel beads for 3 cycles of 2 minutes each at 50 oscillations/second. The homogenized samples were then sonicated at 4°C for ~9 cycles of 5 min each in a Biorupter (Diagenode) on the high setting (15 sec ON, 45 sec OFF) to shear the DNA to ~500 bp length. Immunoprecipitation reactions were carried out as described previously (Chapter 2.3) with 50 µg of chromatin and 2.5 µg of antibody (H3, Abcam ab1791; H3K9me3, Abcam ab8898; H3K27m3, Active motif 39156, or negative-control rabbit IgG, Millipore NG1893918) per IP reaction incubated at 4°C overnight.

Immunocomplexes were then isolated with MagnaChIP magnetic beads, washed 3 times with cold low-salt buffer (150 mM NaCl, 20 mM Tris-HCL, pH 8.1, 2 mM EDTA,
1% Triton X-100, 0.1% SDS, and 1 mM PMSF) and 3 times with cold LiCl wash buffer (50mM HEPES, pH 7.5, 250 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% sodium deoxycholate, and 1 mM PMSF) and once with cold Tris-EDTA buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). The chromatin complexes were eluted twice and combined by adding 90 μl of elution buffer (1% SDS, 0.1 M NaHCO₃) and incubating at 65°C for 10 min rotation for 10 min at room temperature for a total of 180 μl elution volume. DNA was then isolated by reversal of formaldehyde crosslinks, incubation with RNaseA, proteinase K, purification with the QIAquick PCR purification kit (Qiagen), and elution in 50 μl elution buffer twice to yield 100 μl total volume.

**Formaldehyde-assisted isolation of regulatory elements**

A second procedure, formaldehyde-assisted isolation of regulatory elements (FAIRE), was performed on latently HSV-1 infected TGs to assess the degree to which HSV-1 genomes were nucleosome-associated, using a protocol modified from (Giresi et al., 2007). TGs were subjected to formaldehyde crosslinking, homogenization, and sonication as described above for use in ChIP experiments. For FAIRE analysis 15 μg of total chromatin was diluted up to a total volume of 200 μl in ChIP dilution buffer (150 mM NaCl, 10 mM Na₂HPO₄, 2 mM EDTA, 1.1% Triton, 0.1% SDS). Extraction with an equal volume of phenol-chloroform-isoamyl alcohol was used to isolate nucleosome-free DNA in the aqueous phase from protein-bound chromatin. DNA was purified with precipitation in 95% ethanol and 2 μl of glycogen at -20°C overnight. Subsequently, DNA was pelleted and resuspended in water. Input samples were analyzed in parallel.
but treated to reverse formaldehyde-generated histone-DNA crosslinks prior to phenol chloroform extraction. Nucleosome-free DNA was quantified by real-time PCR and expressed relative to input DNA isolated.

**DNA quantification with quantitative PCR**

Quantitative PCR (qPCR) was performed using the Power SYBR Green PCR Master Mix and a Prism 7300 realtime system (Applied Biosystems) according to the manufacturer’s directions in a final reaction volume of 25 µl with 2.5 µl of DNA and 100 nM of each primer. Primer pair specificity was confirmed by running a dissociation curve for each reaction. All DNA samples were run in duplicate, and relative copy numbers were calculated by comparison to a standard curve generated from 10-fold serial dilutions of DNA from HSV-1 infected cells. The fraction of viral DNA immunoprecipitated relative to 1% input was normalized to the fraction of cellular GAPDH DNA immunoprecipitated relative to 1% input from the same reaction. Primers used can be found in Table 2.1.

**RNA isolation and quantification with qRT-PCR**

Trigeminal ganglia from HSV-1 infected mice were isolated and immediately frozen in liquid nitrogen and stored at -80°C. TGs were homogenized in 600 µl of RLT buffer (Qiagen) with β-mercaptoethanol added in the TissueLyser LT (Qiagen) with 5 mm stainless steel beads for 2 minutes, at 50 oscillations/second. The resulting homogenate was divided equally for subsequent RNA or DNA isolation. RNA was
isolated using the RNeasy Kit (Qiagen) and DNA was isolated with the Blood and Tissue DNeasy Kit (Qiagen) according to the manufacturer’s instructions. RNA samples were treated with DNasefree reagent (Ambion) and quantified with qPCR using the Power SYBR® Green RNA-to-CT™ 1-step kit (Applied Biosystems) using primers listed in Table 2.2. DNA was quantified by real-time PCR as described above with *ICP8* and *GAPDH* primers.
3.4. RESULTS

ICP0-null Mutant Viruses Show Reduced Replication in Mouse Ocular Infection

To study the effects of ICP0 on latent chromatin in the mouse model system, we infected mice via corneal scarification with $2 \times 10^6$ PFU/eye of the ICP0-mutant viruses, \( \Delta \text{Prom} \) or \( n212 \), or $2 \times 10^4$ PFU/eye of the WT rescued viruses, PromR or \( n212R \), respectively. To normalize the amount of virus at the ocular site of infection that precedes viral entry into the neurons of the trigeminal ganglia, we varied the inoculum doses of ICP0-mutant and WT virus strains to achieve equivalent titers collected from eyeswabs at days 2-3. To assess acute replication within the eye, we collected eyeswabs daily from 1-5 dpi and titered them on U2OS cells. However, by day 5 post infection WT rescued viruses showed significantly higher levels of virus relative to ICP0-mutant viruses (Figure 3.1A-B). Infected mice were maintained for at least 28 dpi to establish latent infection with HSV-1 virus strains. Survival analysis showed an increased survival of mice infected with ICP0-mutant viruses compared to mice infected with the WT rescued virus strains, with statistically significant differences in survival of \( n212 \) mutant compared to \( n212R \) (p < 0.0001) (Figure 3.1C-D).
Figure 3.1. Acute replication in corneal epithelia and survival of mice infected with ICP0-mutant and ICP0-expressing viruses. (A-B) Viral replication in mouse corneal epithelia. Mice were infected with $2 \times 10^6$ PFU/eye of ICP0-mutant viruses (A) n212 or (B) ΔProm and $2 \times 10^4$ PFU/eye of WT rescued viruses (A) n212R or (B) PromR, respectively. Eyeswabs were collected daily from 1-5 dpi and viral titers were measured by plaque assay on U2OS cells. Statistical significance evaluated with Student's t-test, ($p < 0.05$) is indicated with an asterisk. (C-D) Mouse survival over 28 dpi was compared for mice infected with (C) n212 or n212R and (D) ΔProm or PromR viruses as described above. Statistical significance analyzed with the log-rank Mantel-Cox test ($p < 0.05$) is indicated with an asterisk.
Chromatin Profile of ICP0-Expressing and ICP0-Mutant Viruses

To measure the effects of ICP0 on chromatin association with latent viral genomes, we evaluated the chromatin profile of HSV-1 latent genomes from ICP0-mutant and rescued viruses. Mice were sacrificed after 28 dpi, and TGs were harvested. ChIP experiments were performed on chromatin with antibodies specific for histone H3, or the heterochromatin modifications H3K9me3, or H3K27me3. The association with the viral ICP8 promoter, ICP27 promoter, LAT promoter (LAP), or LAT enhancer relative to the cellular GAPDH promoter was quantified by qPCR.

Contrary to our original hypothesis, we found increased levels of heterochromatin on the WT viruses compared to ICP0-mutant viruses. There was a significant increase in H3K27me3 on the LAT promoter (p < 0.05) (Figure 3.2). The increase in H3K27me3 was also significant for a combined analysis of the lytic promoters (ICP8 and ICP27) from ICP0-mutant viruses compared to WT viruses (p < 0.05). No significant differences were apparent between the two ICP0-mutant virus strains, n212 and ΔProm. These results suggested that ICP0-protein expressing viruses accumulated increased H3K27me3 heterochromatin modifications at lytic promoters and on the LAT promoter during latent infection. The trends of the H3K9me3 modification were similar to those of the H3K27me3 modification, but were not significantly significant.

To confirm these results using an antibody-independent method, we performed another protocol, formaldehyde-assisted isolation of regulatory elements (FAIRE), to measure the proportion of nucleosome-free DNA to nucleosome-bound DNA from ICP0-mutant and WT viruses. FAIRE analysis detected an enrichment of nucleosome-bound
viral DNA in WT viruses relative to ICP0-mutant viruses at both the *ICP8* promoter and *LAP* region (Figure 3.3). These results supported our results from ChIP experiments and suggest that in latency the genomes of ICP0-expressing WT viruses are more nucleosome-bound and associated with repressed heterochromatin.
Figure 3.2. Chromatin profile of HSV-1 genomes during latent infection with ICP0-mutant or WT ICP0-expressing viruses. Chromatin from latently infected TGs was evaluated by ChIP for total histone H3 (A and D), H3K9me3 (B and E), and H3K27me3 (C and F). Specific enrichment of the viral regions LAT promoter (LAP), LAT 5’ exon, ICP8 E gene promoter, and ICP27 IE gene promoter are normalized to enrichment of cellular GAPDH sequences. The ICP0-promoter mutant ΔProm and its WT rescue PromR are compared in Panels A-C, and the ICP0-nonsense mutant n212 and its WT rescue n212R are compared in Panels D-F. Statistical significance was evaluated using Wilcoxon matched-pairs signed rank test, and p < 0.05 are indicated with an asterisk.
Chromatin from latently infected TGs was evaluated by FAIRE to compare the proportion of nucleosome-free DNA relative to total input DNA in ICP0-expressing viruses (PromR or n212R) or ICP0-mutant viruses (ΔProm or n212). FAIRE results were assessed at the ICP8 promoter (A), or LAP (B) regions.

Figure 3.3. FAIRE profile of latent ICP0-expressing and ICP0-mutant genomes.
LAT Expression During Latent Infection with ICP0-Mutant and WT HSV-1 Viruses

We isolated transcripts from mouse TGs latently infected with either ICP0-mutant or rescued viruses, and quantified viral LAT and ICP0 transcripts by qRT-PCR (Figure 3.4). Viral RNA transcripts were measured with virus-specific primers and were normalized to the cellular 18S rRNA control. Viral genomes isolated from the same ganglia were measured by qPCR and normalized to cellular GAPDH. ICP0 transcripts were detected from PromR infected TGs, and were significantly enriched relative to the ΔProm virus, which lacks the ICP0 promoter region sequences (p < 0.05) (Figure 3.4B). ICP0 transcript levels did not vary significantly between n212 or n212R viruses, as was expected due to the mutation in n212 affecting full-length protein expression rather than transcript accumulation (Figure 3.4E). LAT transcripts were approximately 3-fold higher, and significantly enriched in PromR relative to ΔProm viruses (p < 0.05) and approximately 2-fold higher in n212R relative to n212 viruses (Figure 3.4A and D). However, total viral genome analysis demonstrated 2- to 3-fold significant enrichment of viral genomes per cellular genome from WT ICP0+ viruses, n212R and PromR, relative to ICP0-mutant viruses, n212 and ΔProm (p < 0.05) (Figure 3.4C and E). Therefore, the LAT and ICP0 expression per viral genome was not significantly different in ICP0-expressing and ICP0-mutant viruses (Figure 3.5).

We additionally assessed expression of ICP27 and gC viral transcripts; however, levels of the lytic gene transcripts were very low and fell below the linear range of the standard curve. However, samples demonstrated a peak at the appropriate melt temperature, and therefore were not quantified, but rather scored as positive or negative
for detectable transcripts (Table 3.1). ICP27 mRNA was detected from 4 of 13 TGs infected with WT rescued viruses PromR and 4 of 7 infected with r212R, but was not detected in any TGs infected with ΔProm virus, and only one of 9 infected with r212. Results for gC transcripts were similar except for PromR infected TGs which only scored positive in 2 of 13 samples. These results demonstrate a similar trend of expression compared to LAT transcripts. Lytic genes are detected from more ICP0-expressing viruses, approximately in proportion to higher genome accumulation.
Figure 3.4. Accumulation of viral transcripts and genomes from ICP0-mutant viruses and WT rescued viruses in latent infection. Viral transcripts were quantified from latently infected TGs by qRT-PCR using virus sequence specific primers for LAT (A and D), ICP0 (B and E) relative to a cellular 18S rRNA control. Viral genomes were quantified (C and F) by qPCR for viral ICP8 sequences relative to cellular GAPDH. Statistical significance was evaluated with Student’s t-tests, (p < 0.05) are indicated with an asterisk.
Figure 3.5. Relative viral gene expression per genome for ICP0-mutant and WT viruses in latent infection. Viral transcripts per TG are quantified with qRT-PCR primers specific to LAT (A and C) or ICP0 (B and D) are expressed relative to viral genomes. Statistical significance was evaluated with Student’s t-tests.
Table 3.1: TGs with detectable expression of viral ICP27 and gC transcripts during latent infection

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3.5. DISCUSSION

In this study we evaluated the role of ICP0 in modulating chromatin on the HSV-1 genome during latent infection. Previous studies showed ICP0-promoter activation in approximately one-third of latently infected neurons prior to the establishment of latency, VP16-independent ICP0-promoter activation, and low but detectable ICP0 expression during latency (Chen et al., 2002a; Maillet et al., 2006; Proença et al., 2008; Proença et al., 2011). We also found low but detectable ICP0 expression in our study of WT virus infected TGs isolated at day 28. Therefore, we wanted to determine if ICP0 functioned actively in latent infection, despite being generally repressed and traditionally associated with promoting lytic infection.

Previously, chromatin studies of ICP0 in latent infection relied on the 7134 strain ICP0-mutant virus; however, the construction of this virus also eliminates a portion of the primary LAT sequences and expression of the stable LAT 2.0 kbp intron (Halford and Schaffer, 2000). Expression of the adjacent and antisense LAT transcripts can affect chromatin regulation, so our studies were designed to isolate the role of ICP0 from the role of LAT transcripts using two ICP0-mutant HSV-1 viruses that did not disrupt sequences in the LAT intron or enhancer regions. We confirmed that LAT accumulation relative to viral genome copy number was not altered in latent infection with either the ΔProm or n212 ICP0-mutant viruses. However, infection with ICP0-mutant viruses ΔProm or n212 resulted in fewer total latent viral genomes in the TGs relative to infection with WT rescued viruses and resulted in a corresponding proportional decrease in the absolute level of LAT transcript accumulation.
The complementarity of ICP0 and LAT transcripts has been suggested as a potential source of LAT regulation, and modulation of latent infection (Garber et al., 1997). To address whether LAT interaction with ICP0 mRNA mediated latent infection, we compared the ICP0-mutant viruses n212, which affects only the ICP0 protein translation, or ΔProm, which lacks both ICP0 protein and ICP0 RNA transcription. We did not detect any significant differences between these viruses relative to their WT rescued strain viruses. This argues that the effects of ICP0 are related to expression of ICP0 protein rather than the primary ICP0 transcript.

ICP0-expressing viruses show increased replication in the eye during acute infection (Leib et al., 1989b); therefore, we normalized the initial inoculum dose between ICP0-expressing and ICP0-mutant viruses using a previously reported strategy, with modifications, in an attempt to equalize the effect dose of virus that is available to travel to the trigeminal ganglia and establish latent infection (Halford and Schaffer, 2000). However, to avoid unintended effects to our epigenetic phenotypes, we did not attempt normalization using cyclophosphamide-mediated immunosuppression. The 100-fold dose modifications were successfully able to equalize detectable virus shed within the eye at two and three days post infection. However, ICP0-expressing viruses maintained a higher level of replication and by day five had significantly higher levels of virus shed within the eye compared to the ICP0-mutant viruses. Consequently, at day 28, during latent infection, more viral genomes were also measured from the TGs of mice infected with ICP0-expressing viruses relative to ICP0-mutant viruses. This may result from higher viral load transported to the ganglia from the corneal epithelium as
seen previously (Leib et al., 1989b). However, it could also indicate increased acute replication within neurons, or greater success at establishment of latency and maintenance of viral genomes. Mice infected with ICP0-expressing viruses exhibit more severe pathogenic effects and increased mortality. Similarly, this may be a result of ICP0 expression promoting acute infection to facilitate increased replication within neurons. ICP0 also exhibits immune modulatory effects, and expression may alter immune detection or response to prevent viral clearance. These differences in viral genome load at day 28 highlight the need for a neuronal cell culture infection model that accurately represents latent infection, to confirm our assessment of the effects of ICP0 on viral chromatin independently of viral dose.

Surprisingly, in addition to higher levels of latent viral genomes, we found that latent genomes of ICP0-expressing viruses were associated with increased H3K27me3 and H3K9me3 heterochromatin at viral lytic gene promoters and the LAT promoter when compared to their respective ICP0-mutant viruses. The increased heterochromatin detected by ChIP correlated with the increased proportion of nucleosome-bound DNA by FAIRE, and indicated generally greater chromatin silencing during latent infection by ICP0-expressing viruses.

Cell culture-based latency models using quiescent HSV-1 infection of various cell types found that ICP0 expression generally induced active lytic gene expression and enrichment of acetylated histones, while preventing or reducing levels of total histone H3, H3K9me3, HP1γ (Coleman et al., 2008; Ferenczy and DeLuca, 2009; Ferenczy and DeLuca, 2011). While these results appear initially contradictory to our findings, the use
of non-neuronal cells and higher levels of ICP0 expression than are present during in vivo latent infection likely contribute to these differences. Accordingly, deep sequencing analysis of viral lytic gene transcription of neuronal and non-neuronal cells during quiescent infection show significant differences (Harness et al., 2014). Additionally, a neuron-specific host microRNA that targets ICP0 expression and affects latency has recently been identified (Pan et al., 2014). Furthermore, the preference for establishment of latent infection within specific neuronal subsets suggests that latency may be highly dependent on interaction with neuron-specific cellular factors (Bertke et al., 2012).

The establishment of latent infection occurs gradually within neurons during and after the resolution of acute infection. Heterochromatin is progressively accumulated as the virus transitions away from lytic gene expression to latent gene repression (Cliffe et al., 2013; Wang et al., 2005). Early expression of ICP0 during an initial acute phase may function indirectly to facilitate latency establishment by increasing the numbers of viral genomes that are present in the ganglia. Enhancement of viral genome load, lytic gene expression, and/or ICP0 itself may promote activation of a cellular immune response to enhance silencing. HSV-1 also exhibits ongoing low-level expression of viral lytic genes during latency, which has been correlated with changes in neuronal gene expression (Kramer and Coen, 1995; Ma et al., 2014). This suggests that HSV-1 may actively modulate the neuronal environment to facilitate latency. ICP0 could therefore be required to maintain low-level lytic gene expression, while viruses lacking ICP0 may instead be cleared after encountering a hostile neuronal environment refractory to
establishment of latency. Future characterization of lytic gene expression, heterochromatin accumulation, and the association of chromatin modifying complexes during the establishment of latency could indicate how ICP0 promotes heterochromatin accumulation during ongoing latent infection.

Alternatively, increased heterochromatin during latent infection with ICP0-expressing viruses could indicate that ICP0 directly modulates chromatin in a highly context-dependent manner. A recent study identified chromatin modifying factors and demethylases that function in a cell-type specific manner (Oh et al., 2014). Therefore, an alternate repertoire of chromatin modifying factors may be expressed in neurons or may act differently in neuronal tissues. As such, ICP0 could actively promote silencing through encountering a chromatin-modifying factor uniquely expressed or functionally different within neurons. Additionally, the mechanisms by which ICP0 reduces heterochromatin in lytic infection may function differently in latent infection. ICP0 prevents silencing through the degradation of cellular factors IFI16 and PML, and through the disruption of ND-10 bodies or dissociation of HDAC from the repressive HDAC-CoREST-LSD1-REST complex (Everett et al., 1998; Gu et al., 2005; Orzalli et al., 2012). These ICP0 targets operate within complex pathways that may also exhibit cell-type specific activity, and may not prevent silencing in latent neuronal infection. Further studies of cellular pathways and chromatin regulators in a neuronal context may explain an apparent dual role for ICP0 in the lytic-latent balance.

Interestingly, the significant increase in H3K27me3 heterochromatin at the LAT promoter did not appear to correlate with a reduction in LAT transcript accumulation in
ICP0-expressing viruses. The LAP region has been shown previously to contain both repressive H3K27me3 and activating H3K4me3 modifications during latency, suggesting that suppression by H3K27me3 at the LAP may not strictly repress transcription of the LATs (Cliffe et al., 2009). Regions of bivalent cellular chromatin domains have been associated with poised transcription, and likely represent an important regulatory mechanism that may be essential to LAT transcription (Bernstein et al., 2006; Greer and Shi, 2012). Alternatively, the apparent bivalency of chromatin modifications may be due to distinct populations of genomes, or even distinct copies of the LAT region, exhibiting variable chromatin association. In this way, ICP0 may enhance LAT expression from some genomes while facilitating repression from others. Advances in single cell and single molecule analysis of chromatin and gene expression will enable the resolution of these possibilities.

In summary, this study demonstrates that although ICP0 modulates chromatin to reduce heterochromatin and facilitate gene expression in permissive cells during lytic infection, ICP0-expressing viruses are more effective at promoting heterochromatin modifications and establishing latent infection in neurons. Our results argue that ICP0 performs a novel functions in neurons to co-opt cellular chromatin repressive machinery to silence gene expression and enable the virus to remain undetected and persist for the lifetime of the host. ICP0 has therefore emerged as an important regulator of chromatin structure in both lytic and latent infection and may be a useful antiviral target for HSV-1 infection.
Contributions: I constructed the ΔCTRL2 and CTRL2R HSV-1 virus strains. I performed subsequent infections, animal studies, ChIP experiments, and data analyses in collaboration with Priya Raja.
Herpes simplex virus (HSV) establishes latent infection and persists in neurons using a variety of host-derived epigenetic mechanisms to silence its genome. A potential mechanism is through association with the host CCCTC-binding factor (CTCF). As a multifunctional cellular DNA-binding protein, CTCF functions as a mediator of transcriptional control and chromatin organization and has binding sites in the genome of HSV-1 and other herpesviruses. Using an HSV-1 deletion mutant that lacks the CTCF binding sites within the latency-associated transcript (LAT) coding sequences, we found that loss of these CTCF binding sites did not alter lytic replication in the mouse model but increased viral pathogenicity. We also found that CTCF functioned as an insulator in latent infection by preventing the spread of histone H3 lysine 27 trimethyl (H3K27me3) modification from the silenced immediate-early ICP0 gene promoter to the adjacent LAT regulatory region, thus maintaining a distinct chromatin boundary. However, we found that the CTCF binding-site mutant strain also exhibited increased accumulation of the LAT transcripts during latency, indicating that CTCF may regulate transcription through other mechanisms. Furthermore, while the virus was able to establish equivalent levels of viral genomes within the ganglia of mice by day 28, upon explant, these viral genomes were less efficient at reactivation. Cumulatively, these results indicate that CTCF mediates critical functions in regulating a silenced but poised genome during latent infection.
4.2. INTRODUCTION

HSV-1 persists as a latent infection in the sensory ganglia of the host, wherein lytic genes are silenced and the only viral gene products abundantly expressed are a family of noncoding RNAs known as the latency-associated transcripts (LATs) and miRNAs (Kramer and Coen, 1995; Stevens et al., 1987; Umbach et al., 2008). The LAT gene is transcribed as a primary 8.3 kbp transcript from which stable 1.5- and 2.0-kbp introns and a number of miRNAs are processed (Deshmane and Fraser, 1989; Umbach et al., 2008). No strong evidence for proteins encoded by the LATs has been found; however, long non-coding RNAs in other systems are known to mediate assembly of heterochromatin and maintenance through both direct and indirect mechanisms (Drolet et al., 1998). While not essential for the establishment or maintenance of latency, the LATs are believed to mediate gene silencing and increased heterochromatin accumulation at lytic genes and have been associated with a reduction in lytic gene transcripts in both acute and latent infection of neurons (Chen et al., 1997; Garber et al., 1997; Javier et al., 1988; Leib et al., 1989a; Steiner et al., 1989).

During the establishment of latency, the majority of viral genes associate with increasing levels of histones that are hypoacetylated and enriched for markers of heterochromatin such as histone H3 lysine 9 trimethyl (H3K9me3) or H3 lysine 27 trimethyl (H3K27me3) (Cliffe et al., 2013; Cliffe et al., 2009; Deshmane and Fraser, 1989; Kwiatkowski et al., 2009; Wang et al., 2005). Elements of the LAT transcriptional unit, which include upstream regulatory sequences, a neuron-specific promoter, and a downstream enhancer, appear to be the exception to this chromatin phenotype (Batchelor and O'Hare, 1990; Cliffe et al., 2009; Dobson et al., 1989; Kwiatkowski et al., 1990).
The \textit{LAT} gene is the only viral region known to maintain acetylated histones and other markers of active euchromatin, while also maintaining association with markers of heterochromatin (Cliffe et al., 2009; Kwiatkowski et al., 2009). Upon reactivation \textit{in vitro}, this pattern is reversed, with lytic genes associating increasingly with acetylated histones and markers of euchromatin and accumulating transcripts, while the \textit{LAT} gene exhibits a corresponding decrease in euchromatin and transcript levels (Amelio et al., 2006a; Devi-Rao et al., 1994; Kosz-Vnenchak et al., 1993; Spivack and Fraser, 1988). Chromatin control of viral lytic gene expression is therefore thought to act as an essential regulator of the transition between lytic infection, latent infection, and reactivation.

Located antisense to the \textit{LAT} gene is the \textit{ICP0} gene, an immediate-early (IE) lytic gene whose protein product serves many functions that promote lytic infection, including the transactivation of viral genes and repression of the innate immune system (Cai and Schaffer, 1992; Chelbi-Alix, 1999; Chen and Silverstein, 1992; Everett and Murray, 2005; Orzalli et al., 2013; Orzalli et al., 2012). ICP0 counters host-mediated chromatin silencing, intrinsic resistance, and innate immune responses through several mechanisms including the degradation of promyelocytic leukemia (PML) protein in nuclear domain 10 (ND10) bodies (Everett et al., 2006), degradation of interferon-inducible protein 16 (IFI-16) (Orzalli et al., 2013), and through inhibition of the histone deacetylase (HDAC) RE1 silencing transcription factor-corepressor to REST (REST/CoREST)-HDAC repressor complex (Gu et al., 2005; Gu and Roizman, 2007). In latently infected neuronal populations, \textit{ICP0} is largely repressed in latent infection.
Despite proximity to the LAT enhancer sequences and abundant expression from the adjacent LAT region (Proença et al., 2008).

The CCCTC-binding factor (CTCF) is an 11 zinc-finger DNA-binding protein that is essential, ubiquitously expressed, and highly conserved among metazoan species (Baniahmad et al., 1990; Lobanenkov et al., 1990). CTCF was identified independently several times and initially classified as a transcriptional repressor with the ability to bind diverse DNA target sequences (Baniahmad et al., 1990; Köhne et al., 1993; Lobanenkov and Gudvin, 1988; Lobanenkov et al., 1990). CTCF was later recognized as a transcriptional activator as well as an enhancer blocker and insulator (Bell et al., 1999; Filippova et al., 1996; Klenova et al., 1993). Consequently, CTCF emerged as an important chromatin regulator responsible for a wide range of activities also including pausing of RNA polymerase II (RNAPII) and affecting RNA splicing, and directing the specific positioning and phasing of nucleosomes (Fu et al., 2008; Shukla et al., 2011). Recently, the diverse range of CTCF activities have been associated with its ability and create chromatin loops, and to mediate long-range chromatin interactions through manipulation of 3D chromatin architecture (Handoko et al., 2011; Ong and Corces, 2014; Splinter et al., 2006).

Importantly, CTCF is the only identified vertebrate insulator protein. As such, it acts as a boundary element to isolate adjacent domains of active and inactive chromatin by directing enhancer activity to prevent interaction with nearby but inappropriate promoters and by blocking the linear spread of heterochromatin (Bell et al., 1999; Chung et al., 1993; Hark et al., 2000; Kim et al., 2007). Insulators are crucial in the disorganized genomes of both metazoan species and herpesviruses, throughout which
essential active regions of transcription are frequently interspersed with silenced domains.

CTCF binding sites have been identified in a number of herpesviruses and associated with functions such as regulating latent gene expression in the gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesviruses (KSHV), as well as regulating expression of the major immediate-early gene in the betaherpesvirus human cytomegalovirus (HCMV) (Kang et al., 2011; Martinez et al., 2014; Stedman et al., 2008; Tempera et al., 2010). Among the alphaherpesviruses, a number of CTCF binding sites have been identified in HSV-1, including the CTRL2 site, which is located between the LAT and ICP0 promoter regions (Amelio et al., 2006b; Chen et al., 2007). CTCF has been shown to bind within the stable 2.0 kbp LAT intron at CTRL2 during latent infection, where it is hypothesized to regulate distinct expression from the adjacent genetic elements and appears to be lost during reactivation, (Amelio et al., 2006b; Chen et al., 2007; Ertel et al., 2012). Additionally, in transgene assays CTRL2 is capable of enhancer blocking, silencing, and preventing heterochromatin spreading (Amelio et al., 2006b; Chen et al., 2007). Indirect evidence also suggests that removal of CTCF binding sites can alter gene expression from lytic promoters in cell culture latency models (Harness et al., 2014).

We were therefore interested in determining whether the presence of CTCF binding at the LAT intron is indeed critical in establishing and/or maintaining the latent gene expression pattern and chromatin structure in the LAT region during latency. In this study we have eliminated the region of the CTRL2 binding site from HSV-1 (ΔCTRL2) to assess CTCF function in the mouse model system of latency. We have
observed that the *CTRL2* site is required for CTCF binding at the *LAT* intron, and abrogation of CTCF binding enhances pathogenicity of HSV-1 and alters the structure of chromatin at the *LAT* sequences with a specific accumulation of H3K27me3 at the *LAT* promoter and intron region relative to WT rescued virus. Although heterochromatin increases at the *LAT* promoter region with ΔCTRL2 virus infection, we also observed increased *LAT* transcript accumulation, suggesting a complex mechanism of transcriptional control. Furthermore, the total number of viral genomes maintained during latent infection was unaffected, but the reactivation efficiency after *ex vivo* explant was reduced. We have therefore identified the *CTRL2* region as essential for maintaining distinct chromatin regulation at the *LAT* and *ICP0* regions to direct successful latent infection.
4.3. MATERIALS AND METHODS

Cells and Viruses

Vero and primary HFF cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM and supplemented with 5% (v/v) FBS, 5% (v/v) BCS, 2 mM L-glutamine at 37°C.

The HSV-1 ΔCTRL2 mutant virus was constructed through homologous recombination with the HSV-1 WT KOSΔLAT1.8eGFP virus. This KOS-strain virus has an eGFP expression cassette substitution for the 1.8 kbp fragment of the TR$_L$ spanning the major LAT promoter, LAT start site, and approximately 1.0 kbp of the primary LAT transcript (Garber et al., 1997; Leib et al., 1989a). The mutation was constructed using site-directed mutagenesis to introduce a BamHI site into a plasmid containing the LAT intron and its upstream flanking regions. Subsequent HpaI and BamHI digestions yielded a plasmid with a 346 bp deletion across the CTRL2 site, which was linearized and cotransfected into Vero cells with infectious KOSΔLAT1.8eGFP DNA. This removed CTCF binding sites at CTRL2 from within the LAT 2.0 kbp intron of both TR$_L$ repeats without disrupting the nearby LAT intron splice sites, branch points, or the ICP0 polyadenylation signal sequence. The WT rescued CTRL2R was constructed in parallel by co-transfection of the WT DNA fragment. Virus isolates were screened for loss of GFP expression, plaque-purified three times, and confirmed with diagnostic PCR and sequencing. All viruses were propagated and titered in parallel on Vero cells. To assess lytic replication HFF or Vero cells were infected at 3 PFU/cell for 24 h. Cells were scraped in supernatant, frozen, sonicated, and titered on Vero cells.
Mouse Infections

Six-week-old CD1 mice (Charles River Laboratories) were anesthetized by intraperitoneal injection of ketamine hydrochloride (3.4 mg/mouse) and xylazine hydrochloride (0.5 mg/mouse). Mice were the inoculated with $2 \times 10^5$ PFU/eye of virus in a 7.5 ml volume onto scarified corneas as previously described (Tenser and Dunstan, 1979). Eyes were swabbed on days 1 to 5 post infection, and virus collected from tear films was titered on Vero cells as described previously (Coen et al., 1989). Mice were monitored for survival for at least 28 dpi. Mice were housed in accordance with institutional and National Institutes of Health guidelines on the care and use of animals in research. The Institutional Animal Care and Use Committee of Harvard Medical School approved all procedures.

Chromatin immunoprecipitation

Chromatin was prepared from TGs as described previously with modifications (Cliffe et al., 2013). Intact TGs were fixed in 1% formaldehyde for 15 min at 37°C. Formaldehyde was quenched with 0.125 M glycine for 3 min at room temperature. TGs were washed 3 times with cold PBS and then homogenized in PBS with the TissueLyser LT and 5 mm stainless steel beads (Qiagen) at 50 oscillations/sec for 6 min. Subsequently the homogenate was lysed in SDS Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) and sonicated to yield chromatin fragments of approximately 500 bp or smaller using a Biorupter 200 (Diagenode) for 6 cycles of 5 min each (15 sec ON, 45 sec OFF) at a high power setting. IP reactions contained 50 µg of chromatin diluted
10 fold in ChIP dilution buffer (150 mM NaCl, 10 mM Na$_2$HPO$_4$, 2 mM EDTA, 1.1% Triton, 0.1% SDS). From each immunoprecipitation reaction, 1% of the chromatin was removed and reserved for input calculations. Immunocomplexes were incubated overnight at 4°C with antibody as follows; 5-10 µg of anti-CTCF (Millipore, 07-729), 2.5 µg of anti-histone H3 (Abcam, ab1791), 2.5 µg anti-H3K27me3 (Active motif, 39156), 2.5 µg of anti-H3K9me3 (Abcam, ab8580), or normal rabbit IgG (Millipore, 12-370) as a negative control. Antibody complexes were captured with 20 µl Magna ChIP protein A magnetic beads (Millipore) by incubation with IP samples at 4°C for 2 hours.

Immune complexes were washed 3 times with ChIP dilution buffer containing 0.1% SDS and 1 mM PMSF, 3 times with lithium chloride wash buffer (50 mM HEPES, pH 7.5, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% sodium deoxycholate, 1 mM PMSF), and once with Tris-EDTA pH 8.0 buffer. Complexes were eluted from beads twice with the addition of 90 µl of elution buffer (1% SDS, 0.1 M NaHCO$_3$) for 10 min at 65°C. Formaldehyde cross-linking was reversed by treatment with NaCl to a concentration of 200 mM for 30 min at 95°C, and the DNA was purified and isolated by treatment with 1 µg of RNaseA (Ambion) at 37°C for 1 h, proteinase K at 45°C for 2h, and use of the QIAquick PCR Purification kit (Qiagen) according to the manufacturer’s instructions.

**Quantification of DNA with quantitative PCR**

Quantitative PCR (qPCR) was performed as described previously (Cliffe and Knipe, 2008) using Power SYBR green master mix and a Prism 7300 sequence.
detection system (Applied Biosystems) according to the manufacturer’s directions. Reactions were performed in a total volume of 25 µl, with 2.5 µl of DNA, and a final concentration of 100 nM of each primer, listed in Table 2.1. The specificity of each primer pair was determined by running dissociation curves of the PCR products for each reaction. Samples were run in duplicate and relative copy numbers were determine by comparison to a standard curve generated by 10-fold serial dilution of DNA isolated from HSV-1 infected HFF cells. The fraction of viral DNA immunoprecipitated from each reaction was compared to input sample, and presented as percent immunoprecipitated for CTCF, and as fold-enrichment relative to cellular control for histone H3, H3K27me3, and H3K9me3 by normalization to the fraction of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sequences precipitated from the same reaction. ChIP assays were carried out from at least three independent experiments from 3 independent infections with ΔCTRL2 or CTRL2R.

**Quantification of viral gene expression with qRT-PCR**

TGs from infected mice were removed, immediately frozen in liquid nitrogen, and stored at -80°C. TGs were thawed and homogenized in RLT buffer (Qiagen) using a TissueLyser LT and 5 mM stainless steel beads (Qiagen) at 50 oscillations/sec for 2 min, and divided to extract RNA or DNA from the same TG. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s protocol. The viral genomes were quantified by qPCR using the viral ICP8 promoter or cellular GAPDH promoter sequences. RNA was extracted using the RNeasy Kit (Qiagen)
according the manufacturer’s instructions. Any residual DNA contamination was
removed by DNase treatment with the DNA-free kit (Ambion). Equal amounts of DNase-
treated RNAs were then reverse-transcribed and quantified with real-time PCR using the
Power SYBR® Green RNA-to-CT™ 1-Step kit (Invitrogen) and primer pairs listed in
Table 2.2 with protocols described previously (Cliffe et al., 2013; Cliffe et al., 2009;
Orzalli et al., 2013). Relative copy number was determined by comparison to standard
curves of 10-fold dilutions of cDNA normalized to the transcript levels of 18S rRNA
isolated from the same sample. Mock reverse-transcribed samples were analyzed as
negative controls.

**Reactivation from latently infected mouse TGs**

Individual TGs were isolated from mice infected with ΔCTRL2 or CTRL2R HSV-1
viruses at least 28 days prior to collection. Fresh TGs were bisected and explanted over
a confluent monolayer of Vero cells in DMEM supplemented with 10% (v/v) FBS and
0.25 g/ml amphotericin B, in a 6-well plate at 37°C, containing one ganglion per well. At
24 hour intervals for 7 days, 100 µl of the culture overlay media was removed and re-
plated onto a fresh Vero monolayer to detect infectious virus. After 7 days the entire
Vero monolayer and ganglia were collected, frozen, and replated onto a fresh Vero
monolayer to score the number of ganglia that showed detectable infectious virus.
4.4. RESULTS

Construction of an HSV-1 CTRL2 binding site deletion mutant virus

Previous studies identified the CTRL2 DNA element within HSV-1 and demonstrated that CTRL2 was able to bind CTCF in vitro and in vivo, and function as an insulator capable of blocking enhancer-promoter interactions and the spread of heterochromatin in heterologous transgene assays (Amelio et al., 2006b; Chen et al., 2007). To test whether the CTRL2 region has specific effects on in vivo HSV-1 infection, we first constructed a virus with GFP gene in the LAT region, and used this to construct a CTRL2-deletion mutant virus, as described in Materials and Methods, by removing a 346 bp fragment from within the 2.0 kbp LAT intron to generate the ΔCTRL2 HSV-1 mutant virus (Figure 4.1). A WT rescued virus, called CTRL2R was constructed in parallel to restore the intact LAT-CTRL2 region.

To confirm that the deletion of the CTRL2 site did not affect lytic viral replication, we measured growth kinetics in HFF and HeLa cells and found no significant differences between the ΔCTRL2 and CTRL2R viruses (Figure 4.2).
Figure 4.1. Map of the LAT transcriptional unit of an HSV-1 viral mutant with a deletion of the CTCF binding sites from the LAT intron sequences. (A) A schematic map of the HSV-1 genome with an expanded view of the LAT coding region with the LAT promoter (LAP), LAT enhancer (LTE), CTCF binding sites (CTRL2), and ICP0 promoter (ICP0 P) shown. Restriction endonuclease cleavage sites (H, HpaI, B, BamHI) used to generate the ΔCTRL2 virus are indicated. The locations of qPCR primers are indicated with open arrows connected by dashed lines: (I) LAP, (II) LAT Intron, (III) ICP0 P. Below are shown the locations of the primary LAT transcript, stable 2.0 kbp LAT intron, and ICP0 transcript.
Figure 4.2. Analysis of ΔCTRL2 and CTRL2R lytic viral replication. Hela or HFF cells were infected at 0.1 PFU/cell with ΔCTRL2 or CTRL2R viruses. Virus was collected at 12, 24, and 36 hours post infection and titered on Vero cells.
**Analysis of CTCF binding to a ΔCTRL2 mutant virus**

To confirm the loss of CTCF binding upon deletion of the CTRL2 site, we infected mice via corneal scarification with 2 x 10^5 PFU/eye of ΔCTRL2 or CTRL2R virus. After 28 dpi, TGs were removed and processed for ChIP analysis with a CTCF-specific antibody and quantified the immunoprecipitated DNA sequences by qPCR using primers specific for the LAT promoter, LAT intron, and ICP0 promoter (Figure 4.1). ChIP analysis performed in parallel with a nonspecific rabbit IgG confirmed specificity. Statistical analyses were performed on 6 independent ChIP experiments from 4 independent infections. As predicted, CTCF was not enriched at the LAT promoter (LAP) and at the cellular GAPDH pseudogene over nonspecific antibody control with either ΔCTRL2 or CTRL2R virus (Figure 4.3A and D). WT CTRL2R virus showed significant association of CTCF compared to nonspecific antibody control with the LAT intron, with ChIP using primers specific to a region less than 500 bp downstream of the CTRL2 site (p < 0.05) (Figure 4.3B). Surprisingly, WT CTRL2R virus also demonstrated low but not statistically significant CTCF binding within the ICP0 promoter region (Figure 4.3C). This was consistent with a potential CTCF binding site upstream of the ICP0 promoter region. Comparison of the ΔCTRL2 mutant virus to WT rescued CTRL2R showed a significant decrease of CTCF binding at the LAT intron and ICP0 promoter (p < 0.05). Collectively, these results indicated that deletion of the CTRL2 sequences in the ΔCTRL2 virus was sufficient to reduce CTCF association at the LAT intron sequences relative to the WT CTRL2R virus.
Figure 4.3 Comparison of CTCF association at the LAT region during latent infection with ΔCTRL2 or CTRL2R viruses in mice. Mice were infected with equivalent doses of ΔCTRL2 and CTRL2R viruses. At 28 dpi, the mice were sacrificed, and ChIP experiments were carried out on harvested TGs with antibodies specific for CTCF. Three viral regions were queried: LAP (A), LAT Intron (B), and the ICP0 promoter (C). The cellular GAPDH sequences (D) were also analyzed. Percentages of immunoprecipitated DNA are shown as means and standard errors from six independent ChIP experiments performed on chromatin from four independent infections. Asterisks indicate statistical significance (p < 0.05) as evaluated by the Wilcoxon matched-pairs signed rank test.
Acute infection with a \textit{CTRL2} deletion virus in mice

To examine acute infection in mice, we collected shed virus with eyeswabs for the initial 5 dpi and titered the virus on Vero cells. We observed no difference between \textit{ΔCTRL2} and \textit{CTRL2R} virus shedding at days 1 to 4; however, at day 5 virus collected from \textit{ΔCTRL2} infection was slightly but significantly lower compared to \textit{CTRL2R} (\(p = 0.0043\)) (Figure 4.4A). To allow establishment of latent infection, we maintained mice for 28 days. In WT \textit{CTRL2R} infected mice, 90% of mice survived to day 28, and generally did not succumb to infection before day 10. However, \textit{ΔCTRL2} infection resulted in increased mortality beginning at day 7, with 15% fatality by day 10, and overall significantly reduced survival, 81%, by day 28 (Figure 4.4B). Interestingly, these results demonstrated that initial replication within the corneal epithelium was not affected by deletion of the \textit{CTRL2} site; however, the progression of infection after day five suggested that CTCF binding may alter pathogenicity in neuronal tissue.
Figure 4.4. Eyeswab titers and survival of mice infected with ΔCTRL2 or CTRL2R viruses. Mice were infected with $2 \times 10^5$ PFU/eye of ΔCTRL2 or CTRL2R viruses respectively. (A) Viral replication in corneal epithelia. Eyeswabs were collected daily at days 1-5 post infection from five mice infected with each virus, from four independent infections. Collected virus was titered on vero cells, and plotted as mean and standard error for all infections. Significance was evaluated using Student’s t-test and indicated with an asterisk ($p < 0.05$). (B) Infections were allowed to progress for 28 days and survival curves are presented from eight independent infections of groups of 5-20 mice per virus infected in parallel in each infection. Asterisks indicate significance ($p < 0.05$) as evaluated with the log-rank Mantel-Cox test.
Increased H3K37me3 histone modification on the LAT region sequences of a CTRL2-deletion mutant virus.

To examine the potential role of CTRL2 in regulating viral chromatin during latency and its potential function as an insulator, we collected TGs from mice latently infected with ΔCTRL2 or CTRL2R viruses and performed ChIP analysis with antibodies specific for histone H3 or heterochromatin modifications H3K9me3 and H3K27me3. The relative fraction of HSV DNA immunoprecipitated was measured by qPCR using primers specific for the viral LAT promoter (LAP), LAT intron, and ICP0 promoter normalized to the fraction of DNA immunoprecipitated at the cellular control GAPDH sequences. Similar to previous observations, during latent infection with WT CTRL2R virus the LAT promoter was associated with less silenced chromatin relative to the LAT intron or ICP0 promoter regions (Figure 4.5). We observed significantly lower accumulation of total H3 (p < 0.05) (Figure 4.5A), and on average a ~3-5-fold reduction of H3, H3K9me3, and H3K27me3 association at the LAT promoter relative to the ICP0 promoter. However, during latent infection with the ΔCTRL2 mutant virus, the LAT and ICP0 promoters were associated with roughly equivalent levels of histones and heterochromatin modifications, with a mean fold enrichment of less than 1.5-fold at the ICP0 promoter relative to the LAT promoters for all antibodies tested. These results suggested that CTCF binding at the CTRL2 region may limit accumulation of repressive histones and heterochromatin at the LAT promoter.

When we directly compared the ΔCTRL2 virus to its WT rescued CTRL2R virus, we observed a significant increase of the H3K27me3 heterochromatin marker accumulation at the LAT promoter and LAT intron regions (p < 0.05) but not at the ICP0
promoter sequences (Figure 4.5C). However, we did not observe significant differences in total histone H3 or H3K9me3 accumulation. These results suggested that CTCF may prevent the spread of specific heterochromatin markers, such as H3K27me3, to the LAT region.
Figure 4.5. Deletion of CTRL2 increased H3K27me3 accumulation on LAT promoter and intron sequences. Mice were infected with $2 \times 10^5$ PFU/eye of $\Delta$CTRL2 or CTRL2R viruses, respectively. At 28 days mice were sacrificed, TGs harvested, and ChIP experiments carried out with antibodies specific for total histone H3 (A), H3K9me3 (B), or H3K27me3 (C). Three viral regions, the LAT promoter (LAT P), LAT intron, and ICP0 promoter (ICP0 P), were queried and expressed as percent viral chromatin immunoprecipitated relative to immunoprecipitation at the cellular control GAPDH region. Asterisks indicate significance ($p < 0.05$) evaluated using the Wilcoxon matched-pairs signed rank test.
**Increased accumulation of LAT transcripts with ΔCTRL2 virus**

To test whether infection with ΔCTRL2 virus altered latent genome accumulation or gene expression from the adjacent ICP0 and LAT promoters, we measured viral genomes and transcripts levels at 28 dpi in the TGs of latently infected mice. Total viral genomes per TG were measured by qPCR from DNA extracted from the combined TGs from one mouse. Viral genomes were normalized to cellular DNA and showed no significant difference between infections with ΔCTRL2 and CTRL2R viruses (Figure 4.6A), showing that similar numbers of viral genomes established latency at day 28. Total RNA was also extracted from each sample, and measured with primers specific to viral LAT intron, ICP0, and cellular 18S rRNA sequences. Relative amounts of viral RNA were normalized to cellular RNA and total viral genomes for each mouse. We observed a 3-fold increase in levels of LAT transcripts isolated from ganglia infected with ΔCTRL2 virus compared to CTRL2R virus (p = 0.0001) (Figure 4.6B). However, ICP0 transcript accumulation was not significantly different between ΔCTRL2 and CTRL2R infections (Figure 4.6C). Surprisingly, these results argued that accumulation of H3K27me3 at the LAT promoter and intron sequences does not inhibit, but instead may promote accumulation of LAT transcripts during latent infection.
Figure 4.6 LAT transcript expression is enhanced in latent infection with a ΔCTRL2 virus. Five mice were infected with ΔCTRL2 or CTRL2R viruses in each of four independent experiments. At 28 dpi TGs were harvested, subsequently, RNA and DNA were isolated from pooled TGs from each mouse. (A) Viral genomes were measured by qPCR and presented as ICP8 viral DNA normalized to cellular GAPDH DNA control. Viral RNA transcript levels for (B) LAT, and (C) ICP0 were measured by qRT-PCR and normalized to extraction of cellular 18S rRNA and presented as copies relative to viral genomes. Asterisks indicate significance (p < 0.05; Mann Whitney test).
Explant reactivation from TGs infected with a ΔCTRL2 virus is reduced

To determine whether loss of CTCF binding altered the ability of the ΔCTRL2 mutant virus to reactivate, we harvested latently infected TGs and explanted them onto a monolayer of Vero cells. We tested individual ganglia from two independent infections of 5 mice and 10 mice per virus, respectively. We assessed the emergence of infectious virus for 7 days post explant by collecting overlay media, or the underlying Vero cell monolayer by titration on a fresh Vero monolayer. Infectious virus was first detected at 3 days from CTRL2R virus infected ganglia, and appeared first at 4 days from ΔCTRL2 virus-infected ganglia. At 7 days post explant, 77% of CTRL2R virus-infected ganglia produced infectious virus, while only 53% of ΔCTRL2 virus-infected ganglia produced detectable virus (p = 0.0234) (Figure 4.7). These results indicated that despite similar numbers of latent viral genomes present at day 28, genomes from ΔCTRL2 virus infection showed reduced explant reactivation competence.
Figure 4.7 Explant reactivation is reduced with ΔCTRL2 mutant virus. Fifteen mice were infected with $2 \times 10^5$ PFU/eye of ΔCTRL2 or CTRL2R virus. At 28 dpi individual ganglia from each mouse were isolated and explanted onto Vero cell monolayers. Supernatant was collected daily from the overlay media for 7 days. At 7 days the underlying cells were also collected. All samples were re-plated onto fresh Vero monolayers to detect infectious virus. Asterisk indicates significance ($p < 0.05$; log-rank Mantel-Cox test).
4.5. DISCUSSION

The adjacent and overlapping LAT and ICP0 transcriptional units are independently regulated during viral latent infection to maintain suppression of ICP0 expression while allowing persistence of LAT transcription (Stevens et al., 1987). The different expression patterns have been posited to relate to differential chromatin modifications that are found at the LAT and ICP0 promoters and are maintained despite the relative proximity of these genetic elements (Cliffe et al., 2009; Kubat et al., 2004a; Kubat et al., 2004b; Kwiatkowski et al., 2009; Wang et al., 2005). While ICP0 and other lytic gene promoters are generally associated with high levels of heterochromatin during latent infection, the LAT region is the only region to also show enrichment of euchromatin modifications (Cliffe et al., 2009; Kubat et al., 2004a). CTCF is a key mediator of the ability to maintain independently regulated but adjacent chromatin domains within mammalian and viral genomes (Bell et al., 1999; Felsenfeld et al., 2004). In this study we demonstrated that CTCF bound to a region within the LAT intron sequences performed as a functional insulator. Thus, CTCF established an isolated chromatin domain at the LAT promoter and enhancer sequences by blocking the spread of encroaching heterochromatin from silenced lytic genes. Loss of this CTCF binding element resulted in disregulation of LAT expression, altered viral pathogenesis in the mouse model system, and a reduction in reactivation potential. These studies argue that CTCF is an important regulator at many stages in the viral life cycle, including establishment and/or maintenance of latency, as well as reactivation.
CTCF is associated with the LAT intron sequences in a CTRL2-dependent manner. We confirmed that deletion of the CTRL2 region was sufficient to reduce CTCF binding to the LAT intron sequences to background levels. CTCF is known to mediate long-range three-dimensional chromatin interactions through simultaneous association with distant binding sites; therefore, the loss of one binding site has the potential to alter CTCF binding to distant sites (Majumder and Boss, 2010; Ohlsson et al., 2001; Phillips and Corces, 2009; Splinter et al., 2006). Our study identified a reduction of CTCF binding at the ICP0 promoter region after deletion of the CTRL2 site, suggesting that CTRL2 and a region near the ICP0 promoter may bind CTCF cooperatively. An interaction between these sites could form a closed chromatin loop around ICP0 to ensure its isolation from the nearby LAT promoter. However, there are CTCF binding sites at many regions of the HSV-1 genome, and further work is required to characterize these sites, their role in the 3D HSV-1 genome architecture, and their interaction with the LAT region sequences.

Removal of CTCF binding to CTRL2 alters viral infection in a mouse model. Deletion of the CTCF binding site at the CTRL2 region did not affect viral replication in lytic infection of cultured Vero and HFF cells or acute infection at the ocular epithelium for the first four days of infection. These results indicate that initial lytic infection is not affected by CTCF binding to CTRL2. However, we see a reduction in shed virus in the eye of mice at five days post infection upon CTRL2 deletion. We speculate that this may be a consequence of altered acute infection of neurons in the absence of the CTCF
binding site, which may limit the ability of the virus to return from the trigeminal ganglia back to the site of primary infection within the eye. Interestingly, despite reduced viral shedding at the site of primary infection, ΔCTRL2 virus infection increased mortality compared to the WT rescued virus. Mortality is a consequence of viral spread in the brain and encephalitis further supporting the idea that CTCF binding to CTRL2 alters infection within neuronal tissue after infection at the epithelium. The mechanism by which CTCF limits pathogenesis is unknown; however, because latent infection is established during the time we see an altered viral phenotype, we suggest that deletion of CTRL2 may alter progression of genome silencing or may affect the kinetics of LAT transcription, possibly leading to increased neuronal cell death or increased immune activation.

Interestingly, genome copy number within the TGs at 28 dpi is equivalent between ΔCTRL2 and CTRL2 virus infection, indicating that similar numbers of viral genomes establish or are maintained during latent infection. Unfortunately, this assessment is somewhat confounded because it does not account for mice that succumb to infection prior to day 28. These mice likely represent populations of higher viral loads in the ganglia, and the higher proportion of fatality in ΔCTRL2 infected mice may result in a lower-than-expected viral copy number at day 28. Additionally, this measurement is performed on homogenized whole ganglia, and equivalent viral genome numbers may not represent equivalent numbers of neurons harboring virus due to variations in copy number on a single cell level. Techniques that isolate individually
infected neurons and measure viral genome content are needed to resolve this limitation.

Results from the related gammaherpesviruses EBV and KSHV suggest that CTCF has a complex role in genome maintenance during latent infection. Removal of CTCF binding sites from the intron of the LMP2A gene of EBV resulted in a higher viral genome copy number in latent infection, possibly as a result of partial lytic replication or aberrant latent replication (Chen et al., 2014). Surprisingly, results from KSHV indicated that removal of CTCF binding sites from the intron of its major latency-associated transcript reduces latent genome copy number due to reduced viral episome maintenance (Stedman et al., 2008). Given that CTCF binding has such diverse effects among related herpesviruses, it is possible that CTCF binding exhibits both positive and negative regulation on viral genome copy number. To address these questions, future studies that address the progression of latency establishment before 28 days and the maintenance of long-term latent viral genomes are needed to differentiate between initial viral dose encounters within the ganglia, viral replication within the neural tissue, and long-term viral genome maintenance.

Histone modifications at the LAT promoter sequences are affected by CTCF removal. The accumulation of H3K27me3-modified heterochromatin at the LAT promoter is consistent with the hypothesis that CTCF functions as an insulator to block the linear spread of heterochromatic modifications from the lytic ICP0 region to the LAT transcriptional regulatory regions. However, contrary to our initial hypothesis, the
increased H3K27me3 heterochromatin does not correlate with decreased LAT expression. Instead, infection with the ΔCTRL2 virus demonstrates increased LAT transcript accumulation relative to infection with the CTRL2R WT virus. The H3K27me3 histone modification, is also associated with bivalent chromatin domains (Bernstein et al., 2006), and a previous study of HSV-1 found that increased levels of H3K27me3 at the ICP8 promoter relative to the viral U₅48 promoter correlated with higher levels of ICP8 RNA than U₅48 RNA (Cliffe et al., 2013). Therefore, H3K27me3 alone may be insufficient to silence transcription or additional histone modifications, such as H3K4me3, may exert a dominant effect to maintain active transcription. Ultimately, silencing may depend on the proportion and combined effects of many epigenetic modifications, which may be illuminated by expanding future studies to include additional histone modifications.

Alternatively, these results may also be explained by a non-uniform distribution of H3K27me3 among different copies of the LAT gene. Each viral genome contains two copies of the LAT gene, and latently infected neurons harbor multiple viral genomes, resulting in known variation in the level of LAT accumulation per cell (Chen et al., 2002b; Sawtell, 1997). Thus, higher levels of LAT expression may originate from a different population of LAT sequences than those that accumulate increased H3K27me3 at the LAT promoter. Additionally, the H3K27me3 modification accumulates gradually from 10 to 14 days during the establishment of latent infection, and is enhanced by presence of the LAT transcripts (Cliffe et al., 2013; Cliffe et al., 2009). Therefore, higher levels of LAT accumulation during initial infection with ΔCTRL2 may precede and
promote higher levels of H3K27me3 accumulation at the LAT sequences. This could occur before H3K27me3 is able to accumulate sufficiently to silence LAT expression, or on a subset of LAT sequences that is distinct from those expressing LATs. Further investigation with techniques able to resolve individual infected cells and viral genomes will be necessary to resolve the relationship between LAT transcripts and H3K27me3 heterochromatin.

The gammaherpesvirus KSHV also contains CTCF binding sites within its major latency-associated transcript coding sequences, and deletion of these binding sites had a similar effect of elevating latency-associated transcript accumulation (Kang et al., 2013). The increase in latency-associated transcripts concurrently reduced transcription from a distal CTCF binding site. Kang et al., suggest that within the latency transcript region CTCF and nucleosomes compete for binding sites, such that CTCF prevents nucleosome occupancy and affects downstream nucleosome phasing. Alternative nucleosome positioning in turn affects RNAPII and RNAPII-accessory factor association to disrupt transcript splicing and elongation, while gaps in nucleosome spacing block the linear signal propagation of histone modifications from regions downstream of the latency control region (Kang et al., 2013). Although in our experiments total nucleosome occupancy within the LAT region is not different in ΔCTRL2 or CTRL2R infection, our current ChIP protocols lack the resolution to detect subtle changes in nucleosome positioning. Therefore, removing CTCF binding sites could position nucleosome binding to promote RNAPII transcription or splicing of the stable 2.0 kbp LAT intron, while simultaneously allowing the spread of H3K27me3 by creating an unbroken chain of
nucleosomes from a region of recruitment within the *ICP0* sequences to the adjacent *LAT* region.

**The role of CTCF in latent infection.** To establish a successful latent infection, HSV-1 must carefully limit cellular repression to persist in an undetectable inactive but reactivation competent state. Collectively, these results indicate that HSV-1 has exploited the cellular protein CTCF to isolate and maintain control of specific regions within the viral genome. We propose a model in which CTCF bound at the *CTRL2* region restricts the spread of heterochromatin to the *LAT* sequences to facilitate continued LAT expression during latency, while also preventing excess accumulation of LAT transcripts, to prevent excessive accumulation of heterochromatin throughout the genome to maintain reactivation potential. Additional CTCF binding sites outside of the *CTRL2* region may also maintain lytic genes in a poised expression state by limiting both transcriptional activity and heterochromatin accumulation. The loss of CTCF has diverse effects on pathogenesis, latency, and reactivation; therefore, determination of the mechanism of CTCF activity may illuminate the fundamental mechanisms underlying the switch between lytic and latent infection.
Chapter 5. Perspectives and Conclusions
5.1. SUMMARY OF RESULTS

The research presented in this dissertation was directed at understanding how HSV-1 controls epigenetic regulation and how this influences lytic and latent infection. Previously, lytic infection has been characterized by limited histone accumulation and the acquisition of euchromatic modifications to maintain active gene expression. In the second chapter we hypothesized that HSV-1 would also regulate heterochromatin modification during productive infection. We used ChIP techniques to measure the association of total histone H3, as well as H3K9me3 and H3K27me3 modified histones with HSV-1 genomes at hourly intervals following infection. We found that histones and heterochromatin modifications were highly dynamic and independently regulated. Accumulation of total histone H3, H3K9me3, and H3K27me3 peaked in the first two hours post infection, after which total H3 declined steadily throughout infection. Levels of H3K9me3 and H3K27me3 also declined throughout infection; however, the relative proportion of H3 modified with K9me3 or K27me3 decreased rapidly after four hours. Additionally, while the removal of H3 and H3K9me3 was not dependent on viral replication, H3K27me3 removal was inhibited with PAA treatment to block viral DNA synthesis.

We hypothesized that the viral transactivator, ICP0, could mediate transcriptional activation partially through the removal of heterochromatin to relieve epigenetic silencing. Indeed, we found that ICP0 was critical for the removal of histones and heterochromatin during lytic infection, although it did not limit the initial accumulation of histones or heterochromatin. We tested two ICP0-mutant viral strains that were deficient
for ICP0 transcription or ICP0 protein expression and consistently saw no difference between the two strains, arguing that this ICP0 function requires full-length ICP0 protein and not just transcription of its gene.

In the third chapter, we examined the structure of HSV-1 chromatin during latent infection of neurons. In latent infection HSV-1 persists for the lifetime of the host, maintaining limited viral gene expression, and remaining poised for reactivation. Evidence suggests that epigenetic mechanisms define and determine the establishment, maintenance, and reactivation of latent infection. We hypothesized that ICP0 would also limit epigenetic repression of HSV-1 during latent infection. ICP0 promoted acute replication of the virus and accumulation of latent viral genomes. However, surprisingly, we found that, ICP0+ viruses accumulated increased heterochromatin, particularly H3K27me3, relative to ICP0-mutant virus strains. This suggests that ICP0 does not simply remove or prevent epigenetic repression during latent infection.

In the fourth chapter, we tested the insulator capacity of CTCF bound to the LAT intron sequences. We found that removal of the proposed insulator region inhibited CTCF binding, and increased accumulation of H3K27me3 at the adjacent LAT promoter and enhancer sequences. This argues that CTCF prevents the linear spread of heterochromatin from the ICP0 region to the LAT regulatory sequences and supports the role of CTCF as an insulator of the LAT region. However, the accumulation of heterochromatin at the LAT region did not reduce the accumulation of LAT transcripts. We conclude that epigenetic regulation of the LAT sequences is likely dependent on
multiple factors in addition to H3K27me3 and propose that CTCF likely mediates additional functions to affect latent infection.
Eukaryotes use epigenetic modifications to regulate many aspects of their own genome. In metazoan species, epigenetic regulation allows for the differentiation and varied transcription of many cell-types all carrying the same genome. This parallels the ability of epigenetics to differentiate between lytic or latent patterns of HSV-1 gene expression and infection. However, eukaryotes also have evolved defensive epigenetic mechanisms to assemble chromatin onto foreign naked DNA to suppress expression of foreign DNA and inhibit viral infections. In this dissertation, we have described several situations where HSV-1 has evolved to counteract or co-opt cellular epigenetic mechanisms to promote the viral life cycle.

Mechanisms of heterochromatin regulation in lytic infection. In lytic infection, the cellular chromatinization response and the accumulation of heterochromatin are generally described as a barrier to productive infection that must be overcome. To achieve effective lytic infection, HSV-1 must redirect cellular transcription machinery to coordinate the expression of over 80 viral genes in a kinetically ordered cascade. We hypothesize, that rather than an obstacle, epigenetic repression could function as a mechanism to restrict aberrant gene expression and regulate the kinetics of expression through selective derepression. This is consistent with reports suggesting that depletion of the histone chaperone Asf1a reduced histone association with viral IE gene promoters and upregulated IE gene expression, but ultimately resulted in reduced viral growth and replication (Oh et al., 2012). It is therefore possible that HSV-1 has evolved
mechanisms to work with as well as against heterochromatin accumulation early in lytic infection.

We hypothesize that HSV-1 removal of heterochromatin occurs as a multi-step process to carefully fine-tune viral gene expression. In primary human foreskin fibroblasts infected with HSV-1, we find that a viral E gene promoter initially accumulates histones and heterochromatin, presumably as the result of a cellular attempt to silence it. The levels of histone H3, H3K9me3, and H3K27me3 remain proportional for the first four hours post infection, throughout the period of their rapid accumulation and initial removal. Different proteins catalyze the addition and removal of the H3K9me3 and H3K27me3 modifications, making it unlikely that specific targeting of both modifications would result in identical kinetics. Therefore, we believe that initial epigenetic regulation targets histones in their entirety rather than specific histone tail modifications. After the first four hours post infection it appears that H3K9me3 and H3K27me3 are removed independently of total H3 and each other, suggesting specifically targeted modification of chromatin. Interestingly, removal of H3K27me3 at later times is dependent on viral DNA replication. This may indicate that the apparent reduction of H3K27me3 is actually due to dilution with newly synthesized viral genomes. Alternately, H3K27me3 removal may require the disruption and reassembly of nucleosomes during viral DNA synthesis, or transcription of a viral L gene.

The two stages of heterochromatin removal may be biologically as well as mechanistically significant. The first step could reduce or reposition nucleosomes away from viral promoter sequences to expose critical viral sequence elements. This would
facilitate recruitment of the RNAPII pre-initiation complex and other transcription factors to poise genes for expression. If this proves correct, we expect histone mapping to identify regions of preferential nucleosome depletion around viral E gene promoters and transcription initiation sequences. Additionally, we would expect enrichment of transcription factors and the RNAPII pre-initiation complex at these regions, but absent from the downstream gene bodies. The second step targets removal of specific heterochromatin modifications. This could enhance recruitment of transcription factors or initiate transcription and elongation from poised or paused polymerase. At this stage, we would expect transcriptionally active RNAPII complexes to be found at regions downstream of the transcription start site within gene bodies and an accumulation of processed mRNA transcripts. The stepwise removal of repressive chromatin could ultimately serve to regulate expression kinetics by assuring that transcription is poised for synchronous activation and rapid amplification, but dependent on signaling for initiation.

The mechanism of ICP0-dependent regulation of histone methylation. Current data suggests that reversing epigenetic suppression is a critical function of the viral transactivator proteins, VP16 and ICP0. It is known that VP16-mediated transactivation of IE viral gene expression depends on recruitment of the HCF1-Oct1 complex (Gerster and Roeder, 1988; Kristie et al., 1989), which in turn recruits various transcription factors including the histone demethylases, LSD1 and JMJD2, which remove H3K9me2/me and H3K9me3, respectively (Liang et al., 2013b; Liang et al., 2009).
However, the mechanism of ICP0-mediated transactivation of viral genes is less clearly defined. ICP0 can affect epigenetic regulation by disrupting histone deacetylases in conjunction with CoREST (Gu et al., 2005), and by recruiting CLOCK histone acetyltransferases (Kalamvoki and Roizman, 2010). These mechanisms promote acetylation of histone tails to increase transcriptionally active euchromatin and may also destabilize histone-DNA interactions to promote histone dissociation. This agrees with previous reports that identify reduced accumulation of acetylated histones late in infection with ICP0-null viruses (Cliffe and Knipe, 2008), and with our findings that detect reduced histone H3 association as infection progresses.

We argue that ICP0 also mediates the removal of heterochromatic modifications to facilitate gene expression. Histone and heterochromatin levels are independent of ICP0 in the first four hours post infection. After this, during infection with WT HSV-1 expressing full-length ICP0 protein, histones and heterochromatin modifications are removed as infection progresses and transcription is initiated. During infection with ICP0-mutant viruses the chromatin structure remains stable for at least twelve hours, and is unaffected by transcription through the ICP0 gene. This argues that ICP0 is necessary for efficient removal of histones and the H3K9me3 and H3K27me3 histone tail modifications.

The mechanism by which ICP0 regulates histone methylation has yet to be defined, and there is no evidence for direct interaction with histone demethylases. Indirectly, ICP0 degrades the DNA-sensor IFI16 to prevent activation of the intrinsic immune response and downstream accumulation of H3K9me3 (Orzalli et al., 2013).
However, in our experimental system, we see inhibition of removal, but do not see enhanced recruitment of H3K9me3. Additionally, in the absence of ICP0, we see effects to total histone levels, and H3K27me3 levels. This suggests that multiple pathways contribute to ICP0-mediated epigenetic regulation.

To discern mechanisms by which ICP0 regulates the structure of viral chromatin, we will need to identify the specific factors involved and the kinetics of their association at viral gene promoters. However, because the period of interaction with specific elements appears to be brief and varied over the course of infection, identification of the complete set of epigenetic regulators will depend on careful and thorough sampling to detect transient events. We hypothesize that the initial stage of chromatin modification likely employs chromosome remodelers to remove or reposition nucleosomes. VP16 has been reported to recruit remodelers to IE genes, and ICP0 may similarly recruit remodeling factors to viral sequences (Herrera and Triezenberg, 2004). A screen for factors that regulate HSV-1 gene expression identified several chromatin-remodeling factors (Oh et al., 2014). Alternatively, epigenetic regulation may initially depend on histone chaperone proteins to facilitate assembly and disassembly of nucleosomes, and regulate histone-variant selection. Rapid exchange of histones and incorporation of specific histone-variants has been observed in HSV-1 infection (Conn and Schang, 2013; Lacasse and Schang, 2012; Placek et al., 2009) and the histone chaperones HIRA (Placek et al., 2009), Asf1a (Oh et al., 2012), and Asf1b (Peng et al., 2010) have been associated with HSV-1.
Our studies argue that ICP0 participates mainly in the second stage of targeted reduction of methylated heterochromatin. However, the mechanism of ICP0-dependent control of histone-tail methylation is unclear. Isolation of chromatin-modifying factors that are found localized at viral gene promoters during the period of ICP0 transactivation may illuminate pathways by which ICP0 regulates the epigenetic environment. ICP0-null viruses may also help to confirm the two-stage epigenetic derepression model. If our model is correct, ICP0-null viruses will recruit polymerases and transcription factors to viral promoters, but transcript elongation and maturation will be inhibited in the absence of ICP0-dependent removal of H3K9me3 and H3K27me3 heterochromatin.

**Possible mechanisms for ICP0-dependent promotion of heterochromatin in latent infection.** During neuronal infection, host-mediated epigenetic silencing of the viral genome occurs more slowly in the weeks following infection, likely due to low levels of free histones in resting neurons. Ultimately HSV-1 assembles regularly spaced nucleosomes throughout its genome and utilizes epigenetic repression to restrict viral lytic gene expression and persist within neurons. The results presented in chapter three suggest that the presence of ICP0 promotes heterochromatin accumulation in latent infection. This is surprising, given the evidence that the known functions of ICP0 are to enhance lytic gene expression by promoting euchromatin and reducing association histones and heterochromatin.

We propose two alternate but not mutually exclusive models to reconcile the surprising role of ICP0 in latent infection. First, ICP0 could perform a unique function in
latently infected mouse trigeminal ganglia. This is supported by studies that find ICP0-dependent reduction of histones and enhancement of histone acetylation is cell-type dependent (Hancock et al., 2010). While the mechanism of ICP0-dependent histone demethylation has not been defined, this may also exhibit cell-type dependence, potentially to the extent that ICP0 could mediate retention rather than removal of heterochromatin in neurons. Identification of the pathway by which ICP0 removes heterochromatin in lytic infection will allow us to assess cell type specific differences. ICP0 may also interact with a unique factor expressed in neurons, but not epithelial cells. Such a partner could compete for the limited supply of ICP0 and abrogate its ability to reduce epigenetic silencing. Proteomic studies of ICP0-interacting proteins in neurons may therefore reveal the true function of ICP0 in latent infection.

A second possibility is that the cellular response could fail to assemble or maintain heterochromatin in the absence of ICP0. Neurons are non-dividing, and consequently have a smaller free pool of histones. Consequently, histone mobilization or the recruitment of epigenetic silencing factors may have a higher threshold of activation in neurons. In this study we find that ICP0-null viruses are replication defective, less pathogenic, and establish fewer latent viral genomes in mouse TGs. Therefore, reduced viral load and limited neuronal replication may limit immune sensing of naked viral genomes. Additionally, ICP0 is itself immunogenic, and can also promote expression of additional viral genes that may further activate an immune response. The absence of ICP0 may therefore reduce the inflammatory or cellular adaptive immune responses. Consequently neurons may not be primed to activate an initial antiviral
response, or may be insufficiently stimulated to maintain epigenetic silencing during long-term latent infection. Other studies have also suggested that viral gene silencing in neurons depends on IFN activation (Zerboni et al., 2013) and that maintenance of latency depends on immune effector cells including dendritic cells (Mott et al., 2014) and CD8\(^+\) T cells (Liu et al., 2000). To test this hypothesis, the neuronal response to infection with an ICP0-null virus will need to be examined. This will include measuring changes to the neuronal gene expression profile throughout the establishment and maintenance of latency to provide evidence for differences in intrinsic or innate immune signaling, and additional support through assessment of the cytokine profile or cellular immune response. Alternately, construction of ICP0-null viruses that have an additional TK-null mutation could compensate for differences in neuronal pathogenesis.

Ultimately, ICP0 appears to be an important regulator of both lytic and latent HSV-1 infection and may therefore also be an ideal target for the development of therapeutic inhibitors. Our results suggest that inhibiting ICP0 could both limit the productive replication of HSV-1 at epithelial sites by promoting epigenetic repression, while limiting the ability of ICP0 to sustain a latent infection within neurons.

**Possible mechanisms of CTCF dependent regulation of latent infection.** Previous studies have focused predominantly on HSV-1 manipulation of the balance of histone association with heterochromatic and euchromatic modifications. Herpesviruses also exploit the multifunctional cellular insulator binding protein, CTCF, to regulate viral epigenetics. CTCF has an exceptional number of defined activities and functional
mediators. Therefore, we can only so far speculate on the mechanism of CTCF regulation in HSV-1 latent infection. Deletion of the *CTRL2* site results in local changes including heterochromatin accumulation at the *LAT* sequences, and increased *LAT* transcript accumulation, as well as general effects such as increased pathogenesis and reduced reactivation. One possible explanation to explain this phenomenon may not rely on histones or heterochromatin regulation, but may depend on CTCF interaction with RNAPII. CTCF can affect RNAPII recruitment, and can also regulate transcriptional elongation and pausing (Ong and Corces, 2014). Delayed elongation can facilitate intron splicing, affect RNA secondary structure, and influence other post-translational RNA processing events, including the processing of miRNAs. Several miRNA species can be spliced from the *LAT* transcripts, and have been shown to reduce lytic gene expression (Umbach et al., 2008). Therefore, the effects of *CTRL2* deletion may be related to changes in viral miRNAs. To test this hypothesis, we will need to measure the accumulation of viral miRNA species in latent infection. Additionally, assessing RNAPII recruitment, activation, and pausing near the *CTRL2* site would support this hypothesis.

Ultimately, to more fully understand the effects of *CTRL2* deletion on epigenetic regulation, a thorough characterization of the epigenetic modifications that are affected throughout the entire genome, using ChIPseq, as well as comparison to different stages of viral infection including establishment and reactivation of latent infection is needed. These studies in concert with complete profiling of viral transcription throughout neuronal infection may further explain the downstream effects we see to viral pathogenesis, and reactivation.
Assessment of the entire genome is additionally valuable because it is increasingly evident that epigenetic regulation relies on the topology of chromatin. We hypothesize that $CTRL2$ may partner with additional CTCF binding sites within the HSV-1 genome to form chromatin loops. Chromosome conformation capture (3C), and subsequently developed high-throughput methods, can detect long-range chromatin interactions and can confirm this hypothesis. If this proves true, we anticipate that removal of $CTRL2$ may also result in epigenetic changes at distal regions of the viral genome that are adjacent to partner insulator sites. Additionally, association of the $LAT$ regulatory sequences with distal sites and the genes located near them may help to explain the effects we see to chromatin, gene transcription, pathogenesis, and reactivation after $CTRL2$ removal. Interrogating the role of additional CTCF binding sites throughout the genome, both independently and in combination with $CTRL2$ site, will likely support the significance of CTCF to regulation of HSV-1 latent infection.
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