REGULATION OF THE HERPES SIMPLEX VIRUS TYPE-1 (HSV-1) LATENCY-ASSOCIATED TRANSCRIPT (LAT)

By

NICOLE V. GIORDANI

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2007

ACKNOWLEDGMENTS

First and foremost, I thank my parents and family for encouraging me to follow whatever career path I chose. I wouldn't be where I am today without their support and love, and I will always be grateful.

I thank my undergraduate mentor, Richard Hallick, for giving me the chance to get my start in a lab. In addition, I thank his post-doctoral associate, Elena Sheveleva, for her patience and kindness during her days of working with me.

I especially thank Dave for his guidance over the past few years. He has taught me a lot about many aspects of science and has always provided encouragement, even when I was not sure that I could do it. I have always appreciated the enthusiasm with which he approaches science, and I hope some of it has worn off on me.

I thank all of the graduate students (Niki, Jerry, Anne, Lee, Tony, Zane, and Dacia) that I worked with over the years for each teaching me something and helping me whenever I needed it. To list what I have learned from each of them would be impossible, since the knowledge they have passed on to me has been insurmountable. I also thank Dacia for critical reading of this dissertation and for helping me to deal with the stress. I thank Peterjon ("the best tech ever") for all of his computer and cell culture assistance and for helping me with so many other things here and there. I thank Joyce for her expertise with cloning and for always offering to help when she sees the work piling up.

I thank my committee—Jorg Bungert, Rich Condit, Bert Flanegan, and Dick Moyer—for their helpful suggestions and for keeping me on track.

Many other people have helped shape me as a scientist, and I thank all of them.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

REGULATION OF THE HERPES SIMPLEX VIRUS TYPE-1 (HSV-1) LATENCY-ASSOCIATED TRANSCRIPT (LAT)

By

Nicole V. Giordani

August 2007

Chair: David C. Bloom
Major: Medical Sciences—Immunology and Microbiology

Herpes simplex virus type 1 (HSV-1) establishes latency in neurons until stimulated by stress to reactivate. The latency-associated transcript (LAT) region is the only portion of the latent genome that is actively transcribed during latency. While the LAT region is known to facilitate reactivation, the exact mechanism involved is not known. The overall focus of this dissertation was to investigate elements in the LAT promoter that respond to stress and alter transcription in this regulatory region.

Here, the chromatin profile of a reactivation-negative HSV-1 LAT promoter mutant, 17ΔPst, was assessed prior to and following adrenergic induction of reactivation in the rabbit ocular model. In contrast to the latent chromatin profile of wild-type 17*syn*+, 17ΔPst showed increased enrichment of dimethyl H3 K4 just upstream of the deleted region, suggesting that deletion of the LAT promoter causes a loss of a regulatory element required for initiation of reactivation. Post-induction of reactivation, no apparent chromatin remodeling occurred for either virus during early times (1, 2, 4 hours).

To investigate a possible regulator of the LAT promoter, a cAMP response element, located 83 nucleotides upstream of the LAT transcriptional start site (-83CRE) was examined. A recombinant virus with mutation of the -83CRE was created and analyzed for alterations to the

acute, latent, and reactivation phases of the viral lifecycle. The -83CRE recombinant was found to have a second site mutation somewhere in the genome. During acute infection, the -83CRE recombinant displayed attenuated virulence in mice when infected via the footpad, and in cell culture the virus showed increased replication in epithelial cells and decreased replication in neuronal cells, suggesting that the mutations in the -83CRE recombinant play a role in viral replication. The -83CRE recombinant virus was also found to establish latency, express LAT, and reactivate from latently-infected rabbits.

These results indicate that the LAT promoter contains elements that regulate transcription at the level of chromatin, which may play a key role in facilitating stress-induced reactivation. In addition, data from analyses of the -83CRE recombinant suggest that a compensatory mutation generated during viral construction plays a role in virulence and may interact with the -83CRE site to regulate lytic infection. Overall, this study invokes a model where the LAT promoter acts as complex regulatory switch that modulates gene expression in a tissue-specific manner both during the acute and latent periods of HSV-1 infection.

CHAPTER 1
INTRODUCTION

Basic Overview

The virus family *Herpesviridae* is composed of a number of double-stranded DNA (dsDNA) viruses that share patterns of gene expression and the ability to undergo latency, a state in which the viral genome is virtually shutdown until reactivated (reviewed in Wagner and Bloom, 1997). There are herpesviruses that infect a wide range of hosts, from mollusks to mammals. In humans there are eight known herpesviruses, with different primary sites of infection and cell types in which they can become latent. Herpes Simplex Virus type-1 (HSV-1) is a member of the α-herpesvirus subfamily, a group whose members—HSV-1, HSV-2, and Varicella-Zoster Virus—have a tropism for neuronal cells during latency.

HSV-1 is very common in the population, with approximately 60-90% seropositivity in the adult U.S. population (Smith and Robinson, 2002). While the virus usually only causes painful, inconvenient cold sores, it can also cause herpes keratitis, the leading cause of infectious blindness in the U.S. (reviewed in Biswas and Rouse, 2005), and in rare instances, can cause encephalitis and even death (reviewed in Higgins et al., 1993). There is no cure for HSV-1, and while drugs affecting specific viral targets during the periods of active replication (i.e., aciclovir interferes with HSV-1 DNA replication) can limit productive infection, they are not effective against the latent virus. Thus, by studying latency and reactivation, we can better our understanding of HSV-1, which can eventually lead to better treatment options and possibly yield a way to prevent reactivation.

Productive Infection

An infectious herpes virion consists of a glycoprotein-spiked envelope surrounding the amorphous tegument, an icosahedral capsid, and the genome-containing core (Figure 1-1). The

HSV-1 genome is large when compared to other viruses (approximately 152 kilobases (kb)) and encodes approximately 80 gene products during productive infection of the mucosal epithelium, the usual site of entry. The expression of genes is temporally ordered, with immediate-early genes being made before early genes which are made before late genes. Virions can also enter sensory neurons, where the virus can enter latency as a nucleosome-associated, nonreplicating episome (Rock and Fraser, 1985; Deshmane and Fraser, 1989) until stressing stimuli induce the viral genome to reactivate from its quiescent state. At this time the virus can be transported by anterograde axonal transport to once again cause productive infection at the epithelial surface.

The HSV-1 virion first enters its host cell through glycoprotein–receptor fusion, leaving some tegument proteins, including viral host shutoff (vhs, which degrades cellular and viral RNA), in the cytoplasm, while the nucleocapsid travels to the nucleus. At the nuclear pore, the viral DNA is released into the nucleus. Accompanying the viral genome into the nucleus is the tegument protein, VP16, which interacts with cellular factors to enhance transcription of the virus's immediate early genes.

Once the viral genome is in the host cell nucleus, the lytic cascade of transcription can commence. Each class of genes must be transcribed in order for transcription of the next class to begin. The first group of genes made is the immediate-early (IE or alpha) genes. There are five of these genes (ICP4, ICP0, ICP27, ICP22, and ICP47), and this class provides the gene products necessary for the expression of the next gene class, the early (E or beta) genes. The early genes are crucial for replication and include polymerase, proteins for DNA and ORI binding, and the helicase/primase complex. Expression of this class is down-regulated after the start of replication of the viral genome. The final group of genes, the late (L or gamma) genes, provides the more than 30 gene products coding for structural components of the virion.

Once all classes of gene products have been made, the viral capsid assembles in the nucleus and buds through the inner nuclear membrane, acquiring the tegument layer and an envelope. The virus particle is de-enveloped at the outer nuclear membrane and reacquires an envelope at the Golgi apparatus. Once the mature virion is made, virions are spread by cell-to-cell fusion, or they are released from the cell by exocytosis to initiate a second round of infection.

Latency and Reactivation

Animal Models

Studies performed in cell culture have provided much information about the biology of HSV-1, particularly molecular details of gene regulation during the acute infection. However, due to the virus's ability to undergo latency in neurons, studies in cell culture have been limited. While several groups have managed to induce a shut-down state of the viral genome in cell culture (O'Neill et al, 1972; Preston et al, 1991; Moriya et al, 1994), none have been shown to sufficiently mimic *in vivo* latency, so the most relevant means to study HSV-1 latency and reactivation is through the use of animal models. Both the mouse and the rabbit have been invaluable to the advancement of the understanding the latent phase of HSV-1 infection.

Mouse footpad model. There are several models of latency and reactivation that employ the mouse. Once the mouse is infected through the eye, footpad or other route of epithelial inoculation, the virus will establish a latent infection within the sensory ganglia that enervate the region of primary infection. Reactivation is most often induced by hyperthermia or explant of ganglia. Clinical reactivation or virus shedding at the site of initial inoculation does not reproducibly occur, thus placing a limitation on the relevance of the mouse models to human infection. However, mouse systems are more relevant than cell culture, are cost-efficient, and

while reactivation occurs in an *in vitro* manner, the mouse provides useful insight into the molecular events related to latency and reactivation.

For the purposes of this dissertation, of the existing mouse models, only the mouse footpad model has been used for the experiments herein, and therefore, this will be the only mouse model for latency and reactivation that is discussed; this is not intended to trivialize the other murine systems but only to provide the background for later discussion.

In the mouse footpad model, the keratinized epithelia of the rear footpads are first softened by injection of saline. The footpad is then abraded with an emery board, allowing a direct route for viral entry into the anesthetized animal. The virus replicates in the footpad and then enters the peripheral nervous system, traveling to the dorsal root ganglia (DRG), the spinal cord and even the brain. If inoculated with a virulent strain of HSV-1, mice succumb to viral encephalitis at approximately day 7 to 10 post-infection. In surviving mice the virus becomes latent in neurons of the DRG, and after 21 to 28 days, there is no longer evidence of acute infection (lytic viral transcription has ceased, the LAT is expressed, and infectious virus is no longer present). At this time the DRG can be dissected from the mouse, and the latent state can be assessed or the DRG can be explanted to supplemented media for a given length of time, in order to examine molecular events that occur in response to stress. Additionally, the latently-infected DRG can be co-cultivated on a cell monolayer to analyze the kinetics or efficiency of explant-induced reactivation.

While these methods are not considered to necessarily mirror *in vivo* or clinical reactivation, DRG explant still provides useful information and can serve as a starting point for investigation of this process.

Rabbit eye model. In human HSV-1 infection, latency occurs in the trigeminal ganglia (TG), and reactivation occurs at the site of primary infection, commonly the mucosal epithelia. This is similar to what is observed in the rabbit eye model—rabbits are infected through the corneas, latency occurs in the TG, and upon reactivation infectious virus can be recovered at the site of initial infection. In addition to spontaneous reactivation, it is possible to induce reactivation in latently-infected rabbits. Iontophoresis of epinephrine via a direct current to the eye has been shown to reliably induce viral shedding from latently-infected rabbits at high frequencies (Kwon et al., 1981). Because epinephrine is a hormone released in response to stress, the use of it seems quite relevant. In fact, when rabbits are administered propanolol, a blocker of the β-adrenergic receptors that bind epinephrine, there is a significant decrease in the levels of spontaneous reactivation (Kaufman et al., 1996). At the present time, the rabbit eye model is arguably the most relevant model for reactivation studies.

The Latency-Associated Transcript (LAT)

When HSV-1 becomes latent in a neuron, there is an overall shutdown of the genome, with the exception of the latency-associated transcript (LAT) region (Stevens et al., 1987). The LAT is approximately 8.3–8.5-kb in length (Dobson et al., 1989; Mitchell et al., 1990), and the primary transcript can be spliced to yield 2.0- and 1.5-kb species (Wagner et al., 1988; Wechsler et al., 1988). The 2.0-kb LAT has been demonstrated to be a stable intron (Farrell et al., 1991), with a half-life of almost 24 hours in cell culture (Thomas et al., 2002), while the smaller 1.5-kb appears to be a smaller splice product of the 2.0-kb intron produced only in a subpopulation of neurons that express the LAT. Furthermore, studies performed with a transgenic mouse containing the LAT promoter through the 2.0-kb intron demonstrated that while the transgene was expressed in various tissue types, high levels of 2.0-kb intron accumulation occurred only in

sensory ganglia and appeared to be differentially spliced in those tissues (Gussow et al., 2006). While it seems likely that the 2.0-kb LAT intron is important to the virus when in sensory ganglia, no precise function has been ascribed to the LAT splice products, and the 2.0-kb intron is dispensable for normal reactivation (Jarman et al., 2002).

The LAT during lytic infection

While the LAT is the prominent transcriptional unit during latency, LAT is also detectable in murine TG during lytic infection (Spivack and Fraser, 1988) and is expressed as a late gene in cell culture (reviewed in Wagner et al., 1995). In addition, the LAT promoter is active in both neuronal and non-neuronal mouse tissue during the lytic phase of infection (Jarman et al., 1999), suggesting that simply expressing LAT is not enough to cause latency. Finally, one study demonstrated that in acutely-infected murine ganglia, the virus may simultaneously follow productive and latent pathways, the former showing expression of lytic genes and decreased LAT levels and the latter displaying almost no viral transcription except for the LAT (Margolis et al., 1992). The study also demonstrated that the two pathways occur in two different subsets of neurons, which likely drives the outcome of the infection into being productive or latent (Margolis et al., 1992).

The LAT during latency and reactivation

The exact role of the primary LAT in latency and reactivation is still unknown. Promoter deletion mutants that do not express the LAT retain wild-type levels of establishment and maintenance of latency (Ho and Mocarski, 1989; Javier et al., 1988; Sedarati et al., 1989; Steiner et al., 1989), arguing against a critical role for the LAT in those functions. In contrast, however, establishment of latency by a 1.9-kb deletion mutant of the LAT promoter through the 5' exon was reduced by approximately 75% relative to the parental and rescued virus; a significantly higher number of neurons in the TG were destroyed by the mutant than by the wild-type virus,

suggesting a role for the LAT in neuronal survival (Thompson and Sawtell, 2001). Neuronal survival and anti-apoptotic activities have been attributed to the LAT in several studies (Perng et al., 2000; Inman et al., 2001; Ahmed et al., 2002; Jin et al., 2003), and furthermore, a link between spontaneous reactivation and anti-apoptotic activity has been suggested (Jin et al., 2003). It has also been recently suggested that the LAT 5' exon region encodes a microRNA that regulates apoptosis, in order to permit reactivation of the virus, although the functionality of this microRNA has yet to be demonstrated in the context of an HSV-1 infection (Gupta et al., 2006).

A clear role for the LAT appears to be in reactivation. Promoter deletion mutants that do not express LAT display inefficient *in vivo* reactivation when assessed in the rabbit (Hill et al., 1990; Bloom et al., 1994; Perng et al., 1994). Additionally, through analysis of deletion mutants, it was demonstrated initially that the region necessary for reactivation (reactivation critical region, *rcr*) lies in the first 1.5-kb of the LAT (Bloom et al., 1996; Perng et al., 1996). This region was further mapped to the first 699 bp of the LAT, after mutants containing 2.0-kb intron deletions displayed wild-type levels of induced reactivation (Jarman et al., 2002). Thus, the region of the LAT that is imperative for normal reactivation extends from the core LAT promoter through the 5' exon (Figure 1-2). Additionally, this region has also been demonstrated to possess both enhancer and long-term expression functions that allow the LAT promoter to remain highly active throughout latency (Lokensgard et al., 1997; Berthomme et al., 2001).

Several studies have been performed in order to further map the *rcr*. These have involved the analysis of mutants with subdeletions in the 5' exon region, and several of interest will be discussed here. Mutant 17ΔSty contains a 370-bp deletion of most of the 5' exon (Figure 1-3). This mutant generates the 2.0-kb intron, has normal replication kinetics, and displays a normal

level of recovery when latently-infected mice TG are co-cultivated (Maggioncalda et al., 1994). In contrast, 17ΔSty shows an approximately 40% reduction in epinephrine-induced reactivation of the rabbit (Hill et al., 1996). This demonstrates the importance in validating work performed in the *in vitro* mouse model in the more *in vivo* rabbit eye model. Strangely, in contrast to the reduction in induced reactivation of rabbits by the deletion mutant, spontaneous reactivation does not appear to be altered from wild-type levels when that region is deleted (Perng et al., 1996b). This suggests that there may be more than one pathway for reactivation—one that is stress-responsive and dependent on some element within the 5' exon and one that occurs spontaneously and does not require the region.

To further address the role of the 5' exon region in reactivation, mutant 17Δ348 was studied. This mutant contains a 348-bp deletion that is located 217-bp downstream of the LAT transcriptional start site (Figure 1-3). 17Δ348 expresses LAT and establishes a latent infection in rabbits at a level similar to that of wild-type, but like 17ΔSty, this mutant displays a significantly decreased level of epinephrine-induced reactivation (Bloom et al., 1996). Surprisingly, when mutants containing overlapping subdeletions of the 348-bp region (Figure 1-3) are tested in the rabbit, levels of induced reactivation are near that of wild-type (Bloom et al., 1996; Bhattacharjee et al., 2003). This suggests that there are multiple *cis* elements in the region that play a role in reactivation and several must be deleted in order to detectably alter reactivation. The region is clearly quite complex, and there may be different means for the virus to reactivate, possibly requiring interplay between various components.

LAT Promoter and LAT Transcription

Wild-type expression of the LAT—and therefore, wild-type reactivation in the rabbit—is dependent upon the presence of a 202-bp core promoter (Dobson et al., 1989). In fact, when the core LAT promoter is deleted from wild-type 17*syn*+ to generate 17ΔPst, epinephrine-induced

rabbit reactivation (recovery of infectious virus) is significantly decreased, from approximately 79% of 17*syn*+-infected rabbits positive for infectious virus to about 13.5% of 17ΔPst-infected rabbits positive (Bloom et al., 1994). The difference is not as dramatic when the DRG or TG are explanted from the mouse to cell culture. Approximately 50% of 17ΔPst-infected ganglia reactivate (cause visible cytopathic effect when explanted to a cell monolayer) while nearly 100% of 17*syn*+-infected ganglia reactivate (Devi-Rao et al., 1994). This suggests that the presence of the core LAT promoter may have a slight effect on explant-reactivation but appears to be more important for epinephrine-induced reactivation in the rabbit. While this is likely due to a function of the LAT, it may also be due to a *cis*-acting element contained within the region.

Within the 202-bp of the core LAT promoter are several transcription factor binding sites that appear to be important for the full function of the core LAT promoter. Among the identified sites are a TATA box (Dobson et al., 1989), two cAMP response elements (CREs) (Leib et al., 1991; Kenny et al., 1994), and binding sites for the upstream stimulatory factor (USF) (Zwaagstra et al., 1991; Kenny et al., 1997) and ICP4 (Batchelor et al., 1994) (Figure 1-4).

The TATA box is necessary for normal function of the LAT promoter. When chloramphenicol acetyltransferase (CAT) assays were performed using a plasmid in which the TATA box was mutated or deleted, a reduction in promoter activity was observed relative to wild-type (Rader et al., 1993; Ackland-Berglund et al., 1995). *In vitro* transcription assays also demonstrated that the TATA box is required for full LAT expression (Soares et al., 1996). In addition, it was demonstrated that co-cultivation of cells with murine TG latently-infected with a TATA box mutant virus yielded normal levels of reactivation via this method, even though the mutant displayed significantly decreased levels of LAT expression, as assayed by *in situ*

hybridization (Rader et al., 1993). In other words, the ability of HSV-1 to express LAT does not necessarily correlate with the ability to reactivate from explanted murine ganglia.

The CRE identified in the LAT promoter located approximately 43 base pairs upstream of the LAT transcriptional start site (referred to here as -43CRE) (Leib et al., 1991) was demonstrated to bind the CRE-binding type 1 (CREB-1) protein (Millhouse et al., 1998), a known stress-responsive transcriptional activator. When the -43CRE was deleted and its promoter activity was assayed by CAT assay, a decrease of three- to four-fold relative to wild-type was observed (Ackland-Berglund et al., 1995), even though LAT expression was not affected when assayed by ribonuclease protection assay or by *in situ* hybridization (Rader et al., 1993; Ackland-Berglund et al., 1995). It was therefore suggested that the -43CRE has an inducible rather than basal activity in the context of the LAT promoter (Ackland-Berglund et al., 1995). Interestingly, the spacing of the -43CRE site relative to the TATA box appears to play a role in activity. Insertion of 10 nucleotides between the CRE and the TATA box of the LAT promoter resulted in 2-3 fold more CAT activity compared to that of the wild-type promoter, while removal of 5 nucleotides decreased activity by 6-8 fold relative to wild-type; since the 10 bp spacing reflects a turn of the DNA helix, this suggests a requirement for interaction between factors binding to the two elements (Ackland-Berglund et al., 1995).

Because the cAMP response pathway is activated in response to binding of epinephrine to cell receptors, it seems likely that the CREs in HSV-1 play some role in reactivation. However, when the -43CRE site deletion mutant was tested in the rabbit, epinephrine-induced reactivation was intermediate between wild-type strain 17*syn*+ and promoter-deletion mutant 17ΔPst (Bloom et al., 1997), suggesting that the -43CRE was not the only factor affecting reactivation. Interestingly, LAT expression during latency was similar between the -43CRE mutant virus and

wild-type (Bloom et al., 1997). This may indicate that the ability to express the LAT does not necessarily correlate with reactivation, but instead, elements within the LAT promoter may interact with cellular and viral factors to yield wild-type levels of reactivation.

A second CRE was identified between nucleotides -75 to -83 relative to the LAT transcriptional start site (-83CRE) (Kenny et al., 1994). This -83CRE was demonstrated to bind a repressive form of CRE binding (CREB) protein, CREB-2 (Millhouse et al., 1998), suggesting that this site may play a role in transcriptional repression of LAT. Other findings and speculations on the role of the -83CRE will be discussed further in Chapter 3.

In addition to the CREs, a site capable of binding USF is present in the LAT promoter (Zwaagstra et al., 1991; Kenny et al., 1997). USF binds to the E-box of a promoter and interacts with transcriptional machinery as well as with chromatin remodeling proteins (reviewed in Corre and Galibert, 2005). In bovine leukemia virus (BLV), gene expression of the 5' long terminal repeat appears to be regulated by the exclusion of CREB from a CRE that overlaps an E-box; mutation of the E-boxes appears to increase binding of CREB complexes to the CRE and also increases gene expression (Calomme et al., 2004).

Through electrophoretic mobility shift (EMS) assay, it was demonstrated that the HSV-1 LAT promoter E-box can bind either of the two forms, USF-1 and USF-2 (Kenny et al., 1997). When the -43CRE and E-box of the HSV-1 LAT promoter were mutated simultaneously and examined in an *in vitro* transcription assay, transcription levels appeared to decrease more than when either element was mutated alone, suggesting an interplay between the two (Soares et al., 1996). Neither the potential interactions between the E-box and the -83CRE, nor the effects of these elements on reactivation or LAT transcription in vivo have been investigated.

The LAT promoter, in addition to various transcription factor binding sites, also has a site to which ICP4 can bind (Batchelor et al., 1994). When this ICP4-binding site is mutated to abrogate binding, *in vitro* expression of the promoter occurs at aberrant times, displaying early gene expression, as opposed to the usual late gene expression kinetics (Rivera-Gonzalez et al., 1994). In other words, it appears that the ICP4 site may act to control LAT expression at inappropriate times, such as during productive infection and reactivation.

In summary, the core LAT promoter possesses several binding sites that appear to play some role in regulation of the LAT. It is possible that some of these function in certain cell types and at certain times during the infection to control the region. Additionally, various studies suggest interplay between different elements, indicating that this region is both complex and important.

Eukaryotic Epigenetic and Transcriptional Regulation

HSV-1 gene regulation shares similarities with that of eukaryotes, including an association with nucleosomes during latency (Deshmane and Fraser, 1989) and various eukaryotic transcription factor binding sites throughout the genome. While there are other similarities, for the sake of brevity, only the relevant aspects of epigenetics to HSV-1 transcriptional regulation will be discussed here.

Epigenetic Regulation

Epigenetics can be defined as a modification in gene expression or cellular phenotype that does not change the actual DNA (reviewed in Goldberg et al., 2007). More specifically, protein interactions with DNA are capable of causing changes in gene expression. In order for DNA to readily fit into cells, the genome is compacted into chromatin fibers, which is generally grouped into two different classes, euchromatin and heterochromatin. Euchromatin is a more relaxed structure, which allows access to the DNA by a range of transcription factors and other various

proteins, while heterochromatin is condensed chromatin with DNA that is inaccessible to transcription factors and is therefore transcriptionally inactive. The basic unit of chromatin, both euchromatin and heterochromatin, is the nucleosome, which consists of 147 bp of DNA wrapped around an octamer of four core histones (H2A, H2B, H3, and H4) (reviewed in Kouzarides, 2007). Interactions of nucleosomes and DNA can be altered by three mechanisms: post-translational modifications (PTMs), replacement with histone protein variants, and ATP-dependent chromatin remodeling (reviewed in Bernstein and Hake, 2006). However, since PTMs are more relevant than the others to the work in this dissertation, they will be discussed here, and while a variety of PTMs have been characterized, only those that are most pertinent to the work in this dissertation will be reviewed.

The addition of PTMs to the N- or C-terminal tails of histones can alter the transcriptional permissivity of the nucleosomes. Histone H3 acetylation of lysine residues 9 and 14 (K9, K14) and dimethylation of lysine residue 4 (K4) are both traditionally associated with regions of active transcription (reviewed in Li et al., 2007). A yeast microarray study found that while histone H3 K4 dimethylation was not globally correlated with promoter regions of transcriptionally active genes, there was a statistically significant association between the modification and the coding regions of active genes (Bernstein et al., 2002). In contrast, the study also demonstrated that H3 K9, K14 acetylation was associated with the promoters of active genes, as well as within coding regions, although to a slightly lesser extent. A separate study performed using genome scanning of two human cell lines revealed that of 57 active genes analyzed, 58% displayed enrichment in histone H3 K4 dimethylation and H3 K9, K14 acetylation within 500 bp of transcriptional start sites, while 28% were farther down in the coding region (Liang et al., 2004). Thus, while there may be subtle distinctions between where the PTMs are associated, it is commonly accepted that

histone H3 K9, K14 acetylation and histone H3 K4 dimethylation are markers of transcriptional activity.

Chromatin studies performed on HSV-1 have focused mainly on active marks of chromatin. During lytic infection of cell culture, it was shown that while the DNA is in a partially nucleosomal state, association of the active histone mark, acetylated H3 K9, with viral DNA occurs by one hour post-infection (p.i.) for ICP0, thymidine kinase, and VP16 (Kent et al., 2004). However, contrary to that study, Herrera and Triezenberg (2004) demonstrated that very little histone H3 (nonacetylated or acetylated) is present at the IE gene promoters examined (ICP0, ICP4, ICP27) during early lytic infection (2 h.p.i.), while the thymidine kinase, VP16 and glycoprotein C promoters are associated with acetyl-H3 K9, K14. One interesting difference in these lytic infection experiments is that the former, in which acetylated H3 K9 was associated with viral genes of all classes by 1 h.p.i., was performed in the neuronal SY5Y cell line, while the latter was performed in the more epithelial-like HeLa cells. Differential chromatin patterns may indicate that the chromatin conformation, including the association with histone H3, of the HSV-1 genome differs between cell types, possibly impacting establishment of latency.

The ability of HSV-1 to exist as a repressed episome during latency suggests that viral repression may occur at the level of chromatin. In fact, one study suggested that expression of the LAT may cause increased levels of H3 K9 dimethylation and decreased levels of H3 K4 dimethylation of lytic gene promoters during murine infection (Wang et al., 2005). When the LAT region is examined during latency, increased levels of H3 K9, K14 acetylation are observed for the LAT promoter and 5'exon/enhancer regions relative to lytic genes (Kubat et al., 2004a; Kubat et al., 2004b). Further, explant reactivation of murine DRG appears to induce dramatic changes at early times in both the LAT region's and ICP0's transcriptional permissiveness as

assayed by acetyl-H3 K9, K14, whereby the LAT region seems to decrease in acetylation before ICP0 can begin to increase (Amelio et al., 2006). Thus, the dynamic regulation of chromatin modifications appears to also impact the HSV-1 genome's transcriptional permissiveness at various stages during infection.

Promoter Elements and Transcriptional Regulation

In most eukaryotic cells, an active gene promoter contains a nucleosome-free region (NFR) approximately 150 bp in size surrounding the core promoter (reviewed in Heintzman and Ren, 2006). After the region is hyperacetylated, chromatin remodeling occurs and histone-DNA contacts are lost or nucleosome-unfolding takes place (Boeger et al., 2003; Reinke and Hörz, 2003). This allows for binding and stabilization of the transcriptional machinery to the promoter (reviewed in Heintzman and Ren, 2006).

While there are three types of RNA polymerases—I, II, and III—RNA polymerase II (Pol II) is responsible for transcription of mRNA and other regulatory RNAs and will therefore be discussed here. Transcription can initiate once Pol II is recruited to a gene's core promoter, which surrounds the transcriptional start site and encompasses 70–80 surrounding base pairs that are recognized by the transcriptional machinery, but the surrounding sequences may be part of the proximal promoter, conveying tissue-specificity or acting as a transcriptional enhancer (reviewed in Heintzman and Ren, 2006). There is much variability in the factors that are bound to a specific promoter, but promoters generally function in a similar manner. First, chromatin remodeling allows Pol II and other transcription factors to gain access to the promoter; this preinitiation complex (PIC), once properly positioned, melts the 11–15 bp of DNA around the transcriptional start site for correct interaction of the Pol II with the DNA and then begins transcription (reviewed in Heintzman and Ren, 2006).

Binding factor recruitment is essential for transcriptional activation, but proteins that bind to a promoter may instead function to repress the gene. While active transcriptional repressors target a gene at the level of chromatin, passive repressors can compete with transcriptional activators for binding, bind to activators as inactive heterodimers to inhibit transcription, or bind to coactivators to prevent activation of transcription factors (reviewed in Thiel et al., 2004). One example of a transcriptional repressor is the inducible cAMP early repressor (ICER), which has been suggested to play a role in HSV-1 reactivation through repression of LAT (Colgin et al., 2001). ICER is expressed from the same locus as CREB, a transcriptional activator, but is transcribed from an intronic promoter and does not contain an activation domain (reviewed in Mayr and Montminy, 2001). ICER levels peak at 2–6 hours after cAMP stimulation, and ICER prevents CREB from binding to CRE-containing promoters, including the promoter which drives ICER's own transcription (reviewed in Mioduszewska et al., 2003). Numerous other examples of transcriptional repressors exist in eukaryotes and in conjunction with transcriptional activators, can allow promoters to function as switches for transcription.

Summary

Many questions still remain regarding the HSV-1 LAT's role in regulation of the viral lifecycle. Numerous experiments have demonstrated that one major function appears to be in reactivation, since the LAT promoter and the 5' exon are critical to wild-type reactivation. Other activities have also been ascribed to the region, and a picture is emerging in which LAT may play a larger regulatory role, with some of the various phenotypes attributed to LAT mutants being secondary effects of this regulatory function. Clearly, understanding how LAT is regulated at the level of transcription and in different cell types may provide insight into its larger role on HSV biology. Experiments detailed in this dissertation were aimed to address some of the questions regarding LAT regulation—specifically, (1) what is the importance of

histone tail modifications in the ability to reactivate from latency, and (2) does a stress-responsive CRE in the LAT promoter play a regulatory role in reactivation? The answers to these questions will hopefully provide some indication of the complex regulation of the HSV-1 LAT region, as well as open up avenues for future work.

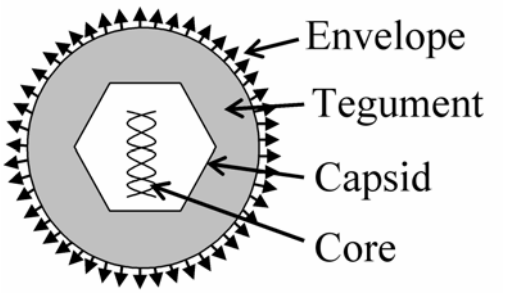

Figure 1-1. Diagram of the HSV-1 virion. The envelope, tegument, capsid, and core are indicated.

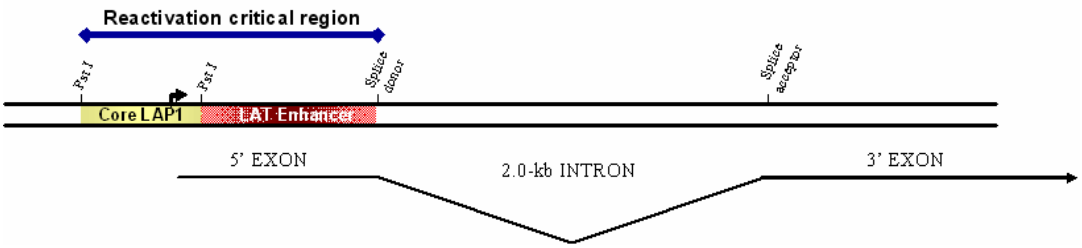

Figure 1-2. Reactivation critical region (*rcr*). The minimal region, mapped by various subdeletions, that is necessary for wild-type *in vivo* reactivation in response to stress is designated by the blue bar. The core LAT promoter (LAP1), shown in yellow, is defined here as the promoter region encompassed by the two *Pst*I restriction enzyme sites. The LAT enhancer, extending through the 5' exon, is shown in red. Note that this is not to scale.

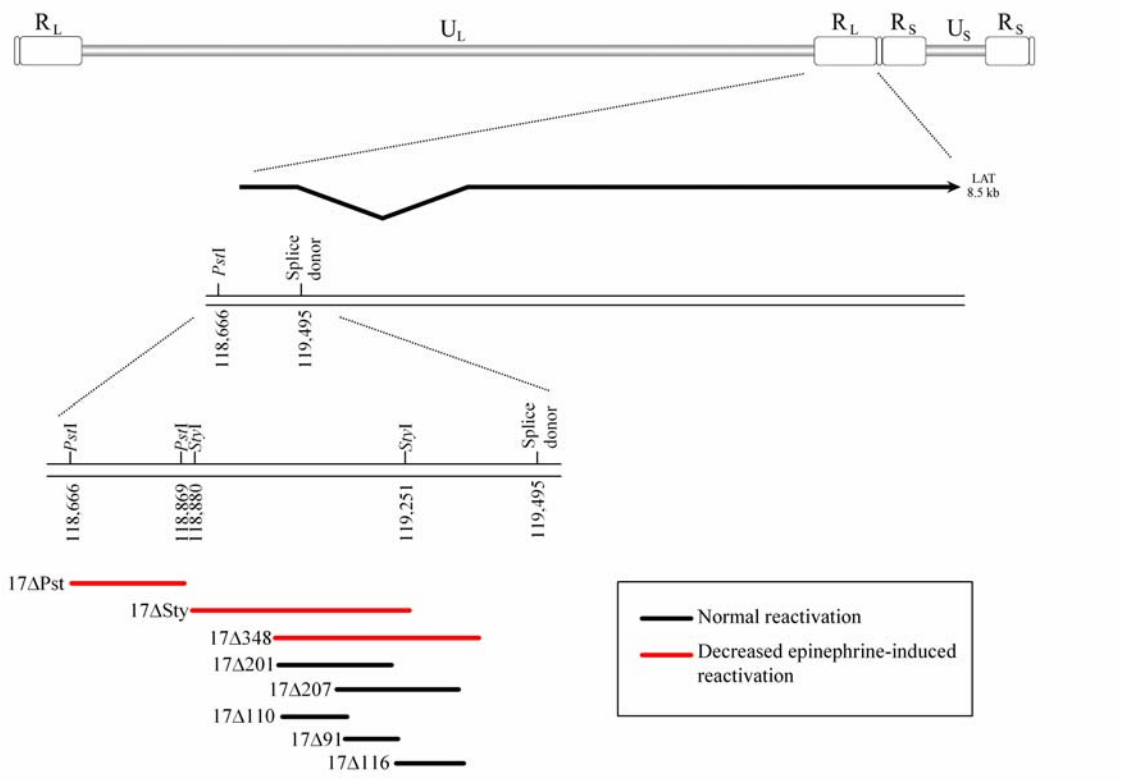

Figure 1-3. Mutants in the reactivation critical region. Normal reactivation, shown in black, refers to the ability to reactivate at wild-type levels in the *in vivo* rabbit reactivation model. Red bars indicate decreased reactivation.

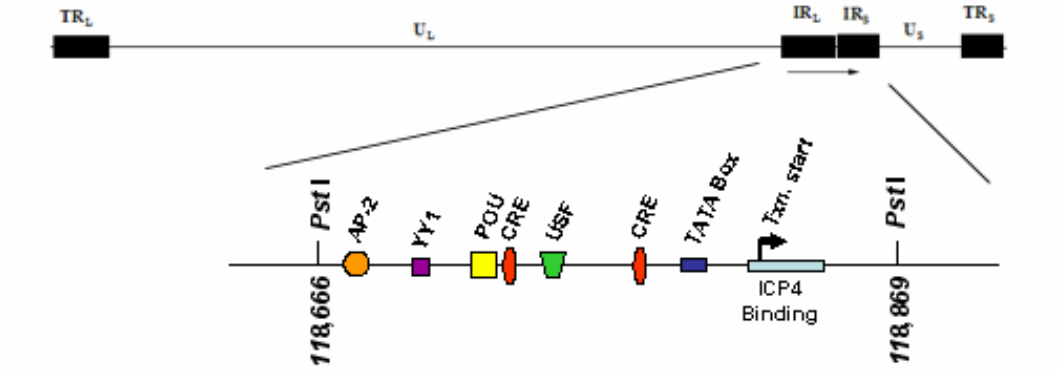

Figure 1-4. Elements of the core LAT promoter (defined as the region encompassed by the two *Pst*I restriction enzyme sites).

CHAPTER 2
CHROMATIN CONFORMATION OF THE LATENT HSV-1 GENOME IN RABBITS

Objective

Preliminary work in the mouse model demonstrated that the HSV-1 genome is associated with specific histone modifications during latency and that there are changes in chromatin permissiveness that occur during explant induced reactivation. The goals of the studies performed here were to investigate patterns of histone modifications in the rabbit, a more relevant model for HSV reactivation and to (1) determine if the histone modifications observed during latency in mice are conserved in the rabbit eye model, (2) determine if epinephrine-induced reactivation results in the same pattern of chromatin changes as observed in the mouse during explant, and (3) determine if the chromatin profile of a reactivation-negative mutant differs from wild-type during latency and/or reactivation (this could suggest that the defect in reactivation is related to inappropriate chromatin configuration affecting promoter accessibility and/or transcriptional permissiveness).

Background and Previous Findings

HSV-1 is maintained as a nucleosome-associated episome during latency (Deshmane et al., 1989). This observation suggested that insight into the transcriptional status and regulatory framework could be determined by analyzing the specific histone modifications that are associated with the different regions of the HSV genome. In order to analyze histone modifications of the latent and reactivating HSV-1 genome, the chromatin immunoprecipitation (ChIP) assay has been used. In the ChIP assay (reviewed in Kuo and Allis, 1999), histones are crosslinked to DNA using formaldehyde, and these complexes are sonicated into fragments of approximately 500–1000 bp. Histones are immunoprecipitated with the antibody of choice and then de-crosslinked from the DNA. The freed DNA is then purified and analyzed by PCR.

As discussed in Chapter 1, there are two primary animal models used for the study of HSV-1 latency and reactivation, the mouse and the rabbit. The mouse footpad model has enabled much of the work related to epigenetic studies of the HSV-1 genome during latency and has also provided some insight into chromatin remodeling that occurs during early DRG explant-induced reactivation.

Using the mouse footpad model, it was determined that DNA methylation does not play a role in the repression of the latent HSV-1 genome (Kubat et al., 2004). Instead, since the latent genome is associated with nucleosomes (Deshmane et al., 1989), histone tail modifications appear to provide some indication as to transcriptional permissiveness (Kubat et al., 2004). Specifically, during latency the LAT promoter region is 2–3.5 fold more enriched in acetylated histone H3 K9, K14, a marker of transcriptional permissiveness, than the lytic genes, ICP27 and ICP0 (Kubat et al., 2004). Upon further assessment of the LAT region—specifically the enhancer located in the 5' exon—it was observed that the enhancer region is more acetylated (approximately 3.5 fold) than the LAT promoter, while lytic genes exist in a hypoacetylated, or less transcriptionally permissive, state (Kubat et al., 2004). The same effect was seen for a LAT promoter deletion virus, which makes no detectable LAT. With this mutant the LAT enhancer was still hyperacetylated relative to the LAT promoter, which was still more acetylated than the nearby lytic genes (Kubat et al., 2004*).* In summary, this previous work indicates that the LAT region is maintained in a transcriptionally permissive state independent of LAT transcription, while the lytic gene regions of the HSV-1 genome exist in a less transcriptionally permissive state during latency (Figure 2-1).

The findings for the latent genome's chromatin configuration were extended to the mouse explant model, which provides information about the molecular events in reactivation. The

question addressed was whether specific regions of the HSV genome undergo chromatin remodeling in response to explant-induced stress. If so, this could indicate what key changes occur early in reactivation. Latently-infected DRG were removed to media for times ranging from 0 hours post-explant (h.p.e) to 4 h.p.e. and then processed for ChIP. The LAT 5' exon/enhancer displayed at least a five-fold decrease in H3 K9, K14 acetylation occurring within the first hour of explant, while the ICP0 promoter exhibited an increase in acetylation between 2 and 3 h.p.e. (Amelio et al., 2006). This study additionally found that there was a dramatic decrease in LAT RNA abundance between 2 and 3 h.p.e. (Amelio et al., 2006). Overall, these findings suggest that there is a remodeling of the LAT region during early explant, whereby both deacetylation of the LAT enhancer and a decrease in LAT levels occur before the ICP0 promoter can become more acetylated.

The mouse explant model is limited by allowing only *in vitro* reactivation studies to be performed. The type of reactivation obtained through DRG explant is relatively LAT-independent, so molecular reactivation occurs regardless of whether a mutant virus does not transcribe LAT. A more relevant model is the rabbit eye model. Epinephrine-induced reactivation of the rabbit occurs *in vivo*, can produce clinical lesions and shed virus at the eye, and reactivation is more LAT-dependent, in that LAT promoter deletion mutant are severely reduced in reactivation relative to wild-type (Hill et al., 1990; Perng et al., 1994). It is for this reason that many of the studies initially performed using the mouse footpad model are validated in the rabbit. The experiments described here utilized the rabbit eye model to determine if the remodeling events observed in the mouse also occur following adrenergic induction of reactivation in the rabbit.

Materials and Methods

Rabbit Infections

One to 2 kg New Zealand White rabbits were infected and housed at the Louisiana State University Health Science Center's Animal Facility. Each rabbit eye received topical proparacaine-HCl anesthesthetic prior to corneal scarification. Rabbits were infected with either 17*syn*+ or 17ΔPst virus inoculum at 50,000 pfu/eye. At days 3, 5, and 7 post-infection (p.i.), the infection of the rabbit eyes was monitored by slit lamp examination for the presence of dendrites on the cornea.

After 28 days p.i., rabbits were sacrificed (latent time point) or epinephrine-induced prior to sacrifice (reactivation time points). If epinephrine iontophoresis was performed, rabbits were anesthetized with isoflurane, and a solution of 0.015% epinephrine was administered to the rabbit eye for 8 minutes at 0.8 mAmps. At 0, 1, 2, or 4 hours post-induction, rabbits were anesthetized with ketamine/xylazine and euthanized with a lethal dose of sodium pentobarbital. After decapitation, rabbit trigeminal ganglia (TG) were removed and processed.

ChIP Assay

ChIP assays were performed at the University of Florida. Rabbit TG were homogenized in 0.5 ml phosphate-buffered saline (PBS) in the presence of protease inhibitors (1 μg/ml aprotinin, 1 μg/ul leupeptin, and 1 mM PMSF). DNA-histone complexes were crosslinked by the addition of 37% formaldehyde to a final concentration of 1%. After the addition of 0.128 M glycine, the sample was pelleted and washed three times with PBS containing protease inhibitors as described above. After the final wash, pellets were resuspended in SDS-lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl), and sonicated (Fisher Sonic Dismembrator 100) to yield fragments of 500-1000 bp (Setting 4, 6 ½ bursts of 40 sec. each). Sonicated samples were pre-cleared with Salmon Sperm DNA/Protein A Agarose beads (Upstate), and histone-DNA

complexes were immunoprecipitated overnight with 3.5 μg/ml of anti-acetyl-Histone H3 K9/K14 (Upstate) or 1 μg/ml of anti-dimethyl-H3 K4 (Upstate). Prior to the wash steps, 25% of the sample was removed and retained as the "unbound" fraction. Complexes were washed with Low Salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), High Salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), LiCl (0.25 M LiCl, 1% Nonidet P-40, 1% Deoxycholate, 1 mM EDTA, 10 mM Tris-HCl), and TE (10 mM Tris-HCl, 1.2 mM EDTA) wash buffers prior to the immune complexes being eluted from the agarose beads with elution buffer (1% SDS, 0.1 M sodium bicarbonate). DNA (bound sample) was de-crosslinked from histones with 10 μl/ml 5 M NaCl and then treated with 20mg/ml RNase A and 40 μg/ml Proteinase K. DNA (bound and unbound fractions) was purified using a QIAquick PCR Purification kit (Qiagen) before analysis by Taqman real-time PCR.

Taqman Real-Time PCR Analysis

Bound and unbound DNA was amplified by real-time PCR using TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) and FAM-labeled TaqMan target-specific primer/probe. Reactions were run in triplicate in concentrations recommended by the manufacturer. Primer and probe sequences are shown in Table A-1. PCR was performed and analyzed using Applied Biosystems 7900HT Sequence Detection Systems. Cycle conditions used were as follows: 50°C for 2 min. (1 cycle), 95°C for 10 min. (1 cycle), 95°C for 15 sec., and 60°C for 1 min. (40 cycles). Threshold values used for PCR analysis were set within the linear range of PCR target amplification. Average cycle threshold (Ct) values were determined, and the relative quantity was calculated using a standard curve specific for the primer/probe set of interest.

Results

The Chromatin Profile of the Latent HSV Genome in Rabbits Latently-Infected with Wild-Type HSV-1 Is Similar to that Observed for Latently-Infected Mice

When the ChIP assay was performed on rabbit TG that were latently-infected with wild-type 17*syn*+, the anti-dimethyl H3 K4 profile revealed an enrichment in the histone modification for the LAT region relative to the lytic genes ICP0 and ICP27 (Figure 2-2A). This was similar to the findings obtained for the latently-infected mouse, in which transcriptional permissiveness of the LAT 5' exon/enhancer was approximately 3.5 times greater than that of the LAT promoter region and almost 40 times greater than that of the lytic genes, ICP0 and ICP27 (Kubat et al., 2004). Thus, it appears that wild-type HSV-1 establishes a similar latent chromatin profile in both the footpad-infected mouse and the ocularly-infected rabbit.

Deleting the Core LAT Promoter Results in Increased H3 K4 Dimethylation of the LAT Promoter Region in Latently-Infected Rabbits

The chromatin profile of HSV-1 genomes in latently-infected mice, in which the 5' exon/enhancer is more transcriptionally permissive than the LAT promoter or nearby lytic genes, is the same for both a wild-type and a promoter-deletion (LAT-negative) mutant (Kubat et al., 2004). The profile observed in latently infected rabbits was strikingly different. As shown in Figure 2-2B, on average the LAT promoter region (amplified by primers approximately 300 bp upstream of the deletion) in the LAT core promoter deletion mutant, 17ΔPst, is 8.5 times more enriched in dimethyl H3 K4 than the wild-type promoter region. The 5' exon/enhancer, ICP0, and ICP27 all show similar levels of enrichment between the two viruses. Since the only region with a dramatic difference between 17*syn*+ and 17ΔPst is the LAT promoter, this suggests that the region deleted in 17ΔPst contains a *cis*-element with repressive activity.

Neither 17*syn*+ nor 17ΔPst Display Dynamic Changes in the LAT Region in Response to Epinephrine Induction of Latently-Infected Rabbits

Since explant of mouse DRG appeared to induce dramatic changes in the chromatin profile at early times post-explant, one would surmise that iontophoresis of rabbits would induce similar changes. However, iontophoresis of rabbits at 0, 1, 2, and 4 h, resulted in no significant change (Figure 2-3). As shown in Figure 2-4, the LAT promoter region of 17ΔPst remains highly enriched in dimethyl-H3 K4 relative to the other targets tested for all times examined. These findings suggest that events related to chromatin remodeling might not occur as rapidly in the rabbit as they do in the mouse or that during latent establishment in the rabbit, the LAT promoter plays a different role than in the mouse in establishing a chromatin profile that is permissive for normal reactivation to occur.

Relative H3 K4 Dimethylation Levels of the LAT Promoter Region in 17ΔPst Are Higher than Those of Wild-type for All Times Examined

The scale of bound/input (B/U) ratios for the 17ΔPst ChIP experiment is much higher than that observed for 17*syn*+, as visible by comparing Figures 2-3 and 2-4. The difference can be appreciated by the comparison of the average values for the times examined. As shown in Figure 2-5, when the ratios of the average 17ΔPst to 17*syn*+ dimethylation levels are determined, the 5' exon/enhancer, ICP0 and ICP27 all show ratios of approximately one, indicating similar levels of dimethylation, while the LAT promoter ratios range from approximately 5 to 22-fold more dimethylation in 17ΔPst than in 17*syn*+. This hyperdimethylation suggests that the region deleted contains an element that normally represses H3 K4 dimethylation in the wild-type virus.

Discussion

Kinetics of Chromatin Remodeling

Upon explant of latently-infected murine DRG, a rapid loss of H3 K9, K14 acetylation of the LAT 5' exon/enhancer is seen within the first hour of explant (Amelio et al., 2006). This

decrease is approximately 5-fold less than what is observed initially at 0 h.p.e. and precedes an increase in acetylation of the ICP0 promoter that occurs between 2 and 3 h.p.e. (Amelio et al., 2006). This timing does not appear to be the same in the rabbit model. When the latent HSV-1 chromatin profile was assessed for rabbit TG infected with wild-type virus, the virus displayed a similar latent profile to that of the latently-infected mouse. However, when the virus's chromatin profile was examined after epinephrine-induced reactivation (1, 2, and 4 hours post-epinephrine induction), it did not mirror the findings from the mouse explant experiments. In fact, little, if any, change is observed at all within the first four hours of epinephrine-induction (Figure 2-3).

Since the timing of chromatin changes does not appear the same as is seen in the mouse, it is possible that there are different mechanisms by which latent HSV-1 can reactivate. The mouse DRG explant model is dependent on a more stressful reactivation stimulus, i.e., the removal of tissue, while the rabbit eye model allows for a more clinically-relevant epinephrine-induced reactivation. A more physiologically relevant stimulus might cause a more gradual change of latent genome configuration. Perhaps at a single-cell level, changes occur quite rapidly, but they do not translate to the level of the tissue until slightly later. For example, since the epinephrine is administered to the eye and reactivation occurs in the ganglia, it might take some time for the epinephrine to reach and stimulate all cells in the ganglia; in explant-induced reactivation, a uniform stressor (ganglion removal from the animal) might stimulate cells to reactivate very rapidly. Thus, it remains possible that if rabbit TG are analyzed for chromatin at later time points, changes might be apparent, and these experiments are underway.

A Repressive Element in the LAT Promoter

When the chromatin profile of the LAT promoter deletion mutant, 17ΔPst, is analyzed, there is a striking increase in transcriptional permissiveness of the region slightly upstream of the deleted region, while the remainder of the viral targets tested show levels of H3 K4

dimethylation that are comparable to wild-type (Figures 2-2). This suggests the presence of a repressive element in the native core LAT promoter that might prevent increased transcriptional permissiveness of the LAT region at inappropriate times, such as during lytic infection. Additionally, since efficient reactivation from latency in the rabbit appears to require LAT expression (Hill et al., 1990; Perng et al., 1994), LAT may play a role in establishing the genome in a configuration compatible with reactivation. It has been proposed by Bloom et al. (1996) that like XIST, a non-coding RNA that silences the inactive X chromosome, the LAT RNA acts in *cis* to silence the HSV-1 genome during latency. Perhaps the LAT functions differently and is less important in the mouse. Since explant of mouse DRG can induce reactivation of promoter-deletion viruses that would not efficiently reactivate in the rabbit, a correct chromatin profile may be less crucial for reactivation in the mouse.

The 202-bp core LAT promoter contains a number of binding sites for factors that may, in turn, bind various chromatin modifiers. Further investigation of this region should yield valuable insight into the mechanisms of HSV-1 reactivation.

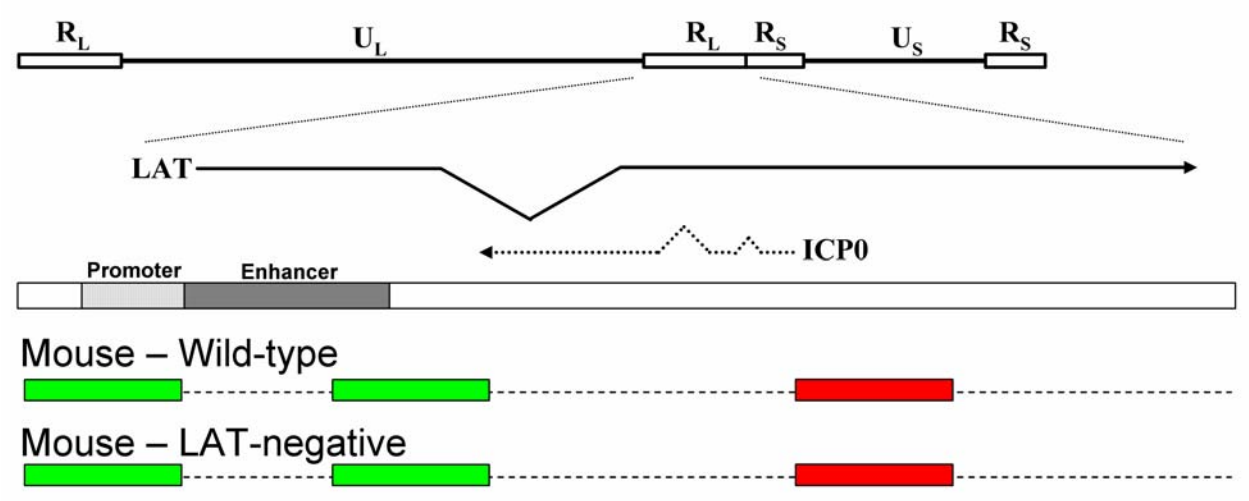

Figure 2-1. Diagram of the chromatin profile of the latent HSV-1 genome in the mouse. The LAT promoter is designated by the light gray box, while the LAT enhancer is shown in dark gray. Green bars indicate regions of higher transcriptional permissiveness, while red bars indicate less transcriptionally permissive regions.

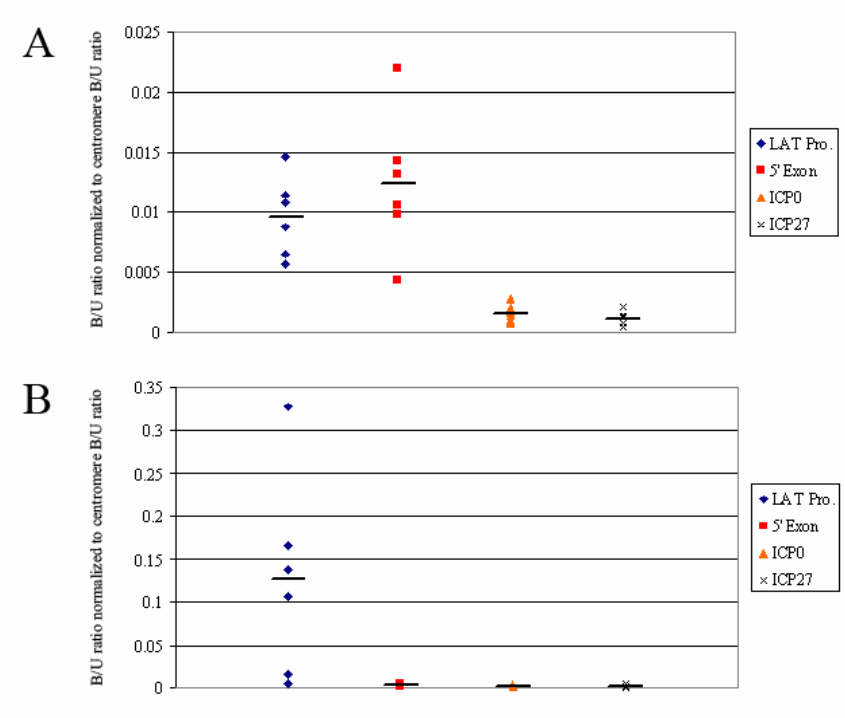

Figure 2-2. LAT region histone H3 K4 dimethylation status of latently-infected rabbits for A) wild-type 17*syn*+, or B) promoter-deletion mutant 17ΔPst. Relative Bound/Unbound, B/U, values are depicted for the LAT promoter, 5' exon/enhancer, ICP0, and ICP27.

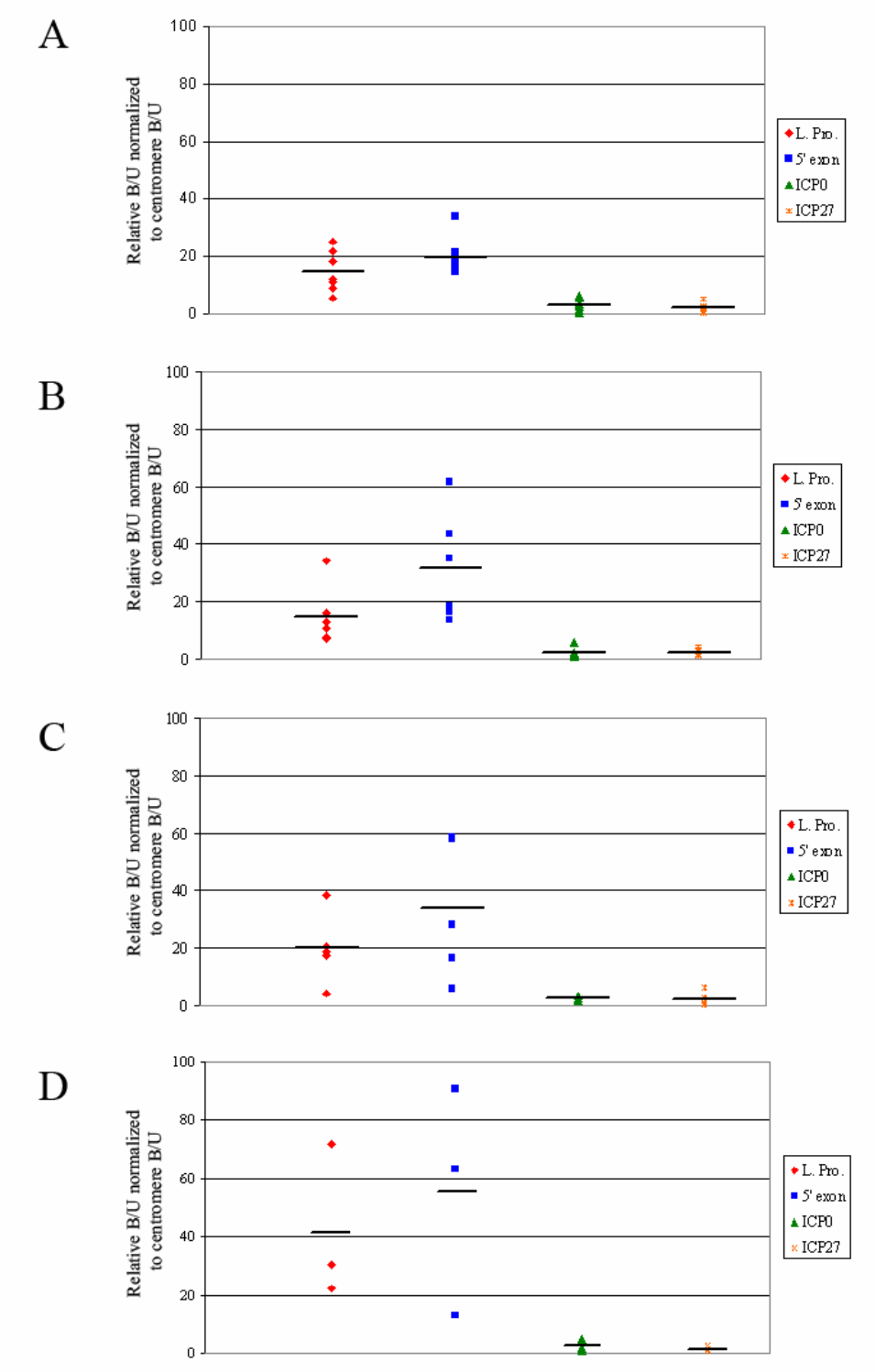

Figure 2-3. Dimethyl H3 K4 status during epinephrine-induced reactivation at A) 0, B) 1, C) 2, and D) 4 hours post-epinephrine (h.p.e.) for wild-type 17*syn*+. Relative Bound/Unbound, B/U, values are depicted for the LAT promoter, 5' exon/enhancer, ICP0, and ICP27.

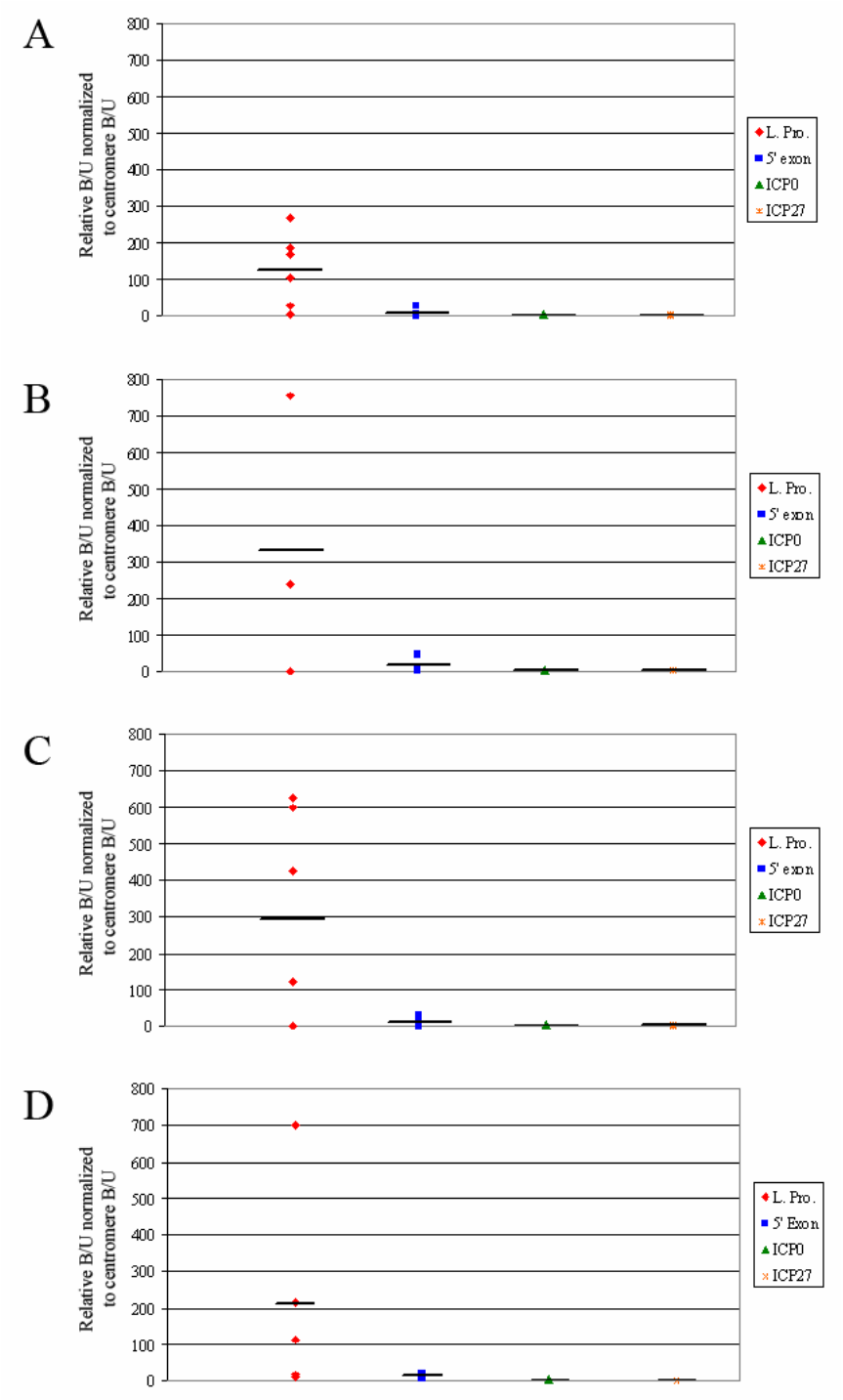

Figure 2-4. Dimethyl H3 K4 status during epinephrine-induced reactivation at A) 0, B) 1, C) 2, and D) 4 hours post-epinephrine (h.p.e.) for promoter-deletion mutant, 17ΔPst. Relative Bound/Unbound, B/U, values are depicted for the LAT promoter, 5' exon/enhancer, ICP0, and ICP27.

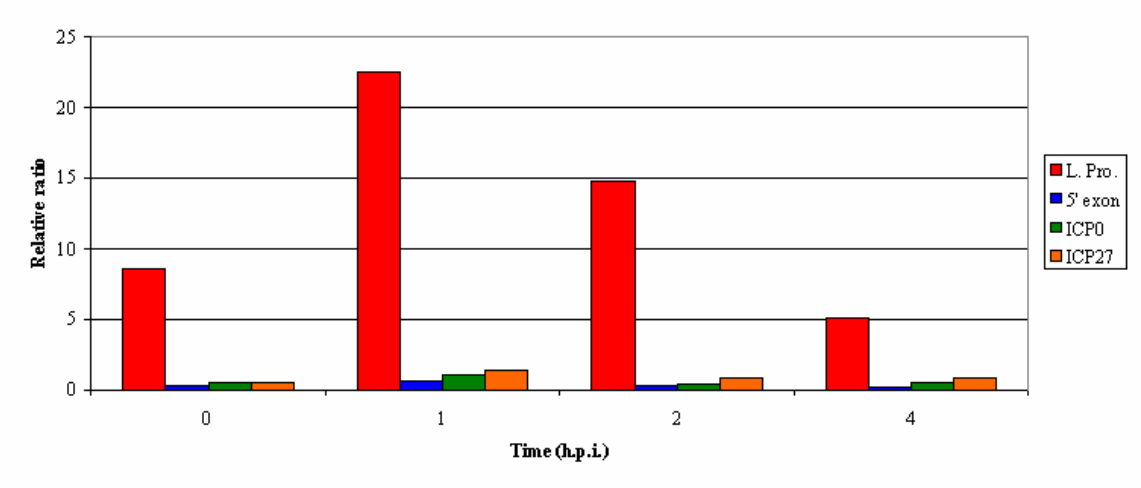

Figure 2-5. Ratios of average relative H3 K4 dimethylation of 17ΔPst to those of 17*syn*+ for epinephrine-induction in rabbits (0, 1, 2, and 4 hours post-induction). Ratios for the LAT promoter (L. Pro.), LAT enhancer (5' exon), ICP0, and ICP27 are shown.

CHAPTER 3
INVESTIGATION OF THE ROLE OF A LAT PROMOTER cAMP RESPONSE ELEMENT (CRE) IN REACTIVATION

Objective

It has been previously shown that LAT abundance transiently decreases following explant-induced reactivation, suggesting that regulation of LAT expression may be an important component of the switch between latent and productive infection. The LAT promoter contains various elements that may be important regulators of the LAT region during latency and reactivation. The studies described here were performed to investigate the role of one of the LAT promoter's cAMP response elements (CREs) in reactivation. To do so, a virus was constructed with a site-directed mutation in the CRE and assessed for (1) *in vitro* replication in cell culture, (2) replication and virulence in the mouse, (3) ability to establish latency and express the LAT, (4) chromatin profile during latency, and (5) ability to reactivate from latency in the rabbit.

Background and Previous Findings

Since reactivation of HSV-1 from latency is a stress-inducible phenomenon, it seems plausible that the stress-responsive cAMP pathway could play a role in regulation, particularly since the LAT promoter contains two CREs (Leib et al., 1991; Kenny et al., 1994). The cAMP pathway's cascade of events is triggered upon epinephrine binding to β-adrenergic receptors of cells. Once binding occurs, adenylyl cyclase converts ATP to cAMP (Tao and Lipmann, 1969), which can then activate the catalytic subunit of protein kinase A (PKA). This subunit translocates to the cell nucleus, where it can phosphorylate members of the cAMP response element binding (CREB) protein family (Montminy and Bilezikjian, 1987; Yamamoto et al., 1988). The CREB activator proteins contain a kinase-inducible domain (KID) flanked by glutamine-rich regions at the amino-terminus and a basic region/leucine zipper (bZIP) domain at

the carboxy-terminus, which is important in DNA binding and nuclear translocation (Dwarki et al., 1990; Waeber and Habener, 1991; Xing and Quinn, 1994). Since the CREB gene undergoes alternative splicing to yield various family members, truncated versions of the protein that lack an activation domain, can cause transcriptional repression rather than activation (Karpinski et al., 1992; Molina et al., 1993).

Once CREB is phosphorylated and activated at a gene's promoter, it facilitates recruitment of CREB-binding protein (CBP) (Chrivia et al., 1993), which in turn can promote transcriptional activation (Arias et al., 1994; Kwok et al., 1994). Although some questions remain as to the exact role of CBP in transcriptional activation, it has been implicated in promoting rapid formation of the preinitiation complex (PIC) to increase the rate of transcription and has also been suggested to facilitate recruitment of mediator complexes to active sites of transcription (reviewed in Vo and Goodman, 2001). Because CBP is a histone acetyltransferase (HAT), it likely contributes to promoter activation through chromatin remodeling (Ogryzko et al., 1996).

Genome-wide characterization of CREB binding to various target genes in human tissues revealed that while CREB occupies approximately 4,000 promoters, only a small subset of those genes are actually activated in response to cAMP, which is likely due to a requirement for coactivator recruitment (Zhang et al., 2005). In other words, CREB binding is constitutive, and regulation is dependent on CBP and possibly, other coactivators. In contrast, one study demonstrated, through the use of chromatin immunoprecipitation (ChIP) assays, that binding is regulated in a cell-specific manner, correlating with the potential for gene expression (Cha-Molstad et al., 2004). This study also found that histone H3 K4 dimethylation corresponds to CREB binding, suggesting either that CREB binding to a promoter is regulated in an epigenetic

manner or that CREB binding is indicative of transcriptional permissiveness (Cha-Molstad et al., 2004).

It was previously determined that the HSV-1 LAT promoter contains a functional cAMP response element (CRE) with complete homology to the proenkephalin CRE (Leib et al., 1991). This element, referred to herein as the -43CRE due to its starting location relative to the LAT transcriptional start site, was shown to be inducible upon application of known modulators of intracellular cAMP levels, and it was also shown to bind the wild-type form of the CRE binding (CREB) protein *in vitro* (Leib et al., 1991; Millhouse et al., 1998). Interestingly, when mobility-shift assays were performed using PC-12 cell nuclear extracts, results suggested that a protein or proteins other than CREB can bind specifically to the LAT -43CRE (Leib et al., 1991); however, this observation was not further explored.

The role of the -43CRE in reactivation was also examined *in vivo* using a recombinant virus, in which the mutated binding site was predicted to provide less than 10% CREB binding (Bloom et al., 1997). When the recombinant virus was tested in the rabbit eye model, epinephrine induction yielded reactivation that was intermediate (58%) between wild-type strain 17*syn*+ (78%) and promoter-deletion mutant 17ΔPst (19%) (Bloom et al., 1997). Spontaneous reactivation levels were similar between the -43CRE mutant virus and 17ΔPst (19% and 16%, respectively) (Bloom et al., 1997). In the rabbit studies, as well as in studies performed using the mouse hyperthermic stress model, the mutation in the -43CRE mutant virus did not appear to affect establishment or maintenance of latency (Bloom et al., 1997; Marquart et al., 2001). Thus, the -43CRE seems to have a role in reactivation. Because epinephrine can stimulate β-adrenergic receptors to begin the cAMP cascade, it seems plausible that the induction of reactivation is tied to the responsiveness of the -43CRE. In fact, when the β-adrenergic receptor-

blocker, propanolol, was injected into mice latently infected with HSV-1, the appearance of infective virus was decreased significantly in the tear film, cornea, and trigeminal ganglia (Gebhardt and Kaufman, 1995), and when it was used to treat latently-infected rabbits, spontaneous reactivation was reduced (Kaufman et al., 1996). Therefore, the cAMP response pathway may play a role in HSV-1 reactivation from latency.

Approximately 40 base pairs upstream of the -43CRE exists a second CRE, which will be referred to as the -83CRE. This element was identified through chloramphenicol acetyltransferase (CAT) activity assays of various promoter deletion mutants and was suggested to convey cell-specificity, as determined through testing in C1300 mouse neuroblastoma cells and L929 mouse fibroblast cells (Kenny et al., 1994). Specifically, when promoter sequences including the -83CRE were added to the neuronal cells in plasmid-based transient expression assays, a three- to fourfold increase in promoter activity was observed, while no effect was seen in nonneuronal cells (Kenny et al., 1994). Upon further examination of the -83CRE, it was determined through electrophoretic mobility shift assays (EMSA) using C1300 cell nuclear extracts that although the site can bind both CREB-1 and CREB-2, it binds CREB-2 with much higher affinity (Millhouse et al., 1998). CREB-2, when over-expressed *in vitro*, causes a significant repression of CRE-mediated transcription due to a lack of phosphorylation sites (Karpinski et al., 1992). Thus, in the HSV-1 LAT promoter, it is possible that the site plays a role in the repression of LAT transcription that is observed during early times post-explant (Amelio et al., 2006).

One might envision the LAT promoter as a molecular switch that controls LAT expression to regulate the transition between latency and reactivation, especially since transcription of ICP0, an IE gene, occurs downstream of the LAT promoter and in an antisense orientation to the LAT,

with the potential for overlap of both transcripts. The ICP0 protein has several defined functions, including transactivation of various cellular and viral genes, host protein degradation, and viral localization to ND10 cellular structures (reviewed in Everett, 2000). ICP0 mutants are viable and can establish latent infection, although they display reduced replication in cell culture at low m.o.i. (Stow and Stow, 1986; Sacks and Schaffer, 1987; Everett, 1989) and inefficient reactivation from latency (Leib et al., 1989; Cai et al., 1993). Interestingly, the inefficient growth by the ICP0 mutants can be restored to near wild-type levels when grown at high m.o.i. in cell culture (Sacks and Schaffer, 1987). The wild-type ICP0 promoter can be activated in response to stress, as demonstrated through stress-stimulating experiments in ICP0 reporter transgenic mice (Loiacono et al., 2003). Additionally, the chromatin surrounding the ICP0 promoter becomes more transcriptionally permissive within four hours of murine DRG explant-induced reactivation (Amelio et al., 2006). While it appears that there may be some role for ICP0 in reactivation, it is not clear whether ICP0 plays a critical role in facilitating IE transcription at very early times during reactivation, or whether it functions as a general transactivator of transcription that enhances the reactivation process.

Because of the potential importance of the -83CRE in regulating the LAT promoter and possibly in facilitating reactivation , the experiments described here were designed to characterize and define the function of the HSV-1 LAT promoter -83CRE site. Creation of a recombinant virus with an eight base-pair mutation in the -83CRE site of the LAT promoter allowed the relevance of the site in the acute, latent, and reactivation phases of the viral lifecycle to be examined.

Materials and Methods

Plasmid Generation, Mutagenesis, and Purification

Plasmid pNG1 was generated through the subcloning of the 1.2 kilobase fragment of the LAT promoter through the 5' exon contained between the *Dra*I and *BstE*II restriction enzyme sites (nt 118,002–119,202) into pBluescript II.

The resulting plasmid was subjected to site-directed mutagenesis of the -83CRE site (AATTACA) to a *Bgl*II restriction enzyme site using Stratagene's Quikchange II Site-Directed Mutagenesis kit. Nucleotides were mutated in groups of four using the following sets of primers: P1Sense—GCA GAC GAG GAA AAT AAA ACA GAA TCA CCT ACC CAC GTG GTG CTG TGG; P1Antisense—CCA CAG CAC CAC GTG GGT AGG TGA TTC TGT TTT ATT TTCCTC GTC TGC; P2Sense—GCA GAC GAG GAA AAT AAA ACA GAT CTT CCT ACC CAC GTG GTG CTG TGG; P2Antisense—CCA CAG CAC CAC GTG GGT AGG AAG ATC TGT TTT ATT TTC CTC GTC TGC. The mutagenesis reaction was performed according to manufacturer's instructions using 50 ng of starting plasmid, and thermal cycler conditions were as follows: 1 cycle of 30 sec. at 95°C and 16 cycles of 30 sec. at 95°C, 1 min. at 55°C, and 4 min. at 68°C. Because the mutated site created a *Bgl*II restriction enzyme site not present in the parental DNA, mutated plasmid DNA was subjected to restriction endonuclease digestion with that enzyme to further verify that the correct mutation was obtained. Additionally, plasmids were sequenced for verification of the correct mutation.

Upon confirmation of the desired mutation, the plasmid DNA was grown in *E. coli* cells and purified using a cesium chloride gradient (Garger et al., 1983). Briefly, the plasmid was grown in 2x YT media containing ampicillin, pelleted, and resuspended in glucose buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM Na_2EDTA). Bacterial cells were lysed in 20 mg/ml lysozyme and lysis solution (0.2 N NaOH, 3% SDS, and water to volume). Potassium

acetate solution (5 M potassium acetate and 11.5% glacial acetic acid) was added and incubated in an ice water bath for 15 min. The sample was centrifuged, the supernatant was filtered and chloroform:isoamyl alcohol (24:1) extracted, and the plasmid precipitated with isopropanol. The pellet was resuspended in 1x TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), combined with 4 g cesium chloride, and 4 mg ethidium bromide. After overnight centrifugation at 44,000 rpm (176,284 x g) in a VTi 65.2 vertical rotor, the plasmid DNA band, visible upon exposure to ultraviolet light, was removed using a syringe and extracted against isoamyl alcohol to remove the ethidium bromide. DNA was precipitated, phenol:chloroform extracted, precipitated again and resuspended in 1x TE.

Cells and Viruses

L7 cells, a Vero cell line containing ICP0 stably transfected (Samaniego et al., 1997), were a gift from the lab of Neal DeLuca. Rabbit skin (RS) and L7 cells were grown at 37°C in the presence of 5% CO_2 in minimal essential media (MEM) supplemented with 5% calf serum (RS cells) or 10% fetal bovine serum (L7 cells), 292 μg/ml L-glutamine, and antibiotics (250 U/ml penicillin and 250 μg/ml streptomycin). Neuro-2A (C1300) cells were obtained from the American Type Culture Collection and grown at 37°C in the presence of 5% CO_2 in MEM supplemented with 10% fetal bovine serum, 292 μg/ml L-glutamine, antibiotics (250 U/ml penicillin and 250 μg/ml streptomycin), and 1 x Non-Essential Amino Acids (Mediatech, Inc.). For transfections, RS cells were subject to overnight serum-starvation at 31.5°C (5% CO_2) with MEM supplemented with 1.5% fetal bovine serum, in addition to L-glutamine and antibiotics as described above.

HSV-1 strain 17*syn*+ was obtained as a low passage stock from J. Stevens. 17ΔPst-Stuffer (replacement of nucleotides 118,666 to 118,869 of 17*syn*+ with the *Kpn*I-*Sac*I fragment

of pBluescript's multiple cloning site [MCS]) was constructed by D. Bloom. -83CRE and F8-1 were constructed as described below.

DNA Isolation for Transfections

17ΔPst-Stuffer virus was used as the backbone for the construction of the -83CRE viral recombinant containing the site-directed mutation of the -83CRE. The -83CRE recombinant was used in the construction of the rescuant, F8-1. In order to prepare the viral DNA used for transfections, virus was cultivated on RS cells at an m.o.i. of 0.01. Upon appearance of cytopathic effect (CPE), infected cells were harvested, centrifuged, and the pelleted cells resuspended in hypotonic lysis buffer (10 mM Tris, pH 8.0, 10 mM EDTA, 0.5% Nonidet P-40, 0.25% NaDOC). After 5 min. incubation on ice, the sample was centrifuged, the supernatant removed and incubated with 1 mg/ml Proteinase K and 1% SDS for 1 h at 50˚C; 1 mg/ml Proteinase K was added after the initial incubation and incubated for 1 h more at 50˚C. The sample was extracted with phenol, chloroform, and isoamyl alcohol. Viral DNA was precipitated by the addition of 0.1 vol 3 M NaOAc, followed by the very slow addition of 2 vol ice cold 100% ethanol. The DNA was spooled, removed, air-dried and resuspended in 1x TE.

Virus Construction and Plaque Purification

Plasmid DNA was transfected with viral DNA to allow for recombination. The mutated -83CRE plasmid DNA was first digested using *BspE*I and *Bsa*BI and was then gel purified. Approximately 4 μg of the purified, linearized plasmid DNA was combined with varying amounts of 17ΔPst-stuffer DNA (2 μg, 4 μg, 8 μg, and 16 μg) in a final volume of 225 μl TNE buffer (10 mM Tris, pH 7.4, 1 mM EDTA, and 0.1 M NaCl) plus 25 μl $CaCl_2$. Rescuant F8-1 was constructed by combining -83CRE viral DNA with plasmid pAatII (nt 4817-9271 of 17*syn*+). The DNA was precipitated by the addition of 2 x HEPES while blowing bubbles through a pipet and allowed to incubate for 20 minutes at room temperature. This transfection

mix was then applied to RS cells which had undergone an overnight serum-starvation (described above), and this was allowed to incubate at room temperature for 30 min. After this incubation, MEM supplemented with 1.5% fetal bovine serum was added to the cells and incubated for 4 hours at 37°C in the presence of 5% CO_2. This incubation was followed by the addition of hypotonic lysis buffer (10 mM Tris, pH 8.0, 10 mM EDTA, 0.5% Nonidet P-40, 0.25% sodium deoxycholate) directly to the monolayer, followed by three washes with media. Supplemented MEM containing 5% calf serum was added to the cells, which were then incubated at 37°C in the presence of 5% CO_2 for 3-4 days (when CPE was observed).

Screening for recombinant viruses was performed by picking plaques followed by dot blot hybridization. The virus resulting from the transfection was diluted to yield well-separated plaques (10^{-4} through 10^{-7}), and 0.5 ml of these dilutions plated onto confluent monolayers of cells in 60-mm tissue culture dishes (two to three dishes per dilution). After 1 hour of adsorption, monolayers were overlayed with 0.75% agarose in 1x supplemented media, and the dishes were then incubated until plaques were visible (2-3 days). To assist in visualization of plaques, dishes were counterstained with a 1:30 dilution of 3.3 g/L Neutral Red (Sigma) in unsupplemented media for approximately 4-6 hours. The liquid overlay was removed, and plaques were picked using sterile Pasteur pipettes. After gently aspirating into the pipette, the plaques were expelled into a 96-well dish containing 150 µl media per well. Once plaques were picked, the dish was frozen at -80°C and then thawed in the tissue culture incubator. 50 µl of the plaque suspensions were inoculated onto confluent cells in a 96-well dish and allowed to adsorb for 1 hour. At the end of the incubation, supplemented media was added to each well, and the dishes were incubated until 100% CPE was observed (approximately 3 days). A Millipore dot-blot apparatus was used to transfer 50 µl of infected cells onto a nylon membrane, which was

washed with 200 µl of a solution containing 1.5 M NaCl and 0.1 M NaOH, followed by 200 µl of a solution containing 0.2 M Tris, pH 7.5, and 200 µl of a solution containing NaCl, NaH_2PO_4, and EDTA. Once the solutions were through the apparatus, the membrane was baked for one hour at 80°C.

Dot blots were probed with radioactively labeled random-primed DNA probes prepared using the *Pst-Pst* fragment of the HSV-1 LAT promoter, the MCS fragment of pBluescript, or a 22-mer encompassing the wild-type -83CRE of the LAT promoter. Probes were labeled with [α-^{32}P]dCTP using the Rediprime II Random Prime Labelling System (Amersham Biosciences), according to the manufacturer's protocol.

To screen for the recombinant -83CRE virus, the membrane was prehybridized for 4–5 hours at 62.5°C with a solution containing 3 M NaCl, 0.3 M sodium citrate, 50 mg/ml nonfat dry milk and 2 µl/ml Antifoam A, the labeled probe added to the prehybridization solution and the dot blot hybridized overnight. The membrane was then washed at room temperature once for 5 min. with a wash solution containing 0.3 M NaCl, 0.06 M Tris-HCl, and 0.002 M EDTA and twice for 5 min. each with a wash solution containing 0.03 M NaCl, 0.006 M Tris-HCl, and 0.0002 M EDTA. The blot was dried and exposed to X-ray film overnight.

To screen for the rescuant virus, F8-1, tetramethyl ammonium chloride (TMAC) hybridization was used. The membrane was prehybridized for 1 hour at 58°C with a hybridization solution consisting of 3 M TMAC, 0.1 M $NaPO_4$, pH 6.8, 1 mM EDTA, pH 8.0, 5 x Denhardt's Solution, 0.6% SDS, and 100 µg/ml denatured Salmon Sperm DNA. For the hybridization, the hybridization solution was replaced with fresh solution, and a labeled 22-mer probe identical to the wild-type promoter region encompassing the -83CRE site was hybridized to the membrane for 24 to 48 hours at 58°C. The membrane was washed twice at room

temperature with Wash #1 (3 M TMAC, 50 mM Tris, pH 8.0, 0.2% SDS), once with Wash #1 at 60°C for one hour, and twice at room temperature with Wash #2 (2 x SSPE, 0.1% SDS, 1 mM EDTA, pH 8.0). The membrane was then dried and exposed to X-ray film. F8-1 was one of two independent plaques identified from the initial screen and purified through 4 rounds of plaque purification.

PCR Analysis

The LAT promoter regions of the recombinants were analyzed by PCR performed on viral DNA that was isolated as described above (see Cells and Virus Cultivation/DNA Isolation for Transfections). Amplification reactions contained 1x GoTaq® Green Master Mix (Promega), 600 ng each of primer Upfragment, 5'—CGA GGA ACA ACC GAG GGG AAC (nt 118,305-118,325) and Downfragment, 5'—CTG AGA TGA ACA CTC GGG GTT ACC (nt 119,179-119,202), 50 ng of viral DNA and nuclease-free water to a final volume of 50 µl. The DNA was amplified using an Ericomp thermal cycler (San Diego, CA) with 2 min. at 95°C (one cycle), 3 min. each at 94°C, 55°C, and 72°C (one cycle), and 1 min. each at 94°C, 55°C, and 72°C (three cycles). PCR products were visualized on 1% agarose gel containing ethidium bromide.

Growth Curves

In order to assess replication of the -83CRE recombinant in various cell lines, the virus was inoculated at a multiplicity of infection (m.o.i.) of 0.001, 0.01, or 5. The virus, along with its rescuant (F8-1) and the wild-type 17*syn*+ virus, was used to infect confluent RS cells, L7 cells, or Neuro-2A cells grown in 35-mm cell culture dishes. Time (hours post-infection) was monitored starting one hour after the inoculum was added to the cells. Infected cells were harvested by gentle scraping of the dishes at 0, 8, 24, 48, 72, and 96 h.p.i. for multi-step growth curves (low m.o.i.) or 0, 8, 24, and 48 h.p.i. for single-step growth curves (high m.o.i.). After one freeze-thaw, viral titers were determined.

Mouse Survival Assay

Six to 8 week old female ND4 Swiss mice (Harlan Sprague Dawley, Inc.) were infected via the footpad with 500 pfu, 5,000 pfu, or 50,000 pfu. Briefly, mice were injected with saline in the rear footpads to soften the keratinized epithelium. Three to four hours later, mice were anesthetized intramuscularly with 0.01 to 0.02 ml of a cocktail of ketamine (30 to 45 mg/kg), xylazine (7.5 to 11.5 mg/kg) and acepromazine (2.5 to 3.75 mg/kg). During this time, the footpads were abraded with an emery board and virus was applied. Viral absorption occurred during the time that the mice remained under anesthesia (approximately 45–60 minutes). The number of mice surviving throughout the acute phase of infection was assessed.

Intracranial Inoculation as an Assay for Neurovirulence

In order to assess the neurovirulence of the recombinant virus, ND4 Swiss mice were anesthetized using isofluorane and inoculated intracranially with 10 µl of dilutions of virus using a 27 ½ gauge needle. Doses given were 10 pfu and 100 pfu. Mice were monitored for survival over the acute phase of infection.

DNA Extraction and Analysis of Course of Infection

Mice were infected via the footpads with 5,000 pfu/mouse (8 hour sample) or 10,000 pfu/mouse (2 and 4 day samples) of either 17*syn*+ or -83CRE virus. At 8 hours, 2 days, or 4 days post-infection, 4-5 mice from each group were sacrificed, and their feet, DRG, and spinal cords were snap-frozen in liquid nitrogen. Tissue samples were homogenized in 4 ml (feet) or 0.2 ml (DRG and spinal cord) TES (10 mM Tris, pH 7.4, 0.1 M NaCl, 1 mM EDTA) with Duall glass dounces and treated with 1% SDS and 1 mg/ml Proteinase K overnight at 50°C. Samples were phenol:chloroform:isoamyl alcohol extracted, chloroform:isoamyl alcohol extracted, and ethanol precipitated. After air-drying, the DNA pellet was resuspended in 1xTE. Spinal cord and DRG samples were diluted 1:10 and foot samples were diluted 1:100 for use in real-time

PCR. HSV-1 genome equivalents were determined through calculation of the relative quantity of PCR amplification of the HSV-1 polymerase gene normalized to the PCR amplification products of the cellular gene target, XIST.

RNA Isolation and Reverse Transcription for Acute RNA Levels in Cell Culture

RS or Neuro-2A cells were seeded for next day confluency in 35-mm tissue culture dishes. Upon confluency cells were infected at an m.o.i. of 0.01 with either 17*syn*+ or -83CRE virus. One ml of viral inoculum in MEM was applied to each dish, allowed to adsorb into the cells for one hour, and then replaced with fresh supplemented MEM. After this point, cells were harvested for RNA at 2, 4, 6, or 8 hours post-infection (with timing started after the application of fresh media) using 1 ml Trizol, which was triturated to lyse cells. Samples were transferred to Eppendorf tubes, incubated at RT for 5 min., and 0.2 ml chloroform was added to each tube, vortexed and incubated at RT for 2 min. Tubes were centrifuged at 12,000xg for 15 min. at 4°C before the aqueous phase was transferred to a new tube and the RNA was precipitated by the addition of 0.5 ml isopropanol. Samples were incubated at RT for 10 min. and centrifuged at 12,000xg for 10 min. at 4°C. After the removal of the supernatant, the RNA pellet was washed with 1 ml 70% EtOH and spun at 7,500xg for 5 min. at 4°C. This supernatant was removed and the pellet was air-dried briefly before being resuspended in 45 µl nuclease-free H_2O.

Upon resuspension of the precipitated RNA, DNase treatment was performed using Turbo DNA-free (Ambion) according to the manufacturer's instruction. Reverse transcriptions were performed with Omniscript reverse transcriptase (Qiagen) in reaction volumes of 20 ul. Briefly, reactions contained Omniscript 1x buffer, 0.5 mM each dNTP, 1 µM random decamer primer (Ambion), 10 units/ul SUPERase-In (Ambion), 1 µg RNA, 8 units Omniscript reverse transcriptase, and RNase-free water to volume. Additionally, RNA controls ("No RT" controls)

were performed using the same concentration of RNA in water. Reactions were incubated at 37°C for one hour.

Taqman Real-time PCR Analysis

cDNA or DNA was amplified by real-time PCR using TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) and FAM-labeled TaqMan target-specific primers/probes (Applied Biosystems, Inc.) (Table A-1). Reactions were run in triplicate in concentrations recommended by the manufacturer. Primer and probe sequences are listed in Appendix A. PCR was performed and analyzed using Applied Biosystems 7900HT Sequence Detection Systems. Cycle conditions used were as follows: 50°C for 2 min. (1 cycle), 95°C for 10 min. (1 cycle), 95°C for 15 sec., and 60°C for 1 min. (40 cycles). Threshold values used for PCR analysis were set within the linear range of PCR target amplification. Average cycle threshold (Ct) values determined in triplicate were averaged, and the relative quantity was calculated using a standard curve specific for the primer/probe set of interest. Briefly, Ct values for 10-fold dilutions of DNA (viral or cellular) of known concentration were determined and graphed as a function of dilution. The equation of the resulting line was used to extrapolate the relative quantity of the sample of unknown concentration.

RNA Isolation and Reverse Transcription for Explant Studies

Mice (infected as described above with 400–500 pfu of virus) were euthanized by an overdose of isoflourane, followed by cervical dislocation. Dorsal root ganglia (DRG) from groups of two mice (8 ganglia per mouse) were removed as quickly as possible (3 to 5 minutes per mouse) and placed in 0.5 ml RNA Later (Ambion). RNA was isolated from the tissue using the method of Chirgwin, et al. (1979). Briefly, DRG were homogenized in guanidine thiocyanate solution and 100 µl removed for DNA isolation. The homogenate was layered on a 5.7 M cesium chloride cushion and centrifuged overnight at 30,000 rpm (111,132 x g) in a SW

41 Ti rotor. The supernatant was aspirated from the RNA pellet, which was briefly dried and then resuspended in nuclease-free water. RNA was precipitated overnight using 0.1 vol 3 M sodium acetate and 2 vol 100% ethanol. The DNA fraction of the sample was isolated as described by Kramer and Coen (1995). Briefly, the DNA was precipitated overnight with 0.1 vol 3 M sodium acetate and 2 vol 100% ethanol. The resulting pellet was resuspended in 50 µl of DNA resuspension solution containing: 0.2 µg/ml proteinase K, 0.02% Tween 20, 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), and 1.5 mM $MgCl_2$. The DNA was incubated at 65°C for 2 h, 80°C for 20 min., and 94°C for 10 min. (to inactivate enzyme). Because of the presence of precipitate, the DNA sample was diluted 1:10 prior to use in real-time PCR analysis.

Upon resuspension of the precipitated RNA, DNase treatment was performed using Turbo DNA-free (Ambion) according to the manufacturer's instruction. Reverse transcriptions were performed with Omniscript reverse transcriptase (Qiagen) as described above (see RNA Isolation and Reverse Transcription for Acute RNA Levels in Cell Culture).

ChIP Analysis

Dorsal root ganglia were removed and pooled from 3 mice per time point. After incubating in media at 37°C for a given amount of time (0, 0.5, 1, 2, or 4 h post-explant), ganglia were homogenized in 0.5 ml phosphate-buffered saline (PBS) in the presence of protease inhibitors (1 µg/ml aprotinin, 1 µg/ul leupeptin, and 1 mM PMSF). DNA-histone complexes were cross-linked by the addition of 37% formaldehyde to a final concentration of 1%. After the addition of 0.128 M glycine, the sample was pelleted and washed three times with PBS containing protease inhibitors as described above. Samples were pelleted, resuspended in SDS-lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl), and sonicated (Fisher Sonic Dismembrator 100) to yield fragments of 500–1000 bp (setting 4, 2 bursts of 40 sec. each

followed by 1 burst of 20 sec.). Sonicated samples were pre-cleared with Salmon Sperm DNA/Protein A Agarose beads (Upstate), and histone-DNA complexes were immunoprecipitated overnight with 3.5 μg/ml of anti-acetyl-Histone H3 (Upstate). Prior to the wash steps, 25% of the sample was removed and retained as the "unbound" fraction. Complexes were washed with Low Salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), High Salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), LiCl (0.25 M LiCl, 1% Nonidet P-40, 1% Deoxycholate, 1 mM EDTA, 10 mM Tris-HCl), and TE (10 mM Tris-HCl, 1.2 mM EDTA) wash buffers prior to the immune complexes being eluted from the agarose beads with elution buffer (1% SDS, 0.1 M sodium bicarbonate). DNA (bound sample) was de-crosslinked from histones with 10 μl/ml 5 M NaCl and then treated with 20mg/ml RNase A and 40 μg/ml Proteinase K. DNA (bound and unbound fractions) was purified using a QIAquick PCR Purification kit (Qiagen) before analysis by Taqman real-time PCR.

Rabbit Reactivation

One to 2 kg New Zealand White rabbits were infected and housed at the Louisiana State University Health Science Center's Animal Facility. Each rabbit eye received topical proparacaine-HCl anesthetic prior to corneal scarification. Rabbits were infected with either 17*syn*+ or -83CRE virus inoculum at 200,000 pfu/eye. At days 3, 5, and 7 post-infection (p.i.), the infections of the rabbit eyes were monitored by slit lamp examination for the presence of dendrites on the corneas. Clinical scores (1 to 5, with 5 being the most severe) were assigned to reflect the relative surface area of the cornea covered with dendrites as a means of assessing the severity of the infection.

After 28 days p.i., rabbits were anesthetized with ketamine/xylazine and a solution of 0.015% epinephrine in sterile water (dissolved by the addition of one drop of HCl) was iontophoresed into the rabbit corneas by applying current for 8 minutes at 0.8 mAmps. At

specified times post-iontophoresis, rabbits were euthanized with sodium pentobarbital and decapitated for removal of TG. The TG were removed as rapidly as possible (5 to 15 min per rabbit).

Results

The -83CRE virus construction was not straightforward. After a mutation was made to the -83CRE site in a plasmid containing the LAT promoter and verified through sequencing, the mutant plasmid was allowed to homologously recombine with DNA from virus 17ΔPst-Stuffer, a LAT-negative mutant containing a bacterial stuffer in place of the core LAT promoter. To ensure that the recombinant used for characterization experiments was not made up of a mixed population of viral genomes (e.g., the parental stuffer and the nascent recombinant), the virus was plaque-purified, which permits selection for purity. Although the plaque purification appeared to produce a pure recombinant virus after approximately seven rounds of purification based on the dot-blot hybridizations, when the promoter region was amplified through PCR (Figure 3-1), two products appeared for the supposed "pure" recombinant (Figure 3-2A). The product of higher molecular weight corresponded to the recombinant, while the lower molecular weight product was representative of the parental virus, 17ΔPst-Stuffer. This suggested that the virus was persisting as a mixed population of recombinants and wild-type virus or more likely as a "single-sided" recombinant, in which only one of the two long repeats (R_L) contained the LAT promoter -83 CRE mutation (see Figure 3-2). Since the lab had never had a recombinant that needed more than 4 rounds of plaque-purification to achieve purity and because the PCR data was consistent with a single-sided virus, this suggested that there might be a bias against making the -83CRE mutation in both copies of the LAT. Because the -83CRE mutation could potentially disrupt regulation of LAT expression and affect ICP0 in the process, the virus was plaque-purified on L7 cells, a Vero-cell derived cell line, in which ICP0 is stably expressed

(Samaniego et al., 1997). Since the parental 17ΔPst-Stuffer virus used in the transfection contained a pBluescript multiple cloning site (MCS) stuffer, the MCS fragment was used as a negative hybridization control. After four rounds of plaque purification on L7 cells, dot blot for the MCS stuffer indicated that the region did not exist in the -83CRE virus (not shown), and the PCR product corresponding to the parental virus was no longer visible, suggesting that complementing ICP0 with L7 cells allowed a virus with the mutated -83CRE in both copies of the LAT promoter to be purified to homogeneity (Figure 3-2B). As will be discussed further later, it was determined upon characterization of the -83CRE virus that a second site mutation also occurred somewhere in the viral genome.

The -83CRE Recombinant's Replication Is Altered during the Lytic Phase of the Infection

After the -83CRE recombinant virus was purified and the correct mutation in the LAT promoter confirmed through PCR analysis and sequencing, it was tested for replication in cell culture. Wild-type 17*syn*+ and -83CRE virus were used to infect RS cells, L7 cells, and Neuro-2A cells at the low m.o.i. of 0.01 to allow for multiple rounds of replication. As shown in Figure 3-3A, the -83CRE displayed approximately 10-fold more efficient replication than 17*syn*+ during the first 8-24 hours of infection when assayed on RS cells, which are epithelial in origin. The opposite was observed for the -83CRE's growth on Neuro-2A cells, which produced yields of approximately 10-fold less than 17*syn*+ throughout the times tested (Figure 3-3B). Since the mutant was purified on L7 cells, it was tested for growth on that cell line as well. Providing excess ICP0 had no major effect on the -83CRE mutant's replication, since relative yields on L7 cells were the same as on RS cells, with the mutant again replicating slightly more efficiently than the wild-type virus early in the infection (Figure 3-3C).

Since the recombinant was not able to be purified until it was plaqued on L7 cells, an ICP0-complementing cell line, the possibility existed that ICP0 function was altered in the

-83CRE. ICP0-deleted viruses show a multiplicity-dependent effect on replication, in which the mutant grows very inefficiently at low m.o.i. but near wild-type levels of replication are restored at high m.o.i. (Sacks and Schaffer, 1987). To determine whether the -83CRE mutation had a multiplicity-dependent effect on replicative yields, single-step growth curves (m.o.i. of 5) were performed using RS and Neuro-2A cells. As shown in Figure 3-4, the -83CRE recombinant shows a similar effect to that observed for low m.o.i. on RS cells but the decreased replication on Neuro-2A cells that was seen for the low m.o.i. infection was not observed. In fact, the -83CRE demonstrated slightly enhanced replication (approximately 5-fold) relative to wild-type at 12 hours p.i. when grown on Neuro-2A cells. Thus, these data suggest a multiplicity-dependent effect of the -83CRE recombinant on viral growth on the Neuro-2A cell line, but not on the RS epithelial cells.

The -83CRE Recombinant Displays Impaired Replication and Spread in the Nervous System of the Mouse

Because the -83CRE mutant virus showed some variation from wild-type in replication assays, it was important to test the virus in the mouse to determine what biological effects the mutation would have. Ten ND4 Swiss mice per dose per virus were infected with 500, 5000, or 50000 pfu of either 17*syn*+ or the -83CRE virus and were monitored closely during the acute phase of infection (first three weeks of infection). The survival of mice at the varying doses is shown in Figure 3-5 and in Table 3-1. While 17*syn*+ resulted in 0–30% survival for the doses tested, mice infected with the -83CRE virus displayed a 90% survival rate for all doses, indicating an LD_{50} of $>5x10^4$ pfu. Because of this dramatic deficit in virulence following infection via the mouse footpad, we sought to determine whether the defect in virulence was a result of decreased replication on the epithelium of the foot and/or within the nervous tissue.

To determine whether the -83CRE virus exhibited a replication defect in nervous tissue, mice were intracranially (i.c.) inoculated with the -83CRE virus to determine its virulence following direct delivery to the CNS. In this assay direct injection of 10 or 100 pfu of virus into the brain of mice was performed. Mice were closely monitored for death and sacrificed once CNS involvement was observed (determined by paralysis, erratic movement, and/or inability to right itself). As shown in Table 3-2, the mutant -83CRE virus exhibited a slight decrease in neurovirulence following i.c. inoculation when compared to 17*syn*+. These results suggest that the -83CRE virus was attenuated in its ability to replicate in the nervous system, but it could kill mice if delivered to the brain directly at higher doses.

The relative avirulence seen following footpad inoculation could have been due to a cumulative effect of slightly less efficient replication at each node of the nervous system in which the virus replicates on its path from the foot to the brain. It also was possible, however, that the virus replicates less efficiently in the epithelium of the foot of the mouse and not just in the nervous tissue. In order to differentiate between these possibilities a tracer study was performed. Mice were infected via the footpad route and sacrificed at 8 hours, 2 days, or 4 days p.i. Foot, spinal cord and DRG were assessed for relative numbers of viral genomes. As shown in Figure 3-6A & B, replication in the foot is equivalent for all times examined. However, once the -83CRE mutant virus reaches the DRG, replication is reduced by approximately 2.5- to 5.5-fold relative to 17*syn*+, while replication in the spinal cord is reduced by approximately 5- to 8-fold (Figure 3-6C, D). These results support the viral growth curve data, as well as the mouse infection data, suggesting that the -83CRE mutant exhibits a reduced ability to replicate within neurons.

The -83CRE Virus Contains a Second Site Mutation

Since the virulence phenotype observed for the -83CRE mutant was quite dramatic, it was necessary to confirm that the -83CRE recombinant virus's phenotype was indeed due to the 8-bp mutation in the LAT promoter. To do this the mutation was rescued by the transfection of wild-type LAT promoter DNA with the -83CRE virus. If the -83CRE site was the only mutation in the virus, restoration of the wild-type LAT promoter should restore virulence to the rescued virus. Mice were inoculated via the footpad with varying doses (500, 5000, 50000 pfu/mouse) of the wild-type, the -83CRE recombinant, or the rescue virus, F8-1. Little to no mortality was observed for the F8-1 rescuant virus at any of the doses tested (Figure 3-7). The similar virulence phenotypes of the F8-1 virus and the -83CRE virus suggested a second mutation somewhere in the mutant virus's genome. Interestingly, when the F8-1 virus was assayed for replication using a multi-step growth curve on RS cells, there appeared to be a delay in replication efficiency, with decreased replication at 8 hours p.i. but levels similar to that of the -83CRE and wild-type at the later times tested (Figure 3-8). This suggests that rescuing the -83CRE mutation may have altered the ability of the virus to replicate on RS cells, but did not rescue a second mutation that seems to primarily affect neuronal replication and virulence *in vivo*. Ongoing experiments using subfragments of the viral genome to rescue the virulence phenotype will map the site of the second mutation.

Mutation of the -83CRE Results in Wild-type HSV RNA Levels in RS Cells during Acute Infection

To further analyze the -83CRE mutant, RS cells were infected at an m.o.i. of 0.01 with either 17*syn*+ or -83CRE. Cells were harvested at acute times for RNA analysis. Random decamer-primed reverse transcription reactions were followed by Taqman real-time PCR to determine the relative quantities of select RNA transcript levels. Relative quantities of viral

targets were normalized to relative quantities of the cellular gene, GAPDH, to account for any variations in cell growth or infectivity. As shown in Figure 3-9, there is no significant difference in abundance of transcripts from any of the gene classes (immediate early, ICP4; early, Polymerase; early-late, LAT; true late, glycoprotein C). Furthermore, inhibition of transcription by treatment with cycloheximide or blockade of replication using phosphonoacetic acid showed no difference in transcript accumulation between the wild-type and -83CRE viruses.

The -83CRE Virus Expresses LAT during Latency

Mice were infected with 400-500 pfu for latency. RNA was isolated from the dissected DRG using guanidine thiocyanate isolation and was reverse transcribed using random decamers. Relative quantities for the LAT 5' exon and ICP4 obtained through Taqman real-time PCR were normalized to the relative quantity for the cellular RNA, XIST. As shown in Figure 3-10, the mutant -83CRE virus expresses the LAT at a detectable level during latency. Additionally, the relative values (HSV-1 polymerase normalized to cellular control XIST) determined for the back-extracted viral DNA are 0.0183 ± 0.0084 and 0.0228 ± 0.0107 for 17*syn*+ and -83CRE, respectively, indicating that levels of establishment of the -83CRE virus are similar to those seen for wild-type 17*syn*+. These data indicate that mutation of the -83CRE site does not abolish LAT expression during latency.

The -83CRE Virus LAT Promoter Region's H3 K9, K14 Acetylation Levels Are Similar to Those of ICP0 during Latency

In order to examine the chromatin profile of the -83CRE virus versus 17*syn*+, mice infected with 400 pfu of virus were sacrificed and their DRG removed and processed for the ChIP assay. Because CREs are able to recruit CREB binding protein (CBP), which is a known histone acetyltransferase (HAT) (Ogryzko et al., 1996), anti-acetyl H3 K9, K14 was used to determine whether mutation of the HSV-1 CRE affects the acetylation levels of the LAT

promoter and/or the LAT enhancer. It was previously shown that the region encompassing the LAT promoter and 5' exon is enriched in H3 K9, K14 acetylation relative to lytic genes (Kubat et al., 2004). As shown in Figure 3-11, that is not observed for the -83CRE virus. In fact, the LAT promoter region is approximately as hypoacetylated as the lytic gene, ICP0. This suggests an abrogation of the ability of CBP, or some other factor affecting transcriptional permissiveness, to bind the LAT promoter when the -83CRE site is mutated.

The -83CRE Virus Reactivates from Latency in the Rabbit with Similar Efficiency as Wild-type 17*syn*+

Rabbits were infected via the ocular route at a dose of 200,000 pfu/eye of wild-type 17*syn*+ or -83CRE. Slit lamp examination of the rabbit eyes at three, five, and seven days post-infection revealed no significant difference in pathology between the viruses (Table 3-3). Furthermore, both viruses had similar mortality, with 12/26 rabbits (46%) surviving the 17*syn*+ infection and 10/20 rabbits (50%) surviving infection with the -83CRE virus. To assess reactivation efficiency, once the viral infections became latent, the rabbit eyes were subjected to epinephrine iontophoresis and swabbed for eight days post-induction. There was no significant difference in reactivation, with the -83CRE recombinant reactivating at near wild-type levels (P=0.4, Table 3-4).

Discussion

Mutation of the -83CRE Is Unfavorable for Recombination

The construction strategy of the -83CRE recombinant should have essentially rescued the LAT promoter deletion of the parental 17ΔPst-Stuffer by replacing the bacterial stuffer fragment with a viral DNA fragment (*Pst-Pst* fragment) containing the LAT transcriptional start site, as well as a mutated -83CRE site. Detection of the bacterial stuffer fragment in the parental virus would suggest that the recombination did not occur. After eight rounds of purification, the

recombinant was fully positive for the *Pst-Pst* fragment but also fully positive for the bacterial stuffer fragment. This suggested that both regions were present in the recombinant virus, and this hypothesis was confirmed when the region was amplified by PCR (Figure 3-2). Recombination, therefore, appeared to occur in only one of the two copies of LAT existing in the viral genome, suggesting that the wild-type -83CRE site is important in virus viability. In other words, a -83CRE recombinant with the mutation in both copies of LAT might prevent normal replication from occurring. The LAT is anti-sense to ICP0 and the 3' ends of the two transcripts overlap. Because of this, one might hypothesize that the -83CRE site normally acts as a transcriptional repressor to control LAT expression at inappropriate times, i.e., times when ICP0 is expressed. When the site is mutated, the virus can no longer replicate because of LAT's interference with ICP0. To address this possibility, the virus was plaque purified on L7 cells. L7 cells are a Vero-based cell line that was stably transfected to express ICP0 (Samaneigo et al., 1997). Interestingly, after only three to four rounds of plaque purification, all plaques were positive for the *Pst-Pst* fragment while none were positive for the bacterial stuffer.

Plaque purification of the -83CRE virus may have been successful on L7 cells and not on RS cells for the reason mentioned above. Since the need for ICP0 complementation was only seen during the plaque purification stage, and not in later experiments, LAT regulation is probably important during recombination. It may be possible that the -83CRE site acts as a repressor of LAT, acting to prevent LAT transcription from occurring at the same time as ICP0. If so, when the -83CRE site is mutated, expression of ICP0 *in trans* by the L7 cells is necessary to overcome the effects of LAT misregulation.

Alternatively, a need for ICP0 in recombination may be caused by the still unidentified second site mutation. If the second mutation affects a region of the virus that is involved in

recombination and ICP0 must be provided in *trans* to overcome the effect, it may suggest that ICP0 and the second site interact during recombination or more likely, that the second site mutation is in ICP0 and that ICP0 itself is important in recombination.

The -83CRE Recombinant Contains a Second Site Mutation that Contributes to the Avirulence Phenotype

When the -83CRE mutant virus was rescued using the wild-type LAT promoter, the avirulence phenotype was not rescued. The mortality rate of the rescuant-infected mice was very low, indicating the presence of another mutation somewhere in the -83CRE mutant's genome. This other mutation, therefore, is likely a major contributor to virulence of HSV-1.

The second site mutation in the -83CRE recombinant may have arisen during the viral construction phase. If the mutation in the -83CRE site was unfavorable to viable virus production, a second compensatory mutation may have occurred, which ultimately allowed the recombinant to be made. An example of a similar situation exists with neurovirulence gene γ34.5 viral recombinants. In eukaryotic cells part of the host defense against viral infection is the activation of protein kinase R (PKR) in response to double-stranded RNA. PKR induction in a cell causes eukaryotic translation initiation factor 2 (eIF-2α) to be phosphorylated, which ultimately shuts down protein synthesis. In wild-type HSV-1, the γ34.5 gene product can dephosphorylate eIF-2α to prevent cellular shutdown of protein synthesis (He et al., 1997). As expected γ34.5 mutants are unable to prevent cellular inhibition of protein synthesis (Chou and Roizman, 1992). Rescue of the γ34.5 mutation led to the isolation of mutants with a second site mutation in the U_s11 gene that causes restoration of the PKR suppressor phenotype observed in wild-type HSV-1 (Mohr and Gluzman, 1996). U_s11 interacts with PKR if present before PKR induction to block eIF-2α phosphorylation and therefore, allows protein synthesis to proceed and

the virus to function (Cassady et al., 1998). The second site mutation in the -83CRE mutant could be functioning in a similar manner, compensating for the -83CRE mutation.

While the second site mutation in the -83CRE virus remains unmapped, one can speculate as to its location. Potentially, the mutation could lie somewhere in ICP0, affecting its normal function. In ICP0-negative mutants, growth in cell culture is inefficient at low m.o.i., but replication is restored to near wild-type levels at high m.o.i. (Sacks and Schaffer, 1987; Chen and Silverstein, 1992). While the -83CRE recombinant did not show the same effect as ICP0 mutants on RS cells, the results from the growth curve assays on Neuro-2A cells suggest multiplicity-dependent growth in neuronal cells. Recall that the -83CRE virus replicated less efficiently than wild-type on Neuro-2A cells at a low m.o.i., but the mutant replicated as well or slightly better than wild-type at the high m.o.i. when grown on Neuro-2A cells. This may indicate that the -83CRE recombinant is functionally deficient in ICP0 in neuronal cells, possibly due to a lack of necessary interplay between effects of the -83CRE mutation, ICP0, and some neuronal-specific factor. When ICP0 is present at high levels, growth in Neuro-2A cells can occur normally. The -83CRE recombinant appeared to grow more efficiently than wild-type during early infection of RS cells at a low m.o.i., suggesting that the -83CRE mutation may compensate for a mutated form of ICP0. The animal experiments performed, in which various tissues were assessed for relative viral DNA levels, support this hypothesis (Figure 3-6). When the -83CRE initially infected the mouse footpad, DNA levels were similar between wild-type and the mutant, but levels of the mutant viral DNA were decreased relative to wild-type in the DRG and spinal cord. This could be due to a lower effective dose of the virus in those tissues; perhaps the -83CRE mutation is no longer an effective means of compensation for whatever defect is present in ICP0. In other words, when the mutant virus is applied to the footpad at a

high dose, it would not appear to act like an ICP0 mutant, in which replication is inhibited at a lower m.o.i. Once the -83CRE virus enters the neuronal tissues, however, the amount of viral production begins to drop. This may be caused by an inability of the mutant virus to utilize some neuronal factor properly and has the end result of decreasing the effective viral dose to the neuronal tissues to one that is not compatible with efficient growth by an ICP0-defective virus.

In the mouse survival experiments, the -83CRE virus was severely attenuated for virulence, corresponding to the finding that ICP0 deletion mutants display decreased pathogenicity in some animal models (Gordon et al., 1990). What is puzzling, however, is the fact that the -83CRE virus established latency and reactivated from latently-infected rabbits with similar efficiency as wild-type 17*syn*+, unlike characterized ICP0 mutants (Wilcox et al., 1997; Halford and Schaffer, 2001). When the chromatin profile was determined for the latent -83CRE in mouse DRG, the LAT promoter and the ICP0 promoter had similar levels of H3 K9, K14 acetylation, which is a marker of transcriptional permissiveness, and both of these regions were less acetylated than the LAT 5'exon. Perhaps the chromatin profile of the -83CRE mutant permitted establishment of latency and reactivation. In other words, even though ICP0 was still hypoacetylated relative to the 5' exon, it may have still been slightly permissive for transcription (which would not be apparent from the qualitative results of the ChIP assay), and this would have allowed reactivation to occur. Marker rescue experiments will aid in determining the site of the second mutation.

Cell-Specific Factors May Interact with the -83CRE Site to Convey Neuronal Tropism to HSV-1

The results from the viral growth curves and the mouse tracer study indicate differential growth efficiencies of the -83CRE recombinant on epithelial cells than on neuronal cells. Specifically, the mutant replicates more efficiently than the wild-type virus on epithelial cells,

while it does not grow as well as wild-type on neuronal cells. This may also explain why the -83CRE is attenuated when mice are infected by the footpad infection route. It seems likely that, although the mutant replicates as efficiently as wild-type at the footpad, the efficiency of replication slows once the mutant reaches the spinal cord and becomes even less efficient once reaching the DRG. By the time it reaches the brain, which is the site of encephalitic infection that leads to mortality in mice, there is little infectious virus left to cause damage.

When the -83CRE virus was used to infect rabbits, the virus did not show the virulence deficit that was observed for mice. The rabbits displayed similar levels of corneal pathology as wild-type, suggesting robust replication in the corneal epithelia by the -83CRE. Because the site of infection (cornea) in the rabbit is close to the site of latency (TG), the virus has less distance to travel and therefore, the -83CRE virus might also replicate less effectively in neurons in the rabbit, as was seen for the mouse, but because of the proximity to the brain, may still produce enough infectious virus to cause death. Experiments to determine viral yields in epithelia versus neuronal tissue during acute infection in the rabbit are in progress.

Mutation of the -83CRE Site Does Not Affect Latency and Reactivation

While mutation of the -83CRE site affected the acute infection of HSV-1, it had no significant effect on the establishment of latency, nor did it affect the ability of the virus to transcribe LAT. This suggests that the -83CRE site, in conjunction with the second site mutation, does not play a role in recruitment of transcription factors to the region during latency. However, when the chromatin profile was determined, the -83CRE mutant virus displayed a profile unlike that seen for wild-type. During latency the -83CRE virus's LAT promoter was less enriched than the 5' exon/enhancer region and was also less enriched than in wild-type 17*syn*+. This hypoacetylation was almost as low as that of the ICP0 promoter, suggesting a

decrease in transcriptional permissiveness in the -83CRE LAT promoter resulting from a loss of the binding site. However, the LAT was still detected by RT-PCR of latently-infected DRG.

Because CRE sites are stress-responsive elements, it was originally hypothesized that the LAT promoter's CREs would play a role in reactivation. However, the -43CRE displayed only an intermediate defect in reactivation when tested in rabbits (Bloom et al., 1997), while the -83CRE virus reactivated at the same frequency as wild-type HSV-1. It remains possible that the two elements act in concert with each other to control reactivation, since the deletion of both elements in 17ΔPst results in a virus that does not reactivate from latency in the rabbit (Hill et al., 1990). However, since the deletion in 17ΔPst is 202 bp, many important factors besides the CREs are deleted, which is likely the cause of the reactivation-negative phenotype. In order to address possible interactions between the two LAT promoter CREs without considering the influence of the other binding sites in the promoter, a double CRE mutant would need to be created. While not detailed in this dissertation, both the -43CRE and -83CRE were mutated and transfected with HSV-1 DNA in order to create a recombinant with both CREs mutated (CREDBL). Like the -83CRE, the CREDBL remained single-sided throughout the plaque purifications on RS cells. Unlike the -83CRE virus, the CREDBL was unable to be purified on L7 cells. Only about 50% of recombinants were positive, although single-sided, and none became pure after several more rounds of purification. This, coupled with the -83CRE viral construction difficulties, implies a need for the wild-type -83CRE in at least the acute, replicative stage of the viral lifecycle, during which recombination would occur. Since the -43CRE recombinant was constructed by Bloom et al. (1997) with little difficulty, this element is likely not as critical as the -83CRE for lytic viral growth.

The Wild-Type -83CRE May Control LAT during the Acute Infection

While the -43CRE showed a slight effect on reactivation, there was no significant effect on reactivation that resulted from mutation of the -83CRE. Instead, the major observations—decreased neuronal replication and attenuated virulence in the mouse—are linked to the acute HSV-1 infection. Since it was previously reported that the -83CRE binds the repressive CREB-2 (Millhouse et al., 1998), the site's main function may be to control LAT. When HSV-1 enters a cell, the cAMP pathway may be induced, since the entry of a virus into a cell is undoubtedly a stressful event. Various CREB family members could be produced, including CREB-2. When this is present at the LAT promoter's -83CRE, the promoter would be repressed. However, in the event that the site is mutated and CREB-2 binding is abrogated, perhaps LAT is no longer controlled and would interfere with ICP0 to affect the acute stage of infection. For this reason, the -83CRE recombinant was not able to be constructed with the mutation in both repeats of the LAT. Instead, a second site compensatory mutation was necessary that allowed for the viability of the -83CRE virus. This mutation may have occurred in ICP0, possibly affecting some function that normally controls ICP0 expression. If LAT interference with ICP0 by the -83CRE mutation needed to be overcome, the likely solution for the virus might be to upregulate ICP0, providing the -83CRE recombinant with a similar environment as that encountered when grown on L7 cells. By characterizing the second site mutation and determining whether it really does occur in ICP0, the importance of the LAT promoter -83CRE site and how it might interplay with the region of the second mutation can be uncovered.

Table 3-1. Survival of mice (number remaining/number infected) infected via the footpad with either 17*syn*+ or -83CRE.

Dose (pfu)	17*syn*+ survival	-83CRE survival
500	3/10	9/10
5,000	1/10	9/10
50,000	0/10	9/10

Table 3-2. Survival of mice (number remaining/number infected) inoculated intracranially with either 17*syn*+ or -83CRE.

Dose (pfu)	17*syn*+ survival	-83CRE survival
10	1/5	4/5
100	0/5	0/5

Table 3-3. Slit lamp examination (SLE) scores of rabbit corneas at 3, 5, or 7 days post-infection.

Day post-infection	17*syn*+ average SLE score	-83CRE average SLE score	P-value*
3	3 ± 1.1	3 ± 1.2	0.82
5	2.8 ± 1.1	2.7 ± 1.0	0.5
7	2.3 ± 1.1	2.3 ± 1.1	0.98

* P-value calculated using the Mann-Whitney Rank Sum test.

Table 3-4. Reactivation of rabbits post-epinephrine induction.

Virus	Percent reactivated eyes (Total positive/total eyes)	Percent reactivated swabs (Total positive/total swabs)
17*syn*+	54.5% (6/11)	26.1% (23/88)
-83CRE	78.6% (11/14)	32.1% (36/112)

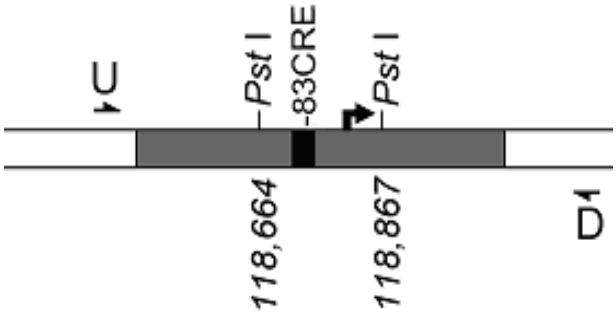

Figure 3-1. Diagram of PCR primer locations used in verification of -83CRE mutant virus. Recombination fragment for virus construction is shown as a gray bar, with the -83CRE site shown as a black box. The LAT transcriptional start site is indicated by an arrow. Primers are designated by arrows and U, Upfragment (nt 118,305–118,325), or D, Downfragment (nt 119,179–119,202).

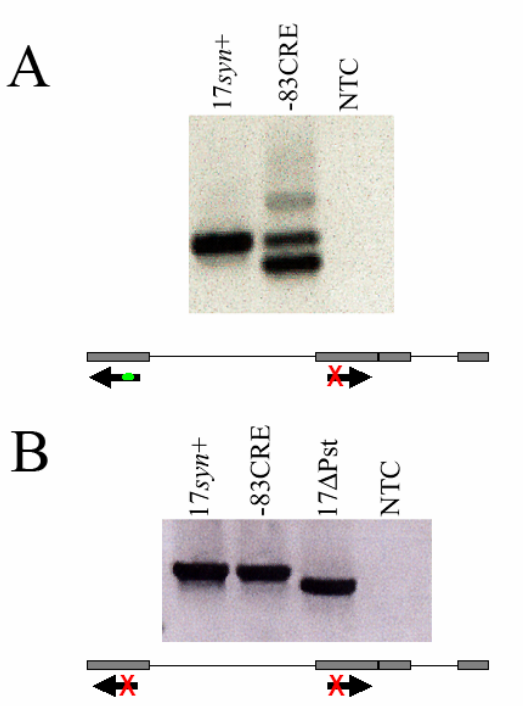

Figure 3-2. Analysis of PCR products amplified from dot blot purification. A) The recombinant virus in an early round of purification shows products corresponding to both the parental and wild-type (17*syn*+) virus. Diagram of the -83CRE recombinant genome shows the two copies of the LAT (arrows) and indicates the presence of the stuffer (green) and the mutation (red "X"). B) The recombinant virus PCR product size corresponds to that of the wild-type virus (17*syn*+) in purification on L7 cells. Note the presence of a single band. Parental virus, 17ΔPst, is also shown. NTC, no template control.

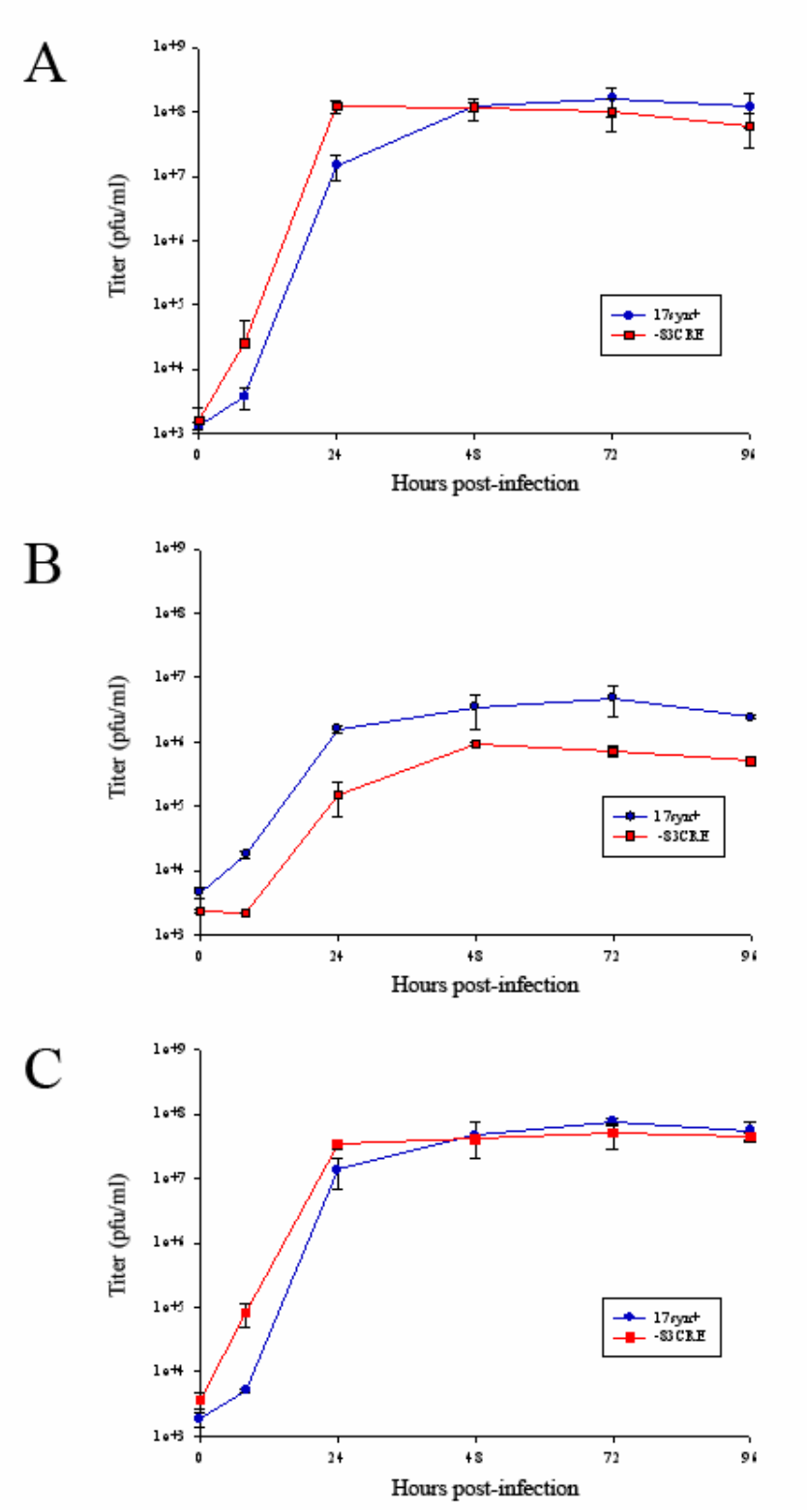

Figure 3-3. Multi-step viral growth curves. 17*syn*+ (wild-type) or -83CRE (recombinant) were inoculated at an m.o.i. of 0.01 to allow for multiple rounds of replication on A) RS cells, B) Neuro-2A cells, and C) L7 cells.

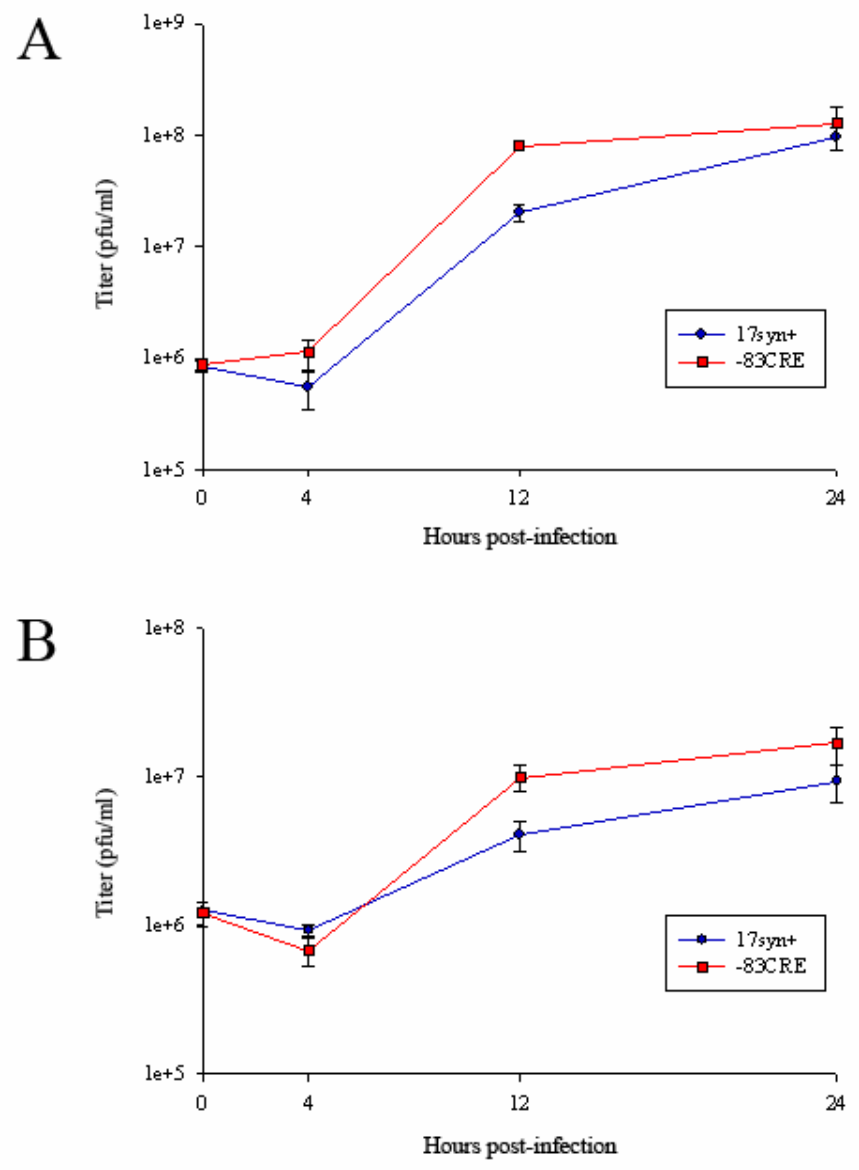

Figure 3-4. Single-step viral growth curves. 17*syn*+ (wild-type) or -83CRE (recombinant) were inoculated at an m.o.i. of 5 to allow for synchronous infection of all cells and a single round of replication on A) RS cells and B) Neuro-2A cells.

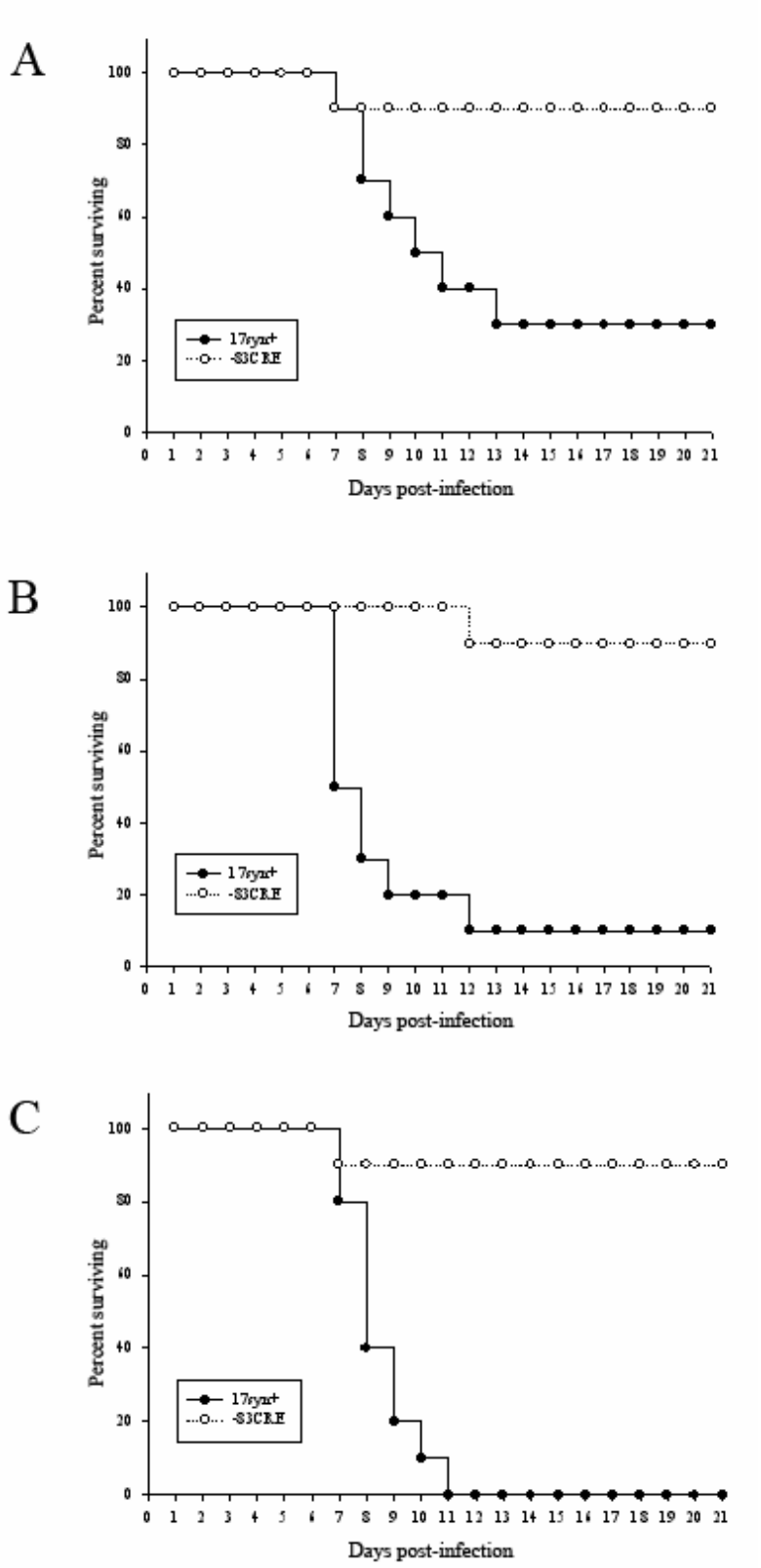

Figure 3-5. Percent survival over the course of acute infection of mice (10 per group) infected with either 17*syn*+ or -83CRE at A) 500 pfu, B) 5,000 pfu, or C) 50,000 pfu.

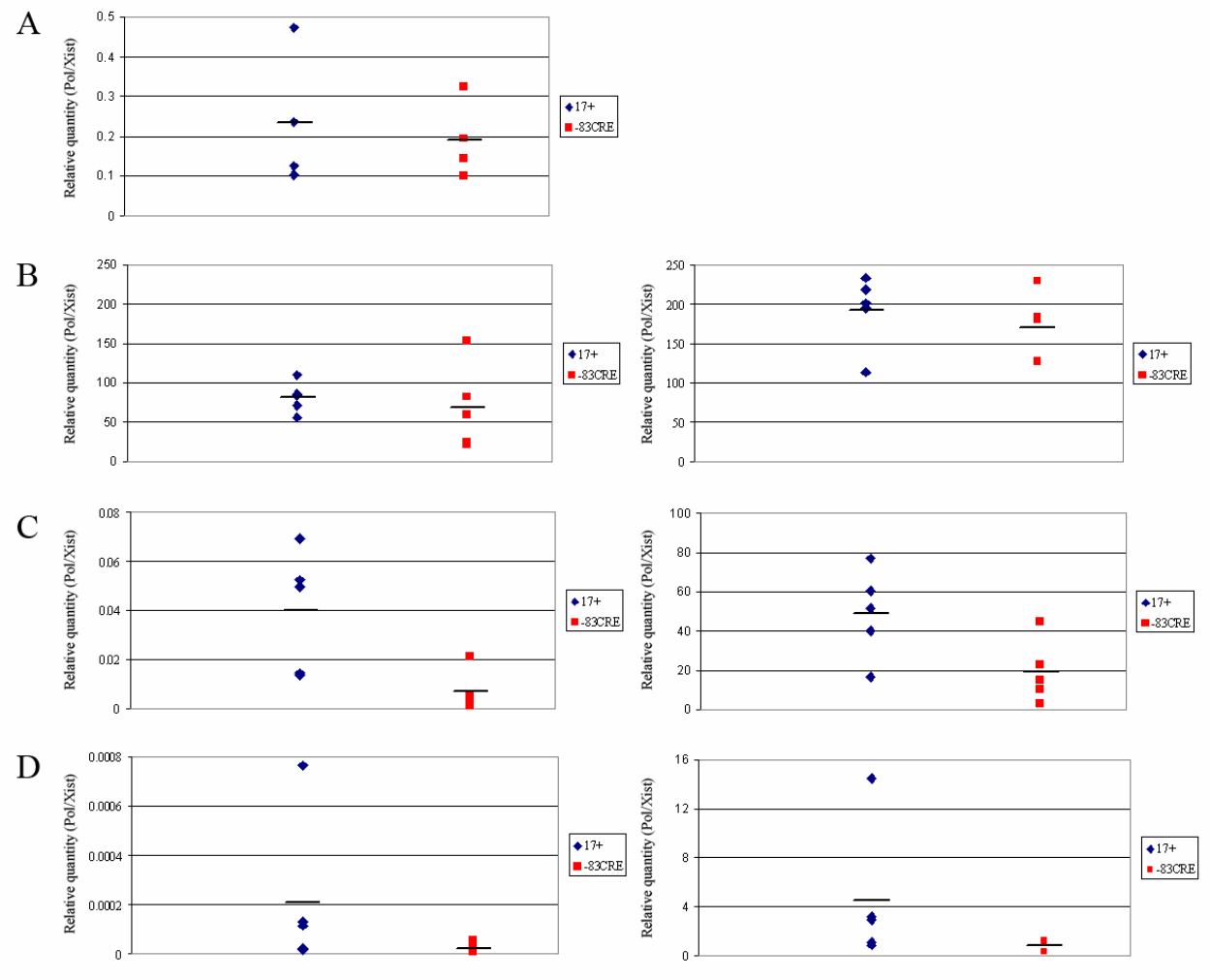

Figure 3-6. Relative viral genomes (Pol, HSV-1 polymerase normalized to cellular gene, *Xist*) for 8 hours, 2 days, or 4 days p.i. of mice. Mice were infected via the footpad with either 17*syn*+ or -83CRE. A) 8 hours p.i. foot samples (n = 4 mice/virus/time), B) foot samples for 2 days p.i. (left panel) and 4 days p.i. (right panel), C) DRG 2 days p.i. (left panel) and 4 days p.i. (right panel) (n = 5 mice/virus/time), D) Spinal cord 2 days p.i. (left panel) and 4 days p.i. (right panel) (n = 5 mice/virus/time).

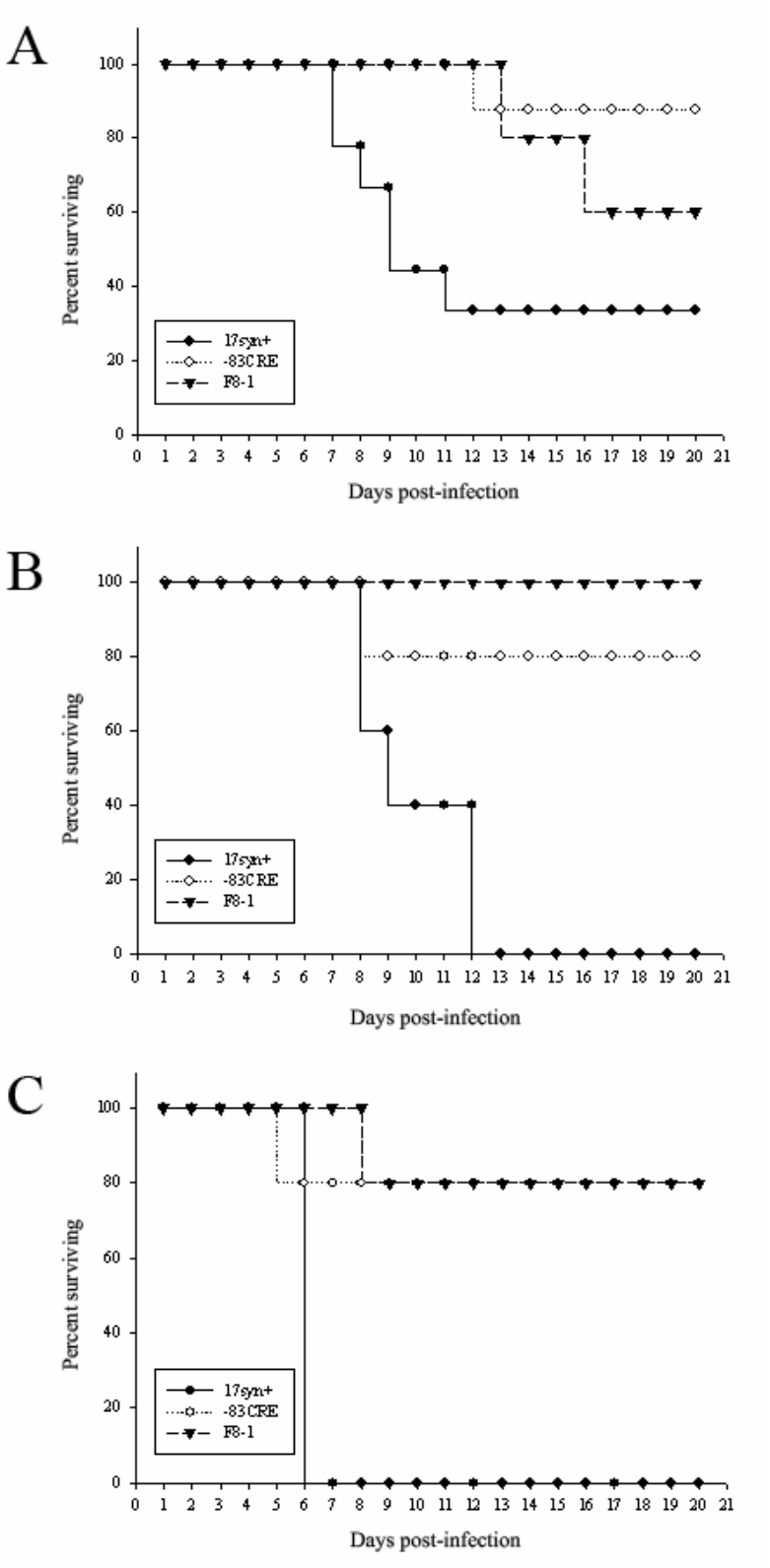

Figure 3-7. Percent survival of mice infected with A) 500 pfu, B) 5,000 pfu, or C) 50,000 pfu of 17syn+, -83CRE, or F8-1 rescuant virus.

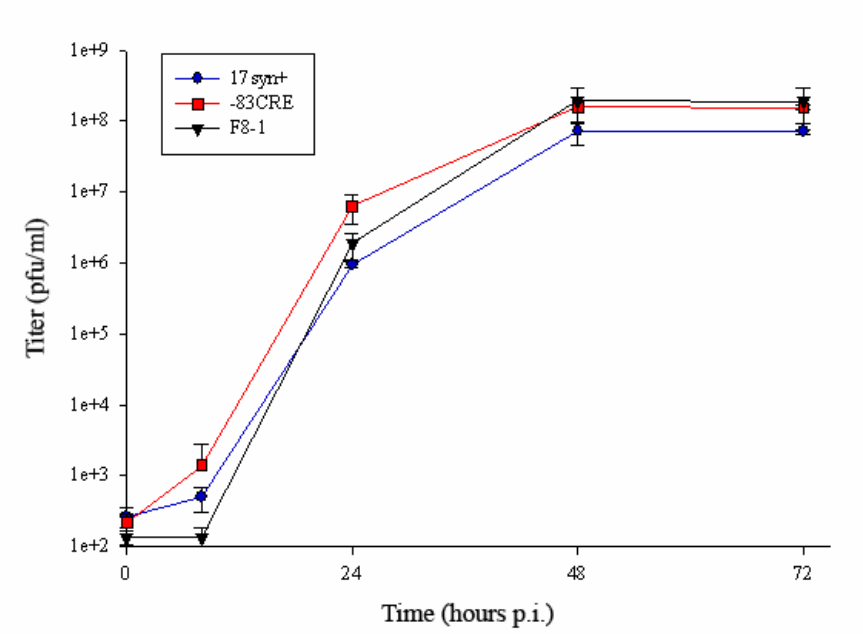

Figure 3-8. Multi-step growth curve (m.o.i. 0.001) on RS cells for 17*syn*+, -83CRE, and F8-1.

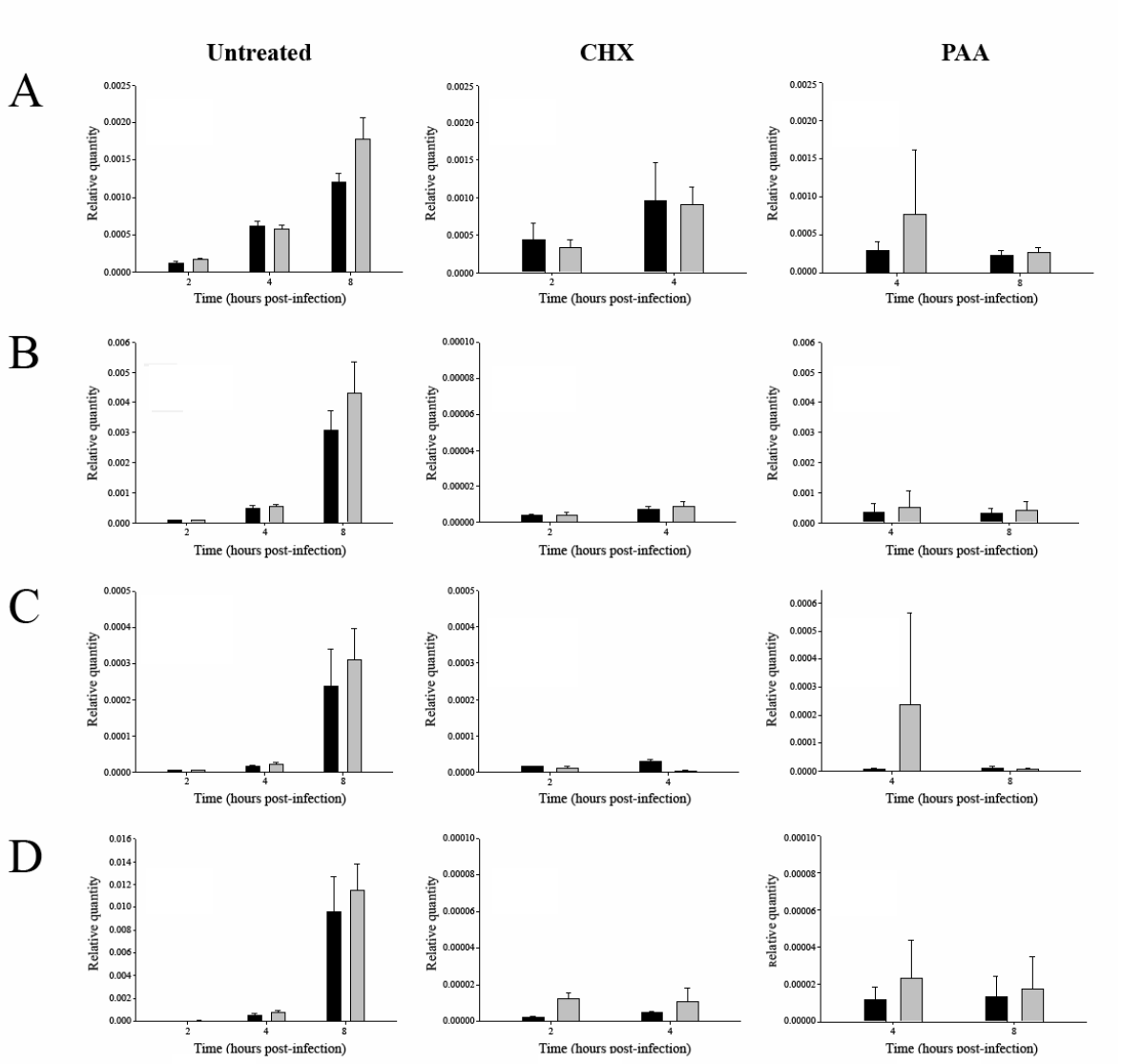

Figure 3-9. Relative RNA transcript levels (target normalized to cellular control, GAPDH) during acute infection of RS cells. A) Immediate-early gene, ICP4, B) early gene, HSV-1 polymerase, C) late gene, LAT, and D) late gene, glycoprotein C (gC). Left panel, no treatment. Middle panel, addition of 50 μg/ml CHX to cells along with infecting inoculum. Right panel, addition of 400 μg/ml PAA to cells with infecting inoculum. Black bars, 17*syn*+. Gray bars, -83CRE recombinant.

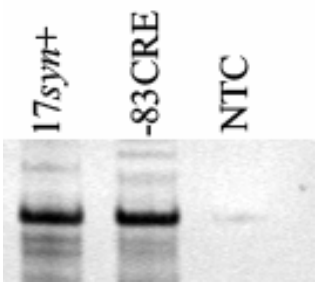

Figure 3-10. LAT expression during latency in mouse DRG infected with the -83CRE mutant virus. RNA was isolated from DRG of latently-infected mice and reverse transcribed using random decamers. PCR was performed using LAT 5' exon primers.

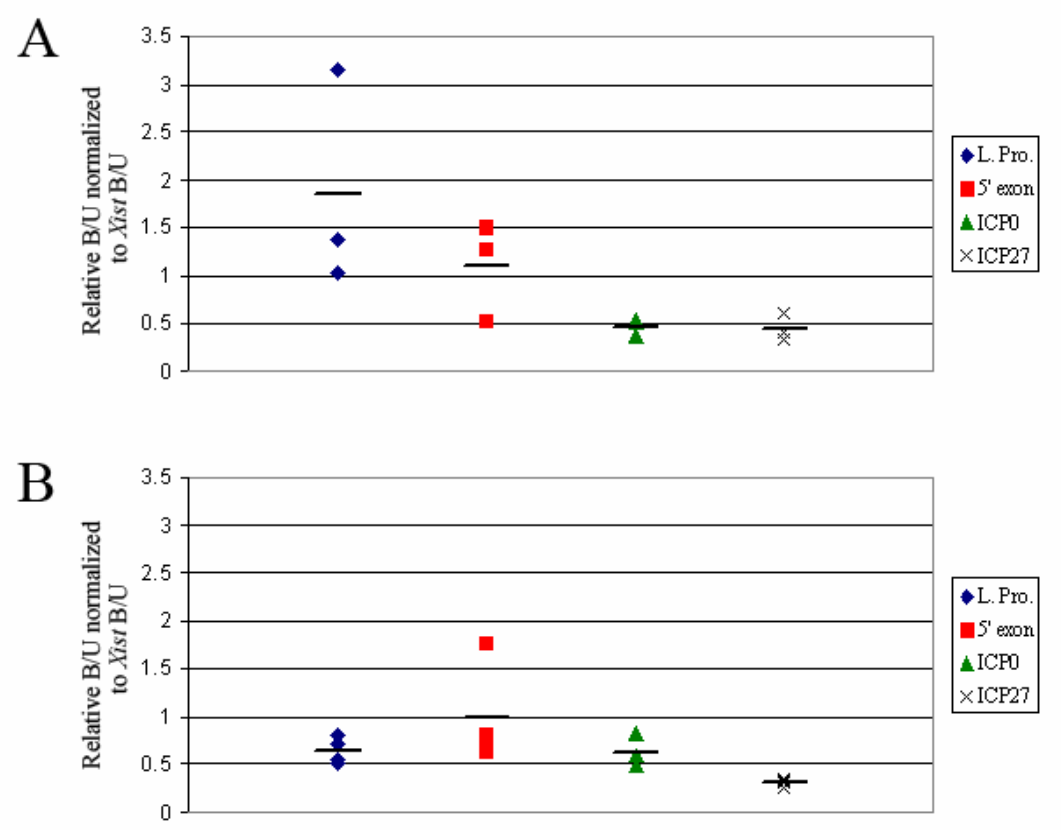

Figure 3-11. The LAT promoter of the -83CRE mutant virus is decreased in histone H3 K9, K14 acetylation relative to wild-type. A) 17*syn*+, B) -83CRE recombinant. Relative bound, B, to unbound, U, ratios normalized to those for *Xist* cellular control are shown for the LAT promoter (L. Pro.), the 5' exon, the ICP0 promoter, and ICP27.

CHAPTER 4
OVERALL DISCUSSION

While the HSV-1 LAT region is known to facilitate reactivation, the exact mechanism involved is not known. The overall focus of this dissertation, therefore, was to investigate regulation of the LAT, particularly at the level of the LAT promoter. Findings suggest that (1) an element or elements in the core LAT promoter are essential for a latent chromatin profile that is compatible with reactivation, (2) epinephrine-induced reactivation of latently-infected rabbits does not cause the same effects on chromatin as explant-induced reactivation of mouse DRG, (3) the -83CRE site of the LAT promoter is dispensable for establishment of latency, ability to express LAT, and capacity to reactivate, even though the latent chromatin profile differs from that of the wild-type virus, and (4) a compensatory mutation generated during the construction of the -83CRE recombinant appears to dramatically affect virulence in the mouse and may be important in regulation of the lytic infection. These data support the developing view that the LAT promoter may be a part of a complex regulatory switch that modulates gene expression in a tissue-specific manner both during the acute and latent periods of HSV-1 infection.

The LAT and Chromatin

During latency transcription from the HSV-1 genome is not repressed through DNA methylation, but instead, histone tail modifications may be a component of gene regulation (Kubat et al., 2004a). In the mouse the LAT region of the latent viral genome shows enrichment in acetylated histone H3 K9, K14, a marker of transcriptional permissiveness, relative to lytic genes (Kubat et al., 2004a; Kubat et al., 2004b). When the LAT promoter is deleted and the virus is unable to transcribe the LAT, the same effect is observed, suggesting that the LAT region contains elements that direct the transcriptionally permissive histone modifications independently of Pol II activity through this region. During early times post-explant of murine

DRG infected with wild-type virus, there is chromatin remodeling of the LAT region to a more transcriptionally non-permissive state and decreased LAT levels between 2 and 3 hours post-explant (Amelio et al., 2006). A major goal of this dissertation project was to extend the results obtained for the mouse to the rabbit eye model, which more closely mimics clinical reactivation of HSV-1 in humans.

In the rabbit, the ability to express at least some regions of the LAT correlates with the ability to reactivate from latency. When the chromatin profile was assessed for a LAT-positive (wild-type) and a LAT-negative (promoter-deletion mutant) virus, two different profiles were observed, unlike what was seen in the mouse. While the wild-type virus displayed a similar latent profile to that of the latently-infected mouse, the LAT-negative virus displayed dramatically more enrichment in dimethylated H3 K4, a marker of transcriptional permissiveness, in the region just upstream of the deletion than the 5'exon/enhancer. Levels of dimethylation in the LAT promoter region were also much higher than that of wild-type, even though the 5'exon/enhancer region showed similar levels. This suggests the presence of a repressive element in the native core LAT promoter that might prevent increased transcriptional permissiveness of the LAT region at inappropriate times, such as during lytic infection. Additionally, because LAT promoter deletion mutants are severely deficient in reactivation in the rabbit, the difference in chromatin profiles between the mutant and the wild-type virus may implicate a requirement for establishment of a certain chromatin profile for normal reactivation and also suggests that deletion of the LAT promoter causes a loss of a regulatory element required for wild-type reactivation.

When the chromatin profiles of wild-type HSV-1 and LAT promoter mutant, 17ΔPst, were assessed following adrenergic induction of reactivation in the rabbit ocular model, no change in

chromatin was evident for either virus between 0, 1, 2, and 4 hours post-induction, times corresponding to those tested in the mouse explant experiments. This finding suggests that chromatin remodeling is not critical to LAT-dependent reactivation or that chromatin remodeling in response to mouse DRG explant is a much faster process than what occurs in the rabbit. For example, since the epinephrine is administered to the eye and reactivation occurs in the ganglia, it might take some time for the epinephrine to reach and stimulate all cells in the ganglia, while in explant-induced reactivation, a uniform stressor (ganglion removal from the animal) might stimulate cells to reactivate very rapidly.

The deletion of the core LAT promoter in the 17ΔPst mutant is 202-bp in size. This suggests that a number of binding sites and *cis*-elements were removed, some of which could normally contribute to controlling the level of transcriptional permissiveness.

LAT Regulation through Promoter Function

To investigate a possible regulator of the LAT promoter, a cAMP response element, located 83 nucleotides upstream of the LAT transcriptional start site (-83CRE) was examined. A recombinant virus with mutation of the -83CRE was created and analyzed for alterations to the acute, latent, and reactivation phases of the viral lifecycle. The -83CRE recombinant was ultimately found to contain a second mutation, which may have been a compensatory mutation that allowed an otherwise unviable recombinant to replicate. During acute infection, the mutant virus displayed attenuated virulence in mice that were infected via the footpad, and in cell culture the virus showed increased replication in fibroblasts and decreased replication in neuronal cells, suggesting a cell-type specific effect of the -83CRE site (or the compensatory mutation) in viral replication. The -83CRE recombinant virus was able to establish latency, express LAT, and reactivate. Interestingly, during latency in mice, the -83CRE mutant virus displayed decreased transcriptional permissiveness at the LAT promoter relative to the wild-type virus. This is in

contrast to the findings for 17ΔPst in the rabbit, in which deletion of the entire promoter (including the -83CRE site) caused dramatically increased transcriptional permissiveness. Thus, the wild-type -83CRE may be important in regulation of the LAT promoter, probably by interacting with another nearby element as well as cellular factors, to maintain the configuration of the LAT region in a reactivation-compatible state. Removal of the -83CRE causes a decreased transcriptional permissiveness of the LAT promoter yet the recombinant virus still reactivates from latency in the rabbit. Since there is another mutation in the recombinant, that second site may contribute to the chromatin configuration. For example, if ICP0 is mutated to a more active form, control of latency might not be as tightly controlled as in the wild-type virus; thus, the -83CRE mutant virus can still reactivate because it is less repressed. Future experiments should address these questions.

Findings suggest that the -83CRE is important in acute phase regulation of HSV-1, likely in concert with some other element(s) of the genome. If the wild-type -83CRE binds a repressive protein to control LAT expression during the lytic infection, deletion of the element would cause misregulation of the LAT, potentially interfering with ICP0 transcription. If this occurred during the construction phase of a mutant -83CRE virus, a second mutation in ICP0 may have occurred to overcome the problem. Characterization of the second mutation in the -83CRE recombinant is ongoing and should provide information about interplay between the LAT promoter and other elements important to the viral life cycle.

A Model for Reactivation

A model has been proposed for steroid receptor function in which random and transient interactions between various factors and a promoter might not actually result in transcription; instead, since those events may be part of a sequential process, in which promoter modification and secondary recruitment of other factors ultimately lead to transcription, ChIP experiments

would indicate only some of the events in a population at a particular time without giving a sense of the dynamic processes occurring in single cells (reviewed in Hager et al., 2006). In other words, the binding of receptor complexes, and probably other transcription factors, is a cyclical process; protein-DNA binding occurs without being stable and long-term, but the end result is usually promoter activation. Support for this comes from experiments performed on single living cells using UV laser crosslinking technology to monitor chromatin remodeling at a promoter; these studies indicated cycle times were less than a minute (reviewed in Hager et al., 2006). The approaches used in the experiments performed in this dissertation used ChIP, thereby limiting observations to events occurring in the collective group of cells in a ganglion.

Perhaps the regulation of HSV reactivation could be modeled like the dynamic process that regulates steroid receptor activation, and at any given time, single "latently-infected" cells are actually producing lytic viral transcripts. Support for this comes from a study performed by Feldman et al. (2002), in which ganglia from latently-infected mice were assayed by *in situ* hybridization for the presence of lytic gene expression and viral replication. Assessment of thousands of neurons led to the finding that a small number of individual neurons actually express lytic viral genes and replicate viral DNA, even though the ganglia as a whole is considered latently-infected. The authors also found indication that the host inflammatory response may prevent the stray neurons from yielding infectious virus (Feldman et al., 2002). Perhaps true reactivation does not actually occur until a group of those cells is synchronously transcribing lytic genes, and enough virus is produced to be infectious. Activation of the cAMP pathway, or another stress-inducible pathway, by a stress stimulus may be sufficient to not only trigger the synchronous initiation of reactivation in a larger number of cells, but also to create the

correct environment (i.e., temporary depression of the local host response) for efficient production of abundant virus.

The entire process of establishment of and reactivation from latency is clearly complex. Findings can vary from system to system and viral strain to viral strain, possibly due to different types of reactivation. If regulation at the level of chromatin is important, maybe a specific chromatin configuration must be established that is compatible with reactivation. If *cis* elements in the LAT *rcr* region are important, maybe timing and synchronicity are critical for reactivation. However, it may be possible that both of these, as well as other regulatory mechanisms, play a role in preventing aberrant transcription of lytic genes and controlling reactivation. HSV-1's ability to persist indefinitely in a host, undetected by the immune system, and periodically reactivate to spread to a new host suggests a very tightly regulated system that may include several ways for reactivation to occur. Work in the field of latency and reactivation will undoubtedly provide interesting and numerous pieces of the puzzle for years to come.

APPENDIX
REAL-TIME PCR PRIMER/PROBE SEQUENCES

Table A-1. Real-time PCR primer/probes.

Target	Sequence (5' to 3')
LAT Promoter (NC_001806: 118,263–118,323)	Forward—CAA TAA CAA CCC CAA CGG AAA GC Reverse—TCC ACT TCC CGT CCT TCC AT Probe—TCC CCT CGG TTG TTC C
LAT 5' Exon (NC_001806: 119,326–119,397)	Forward—GGC TCC ATC GCC TTT CCT Reverse—AAG GGA GGG AGG AGG GTA CTG Probe—TCT CGC TTC TCC CC
ICP0 Promoter (NC_001806: 124,494–124,578)	Forward—CCG CCG ACG CAA CAG Reverse—GTT CCG GGT ATG GTA ATG AGT TTC T Probe—CTT CCC GCC TTC CC
ICP27/UL54 (NC_001806: 113,945–114,034)	Forward—GCC CGT CTC GTC CAG AAG Reverse—GCG CTG GTT GAG GAT CGT T Probe—CAG CAC CCA GAC GCC
ICP4 (NC_001806: 147,941–148,025)	Forward—GAC GGG CCG CTT CAC Reverse—GCG ATA GCG CGC GTA GA Probe—CCG ACG CGA CCT CC
HSV polymerase (NC_001806: 65,801–65,953)	Forward—AGA GGG ACA TCC AGG ACT TTG T Reverse—CAG GCG CTT GTT GGT GTA C Probe—ACC GCC GAA CTG AGC A
Rabbit GAPDH	Forward—GCA CCA CCA ACT GCT TAG C Reverse—CCT CCA CAA TGC CGA AGT G Probe—CTG GCC AAG GTC ATC C
Rabbit centromere	Forward—GCT CCA GAA ACC TGA GAA AAC ATG AT Reverse—TGG AGA AAA GCG CAA TCT TCC T Probe—TTC GGC AAA TGC ATC CAA
Mouse Xist (NR_001463: 857–925)	Forward—GCT CTT AAA CTG AGT GGG TGT TCA Reverse—GTA TCA CGC AGA AGC CAT AAT GG Probe—ACG CGG GCT CTC CA
Mouse APRT	Forward—CTC AAG AAA TCT AAC CCC TGA CTC A Reverse—GCG GGA CAG GCT GAG A Probe—CCA GGG CCT CAC CAC C

LIST OF REFERENCES

Ackland-Berglund, C.E., Davido, D.J., Leib, D.A., 1995. The roles of the cAMP-response element and TATA box in expression of the herpes simplex virus type 1 latency-associated transcripts. Virology 210, 141–151.

Ahmed, M., Lock, M., Miller, C.G., Fraser, N.W., 2002. Regions of the herpes simplex virus type 1 latency-associated transcript that protect cells from apoptosis in vitro and protect neuronal cells in vivo. J. Virol. 76 (2), 717–729.

Alberini, C.M., Ghirardi, M., Metz, R., Kandel, E.R., 1994. C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in Aplysia. Cell 76 (6), 1099–1114.

Amelio, A.L., Giordani, N.V., Kubat, N.J., O'Neil, J.E., Bloom, D.C., 2006. Deacetylation of the Herpes Simplex Virus type 1 latency-associated transcript (LAT) enhancer and a decrease in LAT abundance precede an increase in ICP0 transcriptional permissiveness at early times postexplant. J. Virol. 80 (4), 2063–2068.

Arias, J., Alberts, A.S., Brindle, P., Claret, F.X., Smeal, T., Karin, M., Feramisco, J., Montminy, M., 1994. Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. Nature 370 (6486), 226–229.

Batchelor, A.H., Wilcox, K.W., O'Hare, P., 1994. Binding and repression of the latency-associated promoter of herpes simplex virus by the immediate early 175K protein. J. Gen. Virol. 75 (4), 753–767.

Becker, P.B., Hörz, W., 2002. ATP-dependent nucleosome remodeling. Ann. Rev. Biochem. 71, 247–273.

Bernstein, E., Hake, S.B., 2006. The nucleosome: a little variation goes a long way. Biochem. Cell Biol. 84 (4), 505–517.

Bernstein, B.E., Humphrey, E.L., Erlich, R.L., Schneider, R., Bouman, P., Liu, J.S., Kouzarides, T., Schreiber, S.L., 2002. Methylation of histone H3 lys 4 in coding regions of active genes. Proc. Natl. Acad. Sci. U.S.A. 99 (13), 8695–8700.

Berthomme, H., Thomas, J., Texier, P., Epstein, A., Feldman, L.T., 2001. Enhancer and long-term expression functions of herpes simplex virus type 1 latency-associated promoter are both located in the same region. J. Virol. 75 (9), 4386–4393.

Biswas, P.S., Rouse, B.T., 2005. Early events in HSV keratitis—setting the stage for a blinding disease. Microbes Infect. 7 (4), 799–810.

Bloom, D.C., Devi-Rao, G.B., Hill, J.M., Stevens, J.G., Wagner, E.K., 1994. Molecular analysis of herpes simplex virus type 1 during epinephrine-induced reactivation of latently infected rabbits in vivo. J. Virol. 68 (3), 1283–1292.

Bloom, D. C., Hill, J. M., Devi-Rao, G., Wagner, E. K., Feldman, L. T., Stevens, J. G., 1996. A 348-base-pair region in the latency-associated transcript facilitates herpes simplex virus type 1 reactivation. J. Virol. 70 (4), 2449–2459.

Bloom, D.C., Stevens, J.G., Hill, J.M., Tran, R.K., 1997. Mutagenesis of a cAMP response element within the latency-associated transcript promoter of HSV-1 reduces adrenergic reactivation. Virology 236, 202–207.

Boeger, H., Griesenbeck, J., Strattan, J.S., Kornberg, R.D., 2003. Nucleosomes unfold completely at a transcriptionally active promoter. Molec. Cell 11 (6), 1587–1598.

Cai, W., Astor, T.L., Liptak, L.M., Cho, C., Coen, D.M., Schaffer, P.A., 1993. The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. J.Virol. 67 (12), 7501–7512.

Calomme, C., Dekoninck, A., Nizet, S., Adam, E., Nguyen, T.L., Van Den Broeke, A., Willems, L., Kettmann, R., Burny, A., Van Lint, C., 2004. Overlapping CRE and E box motifs in the enhancer sequences of the bovine leukemia virus 5' long terminal repeat are critical for basal and acetylation-dependent transcriptional activity of the viral promoter: implications for viral latency. J. Virol. 78 (24), 13848–13864.

Cassady, K.A., Gross, M., Roizman, B., 1998. The herpes simplex virus Us11 protein effectively compensates for the γ34.5 gene if present before activation of protein kinase R by precluding its phosphorylation and that of the α subunit of eukaryotic transcription initiation factor 2. J.Virol. 72 (11), 8620–8626.

Cha-Molstad, H., Keller, D.M., Yochum, G.S., Impey, S., Goodman, R.H., 2004. Cell-type-specific binding of the transcription factor CREB to the cAMP-response element. Proc. Natl. Acad. Sci. U.S.A. 101 (37), 13572–13577.

Chen, J., Silverstein, S., 1992. Herpes simplex viruses with mutations in the gene encoding ICP0 are defective in gene expression. J. Virol. 66 (5), 2916–2927.

Chen, X., Schmidt, M.C., Goins, W.F., Glorioso, J.C., 1995. Two herpes simplex virus type 1 latency-active promoters differ in their contributions to latency-associated transcript expression during lytic and latent infections. J. Virol. 69 (12), 7899–7908.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., Rutter, W.J., 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18 (24), 5294–5299.

Chou, J., Roizman, B., 1992. The γ34.5 gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. Proc. Natl. Acad. Sci. U.S.A. 89 (8), 3266–3270.

Chrivia, J.C., Kwok, R.P., Lamb, N., Hagiwara, M., Montminy, M.R., Goodman, R.H., 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365 (6449), 855–859.

Colgin, M.A., Smith, R.L., Wilcox, C.L., 2001. Inducible cyclic AMP early repressor produces reactivation of latent herpes simplex virus type 1 in neurons in vitro. J. Virol. 75 (6), 2912–2920.

Corre, S., Galibert, M.D., 2005. Upstream stimulatory factors: highly versatile stress-responsive transcription factors. Pigment Cell Res. 18, 337–348.

Deshmane, S.L., Fraser, N.W., 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J. Virol. 63 (2), 943–947.

Devi-Rao, G.B., Bloom, D.C., Stevens, J.G., Wagner, E.K., 1994. Herpes simplex virus type 1 DNA replication and gene expression during explant-induced reactivation of latently infected murine sensory ganglia. J. Virol. 68 (3), 1271–1282.

Dobson, A.T., Sederati, F., Devi-Rao, G., Flanagan, W.M., Farrell, M.J., Stevens, J.G., Wagner, E.K., Feldman, L.T., 1989. Identification of the latency-associated transcript promoter by expression of rabbit beta-globin mRNA in mouse sensory nerve ganglia latently infected with a recombinant herpes simplex virus. J. Virol. 63 (9), 3844–3851.

Dwarki, V.J., Montminy, M., Verma, I.M., 1990. Both the basic region and the 'leucine zipper' domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. EMBO J. 9 (1), 225–232.

Everett, R.D., 1989. Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate-early gene 1. J. Gen. Virol. 70, 1185–1202.

Everett, R.D., 2000. ICP0, a regulator of herpes simplex virus during lytic and latent infection. BioEssays 22, 761–770.

Farrell, M.J., Dobson, A.T., Feldman, L.T., 1991. Herpes simplex virus latency-associated transcript is a stable intron. Proc. Natl. Acad. Sci. U.S.A. 88 (3), 790–794.

Feldman, L.T., Ellison, A.R., Voytek, C.C., Yang, L., Krause, P., Margolis, T.P., 2002. Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice. Proc. Natl. Acad. Sci. U.S.A. 99 (2), 978–983.

Garger, S.J., Griffith, O.M., Grill, L.K., 1983. Rapid purification of plasmid DNA by a single centrifugation in a two-step cesium chloride-ethidium bromide gradient. Biochem. Biophys. Res. Commun. 117 (3), 835–842.

Gebhardt, B.M., Kaufman, H.E., 1995. Propranolol suppresses reactivation of herpesvirus. Antiviral Res. 27 (3), 255–261.

Goins, W.F., Sternberg, L.R., Croen, K.D., Krause, P.R., Hendricks, R.L., Fink, D.J., Straus, S.E., Levine, M., Glorioso, J.C., 1994. A novel latency-active promoter is contained within the herpes simplex virus type 1 U_L flanking repeats. J. Virol. 68 (4), 2239–2252.

Goldberg, A.D., Allis, C.D., Bernstein, E., 2007. Epigenetics: a landscape takes shape. Cell 128 (4), 635–638.

Gonzalez, G.A., Montminy, M.R., 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at Ser 133. Cell 59 (4), 675–680.

Gordon, Y.J., Romanowski, E., Balouris, C., Araullo-Cruz, T., 1990. A herpes simplex type 1 ICP0 deletion mutant demonstrates diminished pathogenicity during acute ocular infection in different host animals. Invest Ophthalmol Vis Sci. 31 (4), 681–688.

Guan, Z., Giustetto, M., Lomvardas, S., Kim, J.H., Miniaci, M.C., Schwartz, J.H., Thanos. D., Kandel, E.R., 2002. Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. Cell 111 (4), 483–493.

Gupta, A., Gartner, J.J., Sethupathy, P., Hatzigeorgiou, A.G., Fraser, N.W., 2006. Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript. Nature 442, 82–85.

Gussow, A.M., Giordani, N.V., Tran, R.K., Imai, Y., Kwiatkowski, D.L., Rall, G.F., Margolis, T.P., Bloom, D.C., 2006. Tissue-specific splicing of the herpes simplex virus type 1 latency-associated transcript (LAT) intron in LAT transgenic mice. J. Virol. 80 (19), 9414–9423.

Hager, G.L., Elbi, C, Johnson, T.A., Voss, T., Nagaich, A.K., Schlitz, R.L., Qiu, Y., John, S., 2006. Chromatin dynamics and the evolution of alternate promoter states. Chrom. Res. 14, 107–116.

Halford, W.P., Schaffer, P.A., 2001. ICP0 is required for efficient reactivation of herpes simplex virus type 1 from neuronal latency. J. Virol. 75 (7), 3240–3249.

He, B., Gross, M., Roizman, B., 1997. The γ34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1α to dephosphorylate the α subunit of eukaryotic translation initiation factor 1 and preclude the shutoff of protein synthesis by double stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA 94 (3), 843–848.

Herrera, F.J., Trizenberg, S.J., 2004. VP16-dependent association of chromatin-modifying coactivators and underrepresentation of histones at immediate-early gene promoters during herpes simplex virus infection. J. Virol. 78 (18), 9689–9696.

Higgins, C.R., Schofield, J.K., Tatnall, F.M., Leigh, I.M., 1993. Natural history, management and complications of herpes labialis. J. Med. Virol. Suppl 1, 22–26.

Hill, J. M., Maggioncalda, J. B., Garza, H. H., Su, Y-H., Fraser, N. W., Block, T. M., 1996. In vivo epinephrine reactivation of ocular herpes simplex virus type 1 in the rabbit is correlated to a 370-base-pair region located between the promoter and the 5' end of the 2.0-kilobase latency-associated transcript. J. Virol. 70 (10), 7270–7274.

Hill, J. M., Sederati, F., Javier, R. T., Wagner, E. K., Stevens, J. G., 1990. Herpes simplex virus latent phase transcription facilitates *in vivo* reactivation. Virology 174 (1), 117–125.

Ho, D.Y., Mocarski, E.S., 1989. Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse. Proc. Natl. Acad. Sci. U.S.A. 86 (19), 7596–7600.

Inman, M., Perng, G., Henderson, G., Ghiasi, H., Nesburn, A.B., Wechsler, S.L., Jones, C., 2001. Region of herpes simplex virus type 1 latency-associated transcript sufficient for wild-type spontaneous reactivation promotes cell survival in tissue culture. J. Virol. 75 (8), 3636–3646.

Jarman, R.G., Loutsch, J.M., Devi-Rao, G.B., Marquart, M.E., Banaszak, M.P., Zheng, X., Hill, J.M., Wagner, E.K., Bloom, D.C., 2002. The region of the HSV-1 latency-associated transcript required for epinephrine-induced reactivation in the rabbit does not include to 2.0-kb intron. Virology 292, 59–69.

Jarman, R.G., Wagner, E.K., Bloom, D.C., 1999. LAT expression during an acute HSV infection in the mouse. Virology 262, 384–397.

Javier, R.T., Dissette, V.B., Wagner, E.K., Stevens, J.G., 1988. A herpes simplex virus transcript abundant in latently infected neurons is dispensable for establishment of the latent state. Virology 166, 254–257.

Jin, L., Peng, W., Perng, G., Brick, D.J., Nesburn, A.B., Jones, C., Wechsler, S.L., 2003. Identification of herpes simplex virus type 1 latency-associated transcript sequences that both inhibit apoptosis and enhance the spontaneous reactivation phenotype. J. Virol. 77 (11), 6556–6561.

Karpinski, B.A., Morle, G.D., Huggenvik, J., Uhler, M.D., Leiden, J.M., 1992. Molecular cloning of human CREB-2: an ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response element. Proc. Natl. Acad. Sci. U.S.A. 89 (11), 4820–4824.

Kaufman, H.E., Varnell, E.D., Gebhardt, B.M., Thompson, H.W., Hill, J.M., 1996. Propanolol suppression of ocular HSV-1 recurrence and associated corneal lesions following spontaneous reactivation in the rabbit. Curr. Eye Res. 15 (6), 680–684.

Kenny, J.J., Krebs, F.C., Hartle, H.T., Gartner, A.E., Chatton, B., Leiden, J.M., Hoeffler, J.P., Weber, P.C., Wigdahl, B., 1994. Identification of a second ATF/CREB-like element in the Herpes Simplex Virus type 1 (HSV-1) latency-associated transcript (LAT) promoter. Virology 200, 220–235.

Kent, J.R., Zeng, P-Y., Atanasiu, D., Gardner, J., Fraser, N.W., Berger, S.L., 2004. During lytic infection herpes simplex virus type 1 is associated with histones bearing modifications that correlate with active transcription. J. Virol. 78 (18), 10178–10186.

Kouzarides, T., 2007. Chromatin modifications and their function. Cell 128 (4), 693–705.

Kramer, M.F., Coen, D.M., 1995. Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus. J. Virol. 69 (3), 1389–1399.

Kubat, N.J., Tran, R.K., McAnany, P., Bloom, D.C., 2004. Specific histone tail modification and not DNA methylation is a determinant of herpes simplex virus type 1 latent gene expression. J. Virol. 78 (3), 1139–1149.

Kubat, N.J., Amelio, A.L., Giordani, N.V., Bloom, D.C., 2004. The herpes simplex virus type 1 latency-associated transcript (LAT) enhancer/rcr is hyperacetylated during latency independently of LAT transcription. J. Virol. 78 (22), 12508–12518.

Kuo, M.H., Allis, C.D., 1999. *In vivo* cross-linking and immunoprecipitation for studying dynamic protein:DNA associations in a chromatin environment. Methods 19, 425–433.

Kwok, R.P., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G., Green, M.R., Goodman, R.H., 1994. Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature 370 (6486), 223–226.

Kwon, B.S., Gangarosa, L.P., Burch, K.D., deBack, J., Hill, J.M., 1981. Induction of ocular herpes simplex virus shedding by iontophoresis of epinephrine into rabbit cornea. Invest. Ophthalmol. Visual Sci. 21, 442–449.

Leib, D.A., Coen, D.M., Bogard, C.L., Hicks, K.A., Yager, D.R., Knipe, D., Tyler, K.L., Schaffer, P.A., 1989. Immediate early regulation gene mutants define different stages in the establishment, and reactivation of herpes simplex latency. J. Virol. 63 (2), 759–768.

Leib, D. A., Nadeau, K. C., Rundle, S. A., Schaffer, P. A., 1991. The promoter of the latency-associated transcripts of herpes simplex virus type 1 contains a functional cAMP-response element: role of the latency-associated transcripts and cAMP in reactivation of viral latency. Proc. Natl. Acad. Sci. U.S.A. 88 (1), 48–52.

Liang, G., Lin. J.C.Y., Wei, V., Yoo, C., Cheng, J.C., Nguyen, C.T., Weisenberger, D.J., Egger, G., Takai, D., Gonzales, F.A., Jones, P.A., 2004. Distinct localization of histone H3 acetylation and H3-K4 methylation to the transcription start sites in the human genome. Proc. Natl. Acad. Sci. U.S.A. 101 (19), 7357–7362.

Li, B., Carey, M., Workman, J.L., 2007. The role of chromatin during transcription. Cell 128 (4), 707–719.

Loiacono, C.M., Taus, N.S., Mitchell, W.J., 2003. The herpes simplex virus type 1 ICP0 promoter is activated by viral reactivation stimuli in trigeminal ganglia neurons of transgenic mice. J. Neurovirol. 9, 336–345.

Lokensgard, J.R., Berthomme, H., Feldman, L.T., 1997. The latency-associated promoter of herpes simplex virus type requires a region downstream of the transcription start site for long-term expression during latency. J. Virol. 71 (9), 6714–6719.

Maggioncalda, J., Mehta, A., Fraser, N.W., Block, T.M., 1994. Analysis of a herpes simplex virus type 1 LAT mutant with a deletion between the putative promoter and the 5' end of the 2.0-kilobase transcript. J. Virol. 68 (12), 7816–7824.

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

Marquart, M.E., Zheng, X., Tran, R.K., Thompson, H.W., Bloom D.C., Hill, J.M., 2001. A cAMP response element within the latency-associated transcript promoter of HSV-1 facilitates induced ocular reactivation in a mouse hyperthermia model. Virology 284, 62–69.

Mayr, B., Montminy, M., 2001. Transcriptional regulation by the phosphorylation dependent factor CREB. Nature Rev. 2 (8), 599–609.

Millhouse, S., Kenny, J. J., Quinn, P. G., Lee, V., Wigdahl, B., 1998. ATF/CREB elements in the herpes simplex virus type 1 latency-associated transcript promoter interact with members of the ATF/CREB and AP-1 transcription factor families. J. Biomed. Sci. 5 (6), 451–464.

Mioduszewska, B., Jaworski, J., Kaczmarek, L., 2003. Inducible cAMP early repressor (ICER) in the nervous system--a transcriptional regulator of neuronal plasticity and programmed cell death. J Neurochem. 87 (6), 1313–1320.

Mitchell, W.J., Lirette, R.P., Fraser, N.W., 1990. Mapping of low abundance latency-associated RNA in the trigeminal ganglia of mice latently infected with herpes simplex virus type 1. J. Gen. Virol. 71 (1), 125–132.

Molina, C.A., Foulkes, N.S., Lalli, E., Sassone-Corsi, P., 1993. Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. Cell 75 (5), 875–886.

Montminy, M., 1997. Transcriptional regulation by cyclic AMP. Annu. Rev. Biochem. 66, 807–822.

Montminy, M.R., Bilezikjian, L.M., 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328 (6126), 175–178.

Moriya, A., Yoshiki, A., Kita, M., Fushiki, S., Imanishi, J., 1994. Heat shock-induced reactivation of herpes simplex virus type 1 in latently infected mouse trigeminal ganglion cells in dissociated culture. Arch. Virol. 135, 419–425.

Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H., Nakatani, Y., 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87 (5), 953–959.

O'Neill, F.J., Goldberg, R.J., Rapp, F., 1972. Herpes simplex virus latency in cultured human cells following treatment with cytosine arabinoside. J. Gen. Virol. 14 (2), 189–197.

Perng, G., Dunkel, E.C., Geary, P.A., Slanina, S.M., Ghiasi, H., Kaiwar, R., Nesburn, A.B., Wechsler, S.L., 1994. The latency-associated transcript gene of herpes simplex virus type 1 (HSV-1) is required for efficient in vivo spontaneous reactivation of HSV-1 from latency. J. Virol. 68 (12), 8045–8055.

Perng, G., Ghiasi, H., Slanina, S.M., Nesburn, A.B., Wechsler, S.L., 1996. The spontaneous reactivation function of the herpes simplex virus type 1 LAT gene resides completely within the first 1.5 kilobases of the 8.3-kilobase primary transcript. J. Virol. 70 (2), 976–984.

Perng, G., Slanina, S.M., Ghiasi, H., Nesburn, A.B., Wechsler, S.L., 1996. A 371-nucleotide region between the herpes simplex virus type 1 (HSV-1) LAT promoter and the 2-kilobase LAT is not essential for efficient spontaneous reactivation of latent HSV-1. J. Virol. 70 (3), 2014–2018.

Perng, G., Jones, C., Ciacci-Zanella, J., Stone, M., Henderson, G., Yukht, A., Slanina, S.M., Hofman, F.M., Ghiasi, H., Nesburn, A.B., Wechsler, S.L., 2000. Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript. Science 287 (5457), 1500–1503.

Preston, C.M., Russell, J., 1994. Retention of nonlinear viral DNA during herpes simplex virus latency in vitro. Intervirology 32, 69–75.

Rader, K. A., Ackland-Berglund, C. E., Miller, J. K., Pepose, J. S., Leib, D. A., 1993. In vivo characterization of site-directed mutations in the promoter of the herpes simplex virus type 1 latency-associated transcripts. J. Gen. Virol. 74, 1859–1869.

Reinke, H., Hörz, W., 2003. Histones are first hyperacetylated and then lose contact with the activated PHO5 promoter. Molec. Cell 11 (6), 1599–1607.

Rivera-Gonzalez, R., Imbalzano, A.N., Gu, B., DeLuca, N.A., 1994. The role of ICP4 repressor activity in temporal expression of the IE-s and latency-associated transcript promoters during HSV-1 infection. Virology 202, 550–564.

Rock, D.L., Fraser, N.W., 1985. Latent herpes simplex virus type 1 DNA contains two copies of the virion joint region. J.Virol. 62 (3), 3820–3826.

Sacks, W.R., Schaffer, P.A., 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J.Virol. 61 (3), 829–839.

Samaniego, L.A., Wu, N., DeLuca, N.A., 1997. The herpes simplex virus immediate-early protein ICP0 affects transcription from the viral genome and infected-cell survival in the absence of ICP4 and ICP27. J. Virol. 71 (6), 4614–4625.

Sedarati, F., Izumi, K.M., Wagner, E.K., Stevens, J.G., 1989. Herpes simplex virus type 1 latency-associated transcription plays no role in establishment or maintenance of a latent infection in murine sensory neurons. J. Virol. 63 (10), 4455–4458.

Smith, J.S., Robinson, N.J., 2002. Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review. J. Infect. Dis. 186, S3–S28.

Spivack, J.G., Fraser, N.W., 1988. Expression of herpes simplex virus type 1 latency-associated transcripts in the trigeminal ganglia of mice during acute infection and reactivation of latent infection. J. Virol. 62 (5), 1479–1485.

Steiner, I., Spivack, J.G., Lirette, R.P., Brown, S.M., MacLean, A.R., Subak-Sharpe, J.H., Fraser, N.W., 1989. Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO 8 (2), 505–511.

Stevens, J.G., Wagner, E.K., Devi-Rao, G.B., Cook, M.L., Feldman, L.T., 1987. RNA complementary to a herpesvirus α gene mRNA is prominent in latently infected neurons. Science 235 (4792), 1056–1059.

Stow, N.D., Stow, E.C., 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate-early polypeptide Vmw110. J. Gen. Virol. 67, 2571–2585.

Tao, M., Lipmann, F., 1969. Isolation of adenyl cyclase from *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 63 (1), 86–92.

Thiel, G., Lietz, M., Hohl, M., 2004. How mammalian transcriptional repressors work. Eur. J. Biochem. 271 (14), 2855–2862.

Thomas, D. L., Lock, M., Zabolotny, J.M., Mohan, B.R., Fraser, N.W., 2002. The 2-kilobase intron of the herpes simplex virus type 1 latency-associated transcript has a half-life of approximately 24 hours in SY5Y and COS-1 cells. J. Virol. 76 (2), 532–540.

Thompson, R.L., Sawtell, N.M., 2001. Herpes simplex virus type 1 latency-associated transcript gene promotes neuronal survival. J. Virol. 75 (14), 6660–6675.

Vo, N., Goodman, R.H., 2001. CREB-binding protein and p300 in transcriptional regulation. J. Biol. Chem. 276 (17), 13505–13508.

Waeber, G., Habener, J.F., 1991. Nuclear translocation and DNA recognition signals colocalized within the bZIP domain of cyclic adenosine 3',5'-monophosphate response element-binding protein CREB. Molec. Endocrinol. 5 (10), 1431–1438.

Wagner, E.K., Bloom, D.C., 1997. Experimental investigation of herpes simplex virus latency. Clin. Microbiol. Rev. 10 (3), 419–443.

Wagner, E.K., Devi-Rao, G., Feldman, L.T., Dobson, A.T., Zhang, Y., Flanagan, W.M., Stevens, J.G., 1988. Physical characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol. 62 (4), 1194–1202.

Wagner, E. K., Guzowski, J.F., Singh, J., 1995. Transcription of the herpes simplex virus genome during productive and latent infection. Prog. Nuc. Acid Res. Mol. Biol. 51, 123–168.

Wang, Q-Y., Zhou, C., Johnson, K.E., Colgrove, R.C., Coen, D.M., Knipe, D.M., 2005. Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection. Proc. Natl. Acad. Sci. U.S.A. 102 (44), 16055–16059.

Wechsler, S.L., Nesburn, A.B., Watson, R., Slanina, S.M., Ghiasi, H., 1988. Fine mapping of the latency-related gene of herpes simplex virus type 1: alternative splicing produces distinct latency-related RNAs containing open reading frames. J. Virol. 62 (11), 4051–4058.

Xing, L., Quinn, P.G., 1994. Three distinct regions within the constitutive activation domain of cAMP regulatory element-binding protein (CREB) are required for transcription activation. J. Biol. Chem. 269 (46), 28732–28736.

Xue, Y., Wong, J., Moreno, G.T., Young, M.K., Côté, J., Wang, W., 1998. NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Molec. Cell 2 (6), 851–861.

Yamamoto, K.K., Gonzalez, G.A., Biggs, W.H. 3rd, Montminy, M.R., 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature 334 (6182), 494–498.

Zhang, X., Odom, D.T., Koo, S-H., Conkright, M.D., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E., Kadam, S., Ecker, J.R., Emerson, B., Hogenesch, J.B., Unterman, T., Young, R.A., Montminy, M., 2005. Genome-wide analysis of cAMP-responsive element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc. Natl. Acad. Sci. U.S.A. 102 (12), 4459–4464.

Zhu, J., Kang, W., Marquart, M.E., Hill, J.M., Zheng, X., Block, T.M., Fraser, N.W., 1999. Identification of a novel 0.7-kb polyadenylated transcript in the LAT promoter region of HSV-1 that is strain specific and may contribute to virulence. Virology 265, 296–307.

Zwaagstra, J.C., Ghiasi, H., Nesburn, A.B., Wechsler, S.L., 1991. Identification of a major regulatory sequence in the latency associated transcript (LAT) promoter of herpes simplex virus type 1 (HSV-1). Virology 182 (1), 287–297.

BIOGRAPHICAL SKETCH

Nicole Giordani majored in microbiology at the University of Arizona in Tucson, Arizona from August 1998 to December 2001. During this time, Nicole was part of the Undergraduate Biology Research Program (UBRP) and performed research on the alpha subunit of the RNA polymerase of Euglenoids under the supervision of Richard Hallick, Ph.D. After receiving her Bachelor of Science degree in December 2001, Nicole applied to graduate school and began the University of Florida's Interdisciplinary Program (IDP) in August 2002. Upon earning her Ph.D., Nicole performed post-doctoral work in an academic setting.