ROLE OF HERPES SIMPLEX VIRUS TYPE-1 LATENCY ASSOCIATED TRANSCRIPT (LAT) IN ESTABLISHMENT OF LATENCY AND REACTIVATION

By

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Our study examined the latency-associated transcript (LAT) of herpes simplex virus type-1 (HSV-1) and its contribution to both establishments of latency and reactivation. Experimental analysis includes the two predominant animal models for HSV-1 research: rabbits for studying acute replication kinetics and establishment of latency; and mice for examining LAT's involvement in reactivation.

Results from the rabbit experiments demonstrate that, within our limits of sensitivity, neither acute replication kinetics nor levels of latent genomes are detectably altered by either inoculating dose or LAT genotype. Analyses are extended to indicate the extent to which peripheral replication contributes to levels of latent viral genomes in the trigeminal ganglia.

Results from murine models demonstrate a dramatic decrease in LAT abundance during the first hours of explant co-cultivation of latently infected dorsal root ganglia (DRG). The fact that this decrease is reproducible in both latently infected mice and in
an uninfected LAT-transgenic mouse line indicates that the LAT locus is responsible for relaying cellular reactivation signals independent of exogenous viral factors. These results also offer tentative insight regarding the role of chromatin boundaries in regulating HSV-1’s lytic and latent stages. Finally, while results are unable to demonstrate a direct correlation with LAT kinetics, Immediate Early (IE) transcripts are reliably detected during the early hours post-explant.

Results reported herein address current research topics, revealing molecular events of both establishment of latency and reactivation, while also demonstrating the variability inherent to both model systems.
CHAPTER 1
INTRODUCTION

Experimental approaches to address the fundamental questions of Herpes Simplex Type-1 (HSV-1) should begin by recognizing that herpes viruses represent an evolutionarily ancient virus family, and have undergone co-speciation with their hosts for millennia¹. One should not expect the intimate relationship between an infected cell and its lifelong viral companion to be simple. Herpes viruses’ large and complex genomes clearly indicate an ability to obtain cellular machinery and mechanisms that contribute to their efficient and unique biology. As a virus whose host serves as its only reservoir, HSV-1 must take great care to minimize deleterious effects while maintaining a low immunologic profile. In my opinion, the secrets of HSV-1 will ultimately be revealed by applying advancements in cellular gene regulation to HSV-1 systems. This benefit will ultimately be repaid to cellular biologists when HSV-1 is realized as an ideal ‘mini-chromosome’ for studying an array of eukaryotic gene functions from a much simpler genome.

Herpes viruses infect members of virtually all groups of vertebrates. At least 80 distinct isolates have been identified from a variety of species². Because of the relatively benign effects of an infection in its native host, many isolates tend to be overlooked unless specifically sought; almost all vertebrate species that have been carefully examined are found to support at least one host-specific herpesvirus³,⁴. Humans are clearly no exception to this rule. Since transmission is most efficient in “herd populations,” the social nature of early humans (or their predecessors) provided an ideal
environment for colonization. Descriptions of human infections date to ancient Greece and can be found in the writings of Hippocrates and Shakespeare. The word herpes actually derives from the Greek “to creep,” referring to the ability of lesions to periodically reappear over the course of an individual’s life. Eight discrete human herpes viruses have been identified to date and are mentioned briefly in (Table 1.1).

Two basic features define all herpes viruses regardless of genome size and structure, cell tropism, or replication kinetics. The first feature is their patterns of productive cycle gene expression. As discussed in detail later, acute replication occurs in a well ordered cascade beginning with Immediate Early (IE/α), then Early (E/β), and finally Late (L/γ) transcripts. The second feature is the ability to establish and maintain a lifelong latent infection in their host, from which periodic reactivations may occur in response to a variety of stress-stimuli. Discussion hereafter focuses on the prototypical alphaherpesvirus, HSV-1.

HSV-1 is a nuclear replicating, double-stranded DNA virus, enclosed in both an icosadeltahedral capsid and an envelope. The 152-kilobase (kb) genome is promoter rich, generally using one promoter per protein. Studying a specific gene of interest is typically feasible because of the relative absence of overlapping reading frames. The large genome allows considerable flexibility in accepting transgenes without disrupting neighboring genes; this extensive “payload capacity” makes the construction of ectopic reporter constructs and promoter mutants relatively straightforward. Primary HSV-1 infections in immunocompetent individuals are generally unremarkable, often occurring without production of a detectable peripheral lesion. While periodic reactivations have the potential to produce a recurrent lesion at the initial site of infection, many (possibly
even most) reactivations result in no obvious clinical symptoms. The ability to detect virus from oral swabs by PCR, in the absence of clinical symptoms, underscores the potential for person-to-person spread. While lesions are uncomfortable, HSV-1 is of little medical concern to healthy individuals, even those prone to severe and common reactivations. Immunosuppressed individuals (elderly, neonates, transplant patients, HIV+ patients, etc.) are much more inclined to suffer from a disseminated infection, which may include extreme peripheral lesions and possibly fatal encephalitis.

**Acute Infection**

**Genome Structure and Organization**

HSV-1 is a large, double-stranded DNA virus that, like all herpes viruses, consists of four basic structural elements: 1) a core, containing the 152 kb genome wrapped in a torroid form, 2) a 100-110 nm icosadeltahedral capsid, composed of 162 capsomer components (each component consisting of at least 6 proteins), 3) an amorphous layer of tegument surrounding the capsid, containing 15-20 proteins, and 4) an envelope derived from the nuclear membrane, containing at least 11 different viral encoded glycoproteins (Figure 1.1). The HSV-1 genome has a high GC content (68%) and is organized into two unique sequences (unique long, UL, and unique short, US), each of which is flanked by inverted repeats (repeat long, RL, and repeat short, RS). The architecture of the genome permits four individual isoforms to exist based on the conformation of the RS segments relative to the UL region (Figure 1.2).

The HSV-1 genome encodes 75-80 proteins, most of which are controlled by their own specific promoter. While genes located within the repeat regions are present in two copies, no distinct location is required for a gene to be active. In general, as long as the entire promoter and ORF are included, individual genes can be moved great distances
without affecting their kinetics or the viruses' overall biology. HSV-1’s unique existence is directly linked to its ability to acquire homologues of critical cellular genes that permit its existence in the non-permissive environment of the nervous system.

**Primary Infection**

Since HSV-1 is a non-aerosolized, enveloped virus, it is extremely sensitive to desiccation and requires direct contact of moist mucosal surfaces for its spread. An HSV-1 infection typically initiates within the oral-facial area. The virus contacts mucosal surfaces and replicates within cornified epithelial cells; eventually gaining access to, and replicating within, cells of the basal epithelial membrane. The primary infection is generally unremarkable and resolves within 2-3 weeks after seroconversion. While the virus is ultimately cleared, and the individual makes a full recovery, its true mission has long since been achieved. During replication, the virus gains access to axons of sensory and motor neurons of the peripheral nervous system (PNS) innervating the infected region. Fusion of viral and cellular membranes directed by specific glycoprotein receptors delivers the capsid into the cell. By a process called retrograde fast axonal transport, the viral capsid ascends the axon at a rate of approximately 1 cm/hr. The virion arrives at the soma and delivers its nucleocapsid to the nuclear pores, releasing the viral DNA into the nucleus. Once in the nucleus the virus follows one of two fates: acute genes may be activated to initiate replication and produce progeny virus; or genomes circularize to yield a transcriptionally silent (latent) episome (Figure 1.3). Factors involved in this process, both cellular and viral, are only partially understood and continue to be the focus of research and controversy. For organizational purposes, our discussion continues with the population that “chose” the acute pathway, followed by a detailed discussion of events associated with latency.
Replication Cascade

Alphaherpesviruses have an extremely rapid replication cycle, compared to other herpesvirus family members. In some cell culture systems, infectious progeny can be produced in as little as 8 hours. Replication in vivo may not necessarily proceed this rapidly, it can take 18-20 hrs in neuronal cells\(^7\). Though the specifics of the HSV-1 replication cascade have been determined largely in cell culture, the use of neuronal derived cell lines makes it reasonable to assume that in vivo kinetics would not differ extensively. The replication of all herpes viruses follows a progressive cascade of increasing complexity divided into 3 (general) categories. The first viral genes to be expressed are the Immediate Early (IE/\(\alpha\)) genes. These IE genes mobilize transcriptional machinery and prime the cell for further viral gene expression. The second wave of viral genes expressed is the Early (E/\(\beta\)) genes. The E genes are specifically involved in viral DNA replication. Finally, Late (L/\(\gamma\)) gene expression provides structural proteins for virion assembly and egress. Each gene class is discussed in detail in the next sections.

Immediate Early Genes

HSV-1 encodes five IE genes, all of which are located in or around the repeat regions of the genome. The fact that these important genes lie very near (and in one case anti-sense to) the major locus responsible for reactivation from latency (the latency associated transcript, discussed later) emphasizes the likelihood of this region playing a significant regulatory role. The IE genes are classically identified as capable of being expressed in the absence of prior viral protein synthesis. Ribosomal inhibitors such as cycloheximide will therefore prevent expression of all but these five viral gene products. An interesting point is that while initiation of the acute cascade in infected neurons
requires IE genes for priming, the very first events are actually directed by L gene products carried into the nucleus from the tegument. Two tegument proteins, virion host-shutoff (VHS) protein and virulence protein-16 (VP16), play a crucial role in the early events of acute replication. The VHS is retained in the cytoplasm where it begins the process of shutting down host transcription to allow the virus access to cellular resources, primarily by disrupting polyribosome complexes and degrading viral and cellular RNAs. The VP16 enters the nucleus along with the genome and forms a trimeric complex with cellular proteins Oct-1 and host cell factor (HCF). This complex interacts with specific (TAATGARAT) elements present in the promoters of all five IE genes, activating their transcription and initiating acute replication. A more thorough explanation of VHS and VP16 is given in the discussion of L genes.

Because of their importance in initiating the replication cascade, IE genes tend to possess relatively extensive core promoters that contain more regulatory elements than their E and L counterparts. Of all HSV-1 acute phase promoters, those of IE genes are most similar to cellular RNA pol II promoters. The unifying factor common to all five promoters is the presence of multiple TAATGARAT motifs; the most conserved portion of an extended consensus IE enhancer upstream of the core promoters. This motif is related to the cellular promoter octamer motif (ATGCAACT), which is specific for POU family transcription factors.

The IE genes are regulated at both transcriptional and posttranscriptional levels, and perform distinct functions in the newly infected cell. Only 2 of the 5 IE genes are essential for viral replication in cultured cells, though mutation of nonessential genes may
significantly attenuate viral replication and virulence. A brief description of these genes and their proposed functions is given in (Table 1.2).

Since the IE genes are key regulators of both acute replication and reactivation from latency, and are the focus of much of the research discussed herein, significant detail is provided regarding their structure and function.

**Infected cell polypeptide-4 (ICP4)**

ICP4 is a multifunctional phosphoprotein essential for both viral replication\(^9\) and \(E\) gene transactivation. The inability of ICP4 (-) viruses to replicate renders them incapable of reactivating from latency. The 350 kilodalton (kDa), dimeric, DNA-binding protein\(^9,10\) is found primarily in the nucleus shortly after infection\(^11,12\) and is considered HSV-1’s major regulatory factor. Depending on the promoter, ICP4 can act as either a repressor or activator of viral gene expression. In general, ICP4 increases the transcription rate of all viral gene classes, but actually represses its own promoter\(^12-14\). Transcriptional auto-regulation is (most likely) controlled by an ICP4 binding site within its promoter that spans the transcriptional start site\(^15\), inhibits transcription factor complex formation or blocks access of upstream activators from Sp1 binding sites\(^16\). Transient assays have shown that the repressive effects of ICP4 on its promoter are dominant over VP16 activation\(^14\), and out compete Sp1 for binding during viral infections.

In addition to auto-regulation, the ICP4 promoter is positively regulated by both viral and cellular proteins\(^17\). Numerous cellular promoter-like elements exist, including 2 cis-acting TAATGARATs, at least 4 Sp1 sites, a CAAT box, and a TATA box\(^7,17\). The ICP4-directed activation of gene expression occurs by enhancing recruitment of TFIID (the TBP subunit responsible for TATA recognition) to the promoter, an early step in pre-initiation complex (PIC) formation\(^18\). Most E gene promoters as well as the LAT
promoter (described later) contain ICP4 binding sites. It is widely believed that ICP4 simultaneously turns IE genes off and turns E genes on to facilitate a smooth transition between kinetic classes.

**Infected cell polypeptide-0 (ICP0)**

The ICP0 is a multifunctional, 775 amino acid, nuclear-phosphoprotein that plays an important role in stimulating both lytic replication and reactivation. Regulatory elements within the ICP0 promoter indicate an ability to respond to both viral and cellular factors. In addition to multiple overlapping composite TAATGARAT motifs for activation by VP16/HCF/Oct-1 complexes, the promoter contains a cyclic-AMP response element (CRE) site. The CREs are common cellular promoter elements that serve as a major target for signal transduction pathways. Though ICP0 is described as dispensable for replication in cell culture, this is only true for high-dose infections, which somehow compensate for its absence. The ICP0 is essential for replication after a low multiplicity of infection (M.O.I), typically in the range of 0.01 to 0.001 plaque-forming units (pfu) per cell.

The ICP0 protein serves two distinct functions: to reorganize the transcriptional machinery of (quiescent) neuronal cells, such that viral gene expression is favored; and to act as a general enhancer of all viral gene classes. Like the tegument protein VHS, ICP0 plays a critical role in re-aligning the transcriptional status of newly infected neurons to favor lytic gene expression. Immunofluorescence analysis of recently infected neurons reveals a punctate localization of ICP0 protein within the nucleus. This distribution is due to ICP0’s affinity for distinct nuclear substructures, called ND10 bodies (also called promyelocytic oncogenic domains or PML bodies), via a specific localizing element. These substructures, associated with the nuclear matrix and important for cellular gene
regulation and transcription, are believed to be the site where IE gene transcription occurs. Proteosome-dependent degradation of ubiquitinated proteins plays a key role in many cellular processes; and the presence of ICP0 at ND10s has been shown to colocalize with enhanced levels of polyubiquitin. Ubiquitination requires an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2), and frequently a substrate-specific ubiquitin-protein ligase (E3). Being one of only five intron-containing viral messages, ICP0’s second and third exons encode distinct domains that can serve as an E3 ubiquitin ligase. Exon 2 contains a zinc-binding RING finger domain (common to ubiquitin ligases), while exon 3 contains a separate ubiquitin ligase domain; both of which cause increased levels of conjugated ubiquitin at ND10 substructures for their degradation. Disrupting ICP0’s RING finger destroys its ability to associate with conjugated ubiquitin and therefore proteosomes. Indeed, a human neuronal teratocarcinoma cell line (NT2) with inherently low levels of ND10s is not readily infected by HSV-1. Proteosome recruitment to ND10 substructures results in their disruption; and is believed to permit global increases in transcriptional activity by relieving transcriptional repression. The ICP0 has also been reported to deliver cyclin-D3 to ND10s for degradation, possibly resetting the cell cycle to favor viral gene expression18.

The second major influence attributed to ICP0 is the ability to function as a promiscuous transactivator of all gene classes, including cellular genes and genes of other viruses. The ICP0 acts at the transcriptional level; and unlike ICP4, does not require specific promoter elements to exert its influence. Though ICP0 actually binds DNA inefficiently, it is capable of activating any promoter that exhibits basal level activity
without requiring any particular cis-acting element. The ICP0 can, therefore, positively influence cellular-like promoters of IE genes (along with the more rudimentary E and L gene promoters, including TATA-less promoters\textsuperscript{19}). It has been proposed that ICP0's ability to induce IE promoters, considered with ICP4's ability to inhibit them, mediates the early "switch" by which individual neurons follow lytic or latent pathways\textsuperscript{14}. Since ICP0 does not transactivate E genes as readily as ICP4 does, it seems likely that ICP0 provides a boost to the productive cascade while ICP4 controls its temporal progression.

The nuclear reorganizing and transactivating functions of ICP0 are clearly critical for acute replication and reactivation. Even though it is possible to overcome the replication restriction of an ICP0 (-) mutant by infecting at a high M.O.I., the mutation has a much greater impact on the ability of the virus to reactivate. Since reactivation is (somewhat) analogous to a very low M.O.I. infection, it is not surprising that ICP0 mutants are deficient in this respect.

\textbf{Infected cell polypeptide-27 (ICP27)}

As shown in Table 1.2, the ICP27 gene is one of the two IE genes essential for viral replication. The ICP27 gene acts as both a positive and negative post-transcriptional regulator; and like ICP4, represses IE genes. One mechanism by which HSV-1 ensures that its mRNAs receive priority over cellular transcripts is via ICP27's ability to prevent intron-containing mRNAs from being exported from the nucleus. Of HSV-1's 75-80 genes, only five contain introns and three of these are IEs (Figure 1.4). While ICP27 positively regulates viral genes by promoting 3'end maturation and nucleocytoplasmic transport, it also seems to be involved in down-regulating IE genes when necessary. It is likely that the activity of ICP4 and ICP27 is crucial for sharpening the IE-to-E gene transition once the stage has been properly set for full-scale (E) gene expression.
Early Genes

Early gene synthesis begins once IE genes have sufficiently mobilized the cellular transcriptional machinery. The primary role of E genes is to provide viral replication machinery; and provide enzymes for increasing deoxynucleotide (dNTP) pools and repairing newly synthesized genomes. Once sufficient levels of these essential components are produced, E genes are shut off and L gene transcription begins. Since E gene synthesis terminates at the start of replication, their classic definition is sensitivity to translation inhibitors without being affected by inhibitors of replication, such as phosphonoacetic acid (PAA).

All E gene promoters have been shown to contain a set of core elements that include both a CAAT and TATA box\(^2\). As a rule, E gene promoters are simpler than IE gene promoters but more complex than L gene promoters. For example, while an IE promoter may contain many Sp1 sites, an E promoter might contain one or two Sp1’s, while L promoters have zero.

A number of E genes are dispensable for replication in cell culture, most likely because cellular homologues are capable of serving as substitutes. In fact, roughly half of HSV-1’s genes are not required for replication in dividing cells\(^7\). The absence of non-essential genes may still have a profound effect on pathogenesis, cell tropism, immune evasion, and replication in specific cells, especially (non-dividing) neurons. Included among the non-essential E genes are those encoding enzymes that provide DNA metabolism precursor genes such as thymidine kinase (TK), ribonucleotide reductase, uracil-DNA glycosylase, and dUTPase (Table 1.3).

Only seven E genes are absolutely necessary and sufficient for viral replication in cell culture (Table 1.4). Replication can ensue once levels of these seven proteins reach
sufficient levels. Replication proceeds via a rolling circle mechanism from three separate origins of replication (Ori), one in the UL and two in the US, to produce concatameric copies of the viral genome which must be resolved into individual linear molecules for packaging.

**Late Genes**

With the replication machinery in place and viral genomes being amplified, the third and final gene class begins to be expressed. Due to their dependence on the initiation of replication, L genes are classified by their sensitivity to replication inhibitors. While it is convenient to place all HSV-1 genes into one of three categories, the delineation between E and L genes is not as clear-cut as it is between IE and E genes. Of the nearly 30 L gene products, some are detectable at low levels prior to the onset DNA replication but do not reach maximum levels until post-replication. These genes are therefore referred to as “leaky-late” (βγ) genes, while L genes that do not exhibit such leakiness are termed “strict-late” (γ) genes. Since the previously diffuse pattern of viral proteins (i.e. ICP4 and ICP8) in the nucleus switches to a punctate pattern upon initiation of replication, it has been proposed that replication occurs within discrete nuclear compartments and that E and L gene transcription occur in distinct environments. This has been proposed to be involved in regulating the switch from E to L gene synthesis.

As mentioned previously, HSV-1 promoters tend to become progressively less complicated over the course of lytic replication. A leaky-late promoter, such as the one controlling the major structural component VP5, contains an Sp1 site, TATA box, and initiator element (INR); while a typical strict-late promoter may consist of only a TATA
box and an INR or a downstream activating sequence (DAS). L gene products predominantly encode structural components involved in capsid assembly and packaging of viral DNA. Capsids assemble in the nucleus and bud through the nuclear membrane, thereby, acquiring an envelope while incorporating tegument proteins (such as VHS and VP16) critical for re-initiating the acute cascade in a new cell. Progeny may be released as free viral particles but are most often transmitted by direct cell-to-cell contact.

**Virulence protein-16 (VP16)**

VP16 is a tegument phosphoprotein that forms complexes with the cellular transcription factor Oct-1 and HCF upon entering the nucleus to activate IE genes at TAATGARAT and GCGCGAA (Sp1) motifs. While the POU family member transcription factor Oct-1 provides the actual DNA binding, VP16 provides an acidic domain that enhances PIC formation. Unlike ICP4, which recruits TFIID, VP16 acts at a much later step by enhancing the binding of general transcription factors (GTFs) TFIIB and/or TFIIA. While many similarities exist between lytic and reactivation cascades, an important difference is that during reactivation VP16 is not present to enhance IE promoters. The manner in which IE genes are activated in the absence of VP16 represents a fundamental difference between lytic replication and reactivation that remains to be addressed.

**Virion host shut-off (VHS) protein**

As mentioned earlier, VHS remains in the cytoplasm during the early stages of the primary infection, disrupting polysomes and rapidly degrading pre-existing mRNAs. VHS also degrades HSV-1 mRNAs, most likely to hasten the transition between kinetic classes of genes. Interestingly, at late times during infection VHS is directly bound by
VP16 to prevent it from unnecessarily degrading viral mRNAs. In the absence of VP16 it has been demonstrated that VHS will ultimately terminate viral protein synthesis.

**Latency**

While lytic replication of HSV-1 requires an intricately organized cascade of highly specialized genes, the true evolutionary marvel of this virus is most evident when it does (almost) nothing at all. By far, the majority of HSV-1’s existence is as a latent episome; even during the exception to this rule, very few of the thousands of latent molecules are ever called to action. Since HSV-1 has only a small chance of infecting a new host during any short time interval, the ability to maintain latent genomes provides a reservoir for the eventual transmission to other individuals. Latency is often described as consisting of three separate phases: 1) establishment of the latent infection, 2) maintenance of latency, and 3) reactivation from latency.

While there are many differences between HSV-1’s lytic and latent cycles, latent genomes are typified by two distinguishing characteristics. First is circularization of the linear genome, which likely prevents degradation due to availability of free DNA ends. The absence of linear genomes in latent tissue was first reported by Southern and restriction enzyme analysis showing that terminal sequences could not be detected and terminal fragments were an uncharacteristic size, respectively. This was followed by the observation that junction specific probes detected terminal fragments but not junction fragments. The second characteristic unique to latency is a profound restriction of viral gene expression. The only exception to this state of complete quiescence is transcription from a distinct region of the R1. The transcript produced during latency is termed the latency-associated transcript (LAT). LAT does not belong to any of the lytic gene classes
and is the focus of considerable research. For organizational purposes, LAT and its effects on all aspects of the viruses' biology will be discussed later.

A central problem to studying latency is the lack of a reliable cell culture model. This requires all latent analysis to be performed using animal models, which re-create many (but not all) of the aspects of this state. Later sections discuss different animal models of latency, including their attributes and shortcomings.

**Establishment**

As mentioned earlier, upon entering the neuronal nucleus viral genomes follow one of two fates, either initiating acute replication or entering latency. The "decision" to enter latency is considered by some to result from a failure of the acute cascade. Others propose that the genome copy number (high vs. low copy) entering an individual neuron dictates the path followed. The complexity of neuronal cell types within ganglia makes it very possible that the cell directs the process, with specific populations of neurons permissive for lytic replication and others more prone to harbor latent genomes.

Establishment of the latent infection is a completely passive event; no gene product is involved, and even replication defective viruses cause latent infection when inoculated at peripheral sites. To date, no viral mutant(s) have been constructed which are unable to establish latency. Further indication that a very early decision determines which path is followed is evident in latent ganglia cross-sections from early times post-infection. Infected mice (eyes or footpads) with an HSV-1 recombinant containing an ectopic LAT promoter driving expression of the β-galactosidase gene (LAT-LacZ) followed by *in situ* hybridization for LacZ (latent population) or acute viral antigen (replicating population) shows that latent neurons appear as rapidly as productively infected neurons, and that the
populations do not overlap \(^{32}\). While latency may represent a failure of the productive cascade, it is unlikely that neurons that support replication contribute to the latent population; replication is actually believed to result in cell lysis and death. Since significant impairment to the host is rare, it seems unlikely that extensive loss of neurons occurs during this phase. By releasing progeny virus, neurons that die may ultimately enhance establishment by re-seeding the periphery (anterograde fast axonal transport) to increase exposure of axonal termini so that other neurons become infected. The inability of replication defective viruses to establish levels of latent genomes equivalent to wild type virus is probably due to less than maximal exposure of axonal termini at the periphery. The importance of extensive exposure to nerve termini for efficient establishment is underscored by the fact that HSV-1 does not spread directly from neuronal soma to adjacent neurons or support cells (glial cells), which are relatively resistant to HSV-1 infection \(^{33}\). Sensory ganglia lack inter-neuronal connections, thus, the ability to migrate to other ganglia is due to connections of sensory and motor neurons of the PNS to the spinal cord.

**Maintenance**

Since levels of replication defective and LAT (-) mutants are not diluted over time, maintenance of latent genomes for the life of an infected individual is (like establishment) considered a passive event. Rather than promoting large-scale transcriptional activity and cellular re-organization, genomes entering latency are best served by maintaining as low a profile as necessary. A major factor contributing to the stability of latent HSV-1 is the tightly wound and extensively chromatinized state of the episome \(^{34}\). Genomes are so well repressed that even though an average latent neuron
contains 10-100 viral copies⁡²⁹,³¹,³⁵ they are undetectable by in situ hybridization. To date, the only method for detecting latent HSV-1 from tissue sections is by in situ PCR.

A second example of the repressed state that occurs during latency is the rapid shut down of viral reporter constructs. A striking illustration of the tight repression of latent HSV-1 is that heterologous reporters such as the cytomegalovirus (CMV) IE or cellular phosphoglycerine kinase (PGK) inserted into the genome are quickly repressed as the virus enters latency. Since unmodified cellular transcription systems have been shown to be capable of activating all kinetic classes of HSV-1 promoters, the absence of transcription from all but the LAT promoter clearly indicates that the status of the latent genome as a transcriptional template is extensively modified. Current progress in our lab has demonstrated that specific histone modifications, rather than DNA-methylation, direct this repression.

**Reactivation**

HSV-1 faces one problem from the strategy that allows existence in a non-dividing, immuno-privileged site, for up to 80 years: how does such a highly repressed molecule covertly re-enter the lytic cycle, especially without the help of tegument proteins to potentiate the IE genes? Reactivation is the process by which latent genomes re-initiate productive cycle transcription in response to a variety of cellular signals. Numerous sporadic stress-mediated events in the host have been attributed to HSV-1 reactivation, such as, stress, exposure to UV light, menses, lactation, malnutrition, fatigue, anxiety, and immunosuppression. Infected individuals are capable of undergoing repeated bouts of reactivation throughout their lives, with each event representing only a minor fraction of the total latent population. Even in the most dramatic reactivation model, explant cocultivation of latent murine DRG, less than one percent of latent genomes reactivate.
Anterograde fast axonal transport along the original axon(s) involved in the primary infection deliver viral progeny to the initial site of infection, potentially causing a renewed lesion. One reason HSV-1 is so common within the general population (>90% of people are seropositive) is that most reactivations are sub-clinical; an individual may not be aware they are even infected, let alone undergoing recrudescence. In fact, only approximately 10% of infected individuals ever develop recurring oral-facial lesions. The severity and duration of lesions due to episodic reactivations tend to progressively diminish and exhibit shorter recovery times; most likely due to a primed immune response’s ability to rapidly bring the infection under control. Though a given reactivation event occurs from only a small percentage of the total latent population, their relatively frequent occurrence over an individual’s life without causing any obvious neurological defects indicates that neurons survive the process. Indeed, it seems illogical for the virus to both reduce its latent pool and draw undue attention by causing cell destruction.

**Role of Neurons in Latency**

HSV-1, like all alphaherpesviruses, is neurotropic with neurons serving as the nominal site of latency. HSV-1 has an overall affinity for the entire nervous system, through which it will readily migrate if presented the opportunity. Human autopsy analysis has shown HSV-1 to be readily detectable in a significant percentage of human brain samples. Interestingly, HSV-2 is also detectable, albeit less frequently, in human brains.

The nervous system is comprised of two basic components: 1) the central nervous system (CNS), consisting of the brain and spinal cord, and 2) the peripheral nervous system (PNS), consisting of neurons and their projections (axons and dendrites). The
role of the PNS is to link peripheral stimuli to the CNS. Two types of neurons comprise the PNS: afferent neurons, and efferent neurons. Afferent (sensory) neurons carry information towards the CNS, linking sensory receptors within the body to processing circuits in the CNS. Efferent (motor) neurons carry information from the CNS back to the periphery in response to sensory input. There are two distinct classes of (efferent) motor neurons: 1) somatic neurons connect the CNS and skeletal muscles, and therefore control voluntary movement, 2) autonomic neurons innervate smooth muscle, cardiac muscle, and glands, and therefore control involuntary processes. A ganglion (ganglia plural) is a local accumulation of neuronal cell bodies and support cells in the PNS. Nerves tracts extending from the ganglia to the periphery are simply bundles of axons from individual neurons gathered together. While motor neuron ganglia reside within the spinal cord, sensory neuron ganglia are found on both sides of the spinal cord in distinct out-pockets called lacunae. Sensory ganglia are located at distinct intervals along the entire length of the spinal cord and are named in accordance with the vertebral sections they innervate. These include the trigeminal ganglia, thoracic ganglia, lumbosacral ganglia, and dorsal root ganglia.

The primary sites of latency in humans are the sensory neurons of the trigeminal ganglia. While somatic ganglia can harbor latent virus, they simply are not as readily infected as the trigeminal ganglia. Trigeminal ganglia have a complex architecture divided into three separate regions, due in large part to their formation by the fusion of two nerve branches (ophthalmic and maxillomandibular) during embryonic development*. Since these neurons supply distinct oral-facial regions (ophthalmic,
maxillary, and mandibular) it should not be too surprising that primary infection of any oropharynx area can result in latency within the trigeminal ganglia.

A typical murine ganglion has been shown to consist of approximately 20,000 cells, of which 18,000 are support cells and 2,000 are neurons. Following inoculation, approximately one-third of these neurons will become latently infected, harboring an average of 10-100 viral genomes per cell. In situ PCR analysis has indicated that only 10-30% of neurons harboring latent virus produce detectable amounts of the LAT. Since LAT is often used as a marker for latent infection, many researchers describe latent neurons as falling into two classes: LAT (+) or LAT (-).

Due to the vast array of functions ascribed to a given ganglion, neuronal populations should not be expected to be homogeneous. Considering the many types (and sub-types) of neurons and the extensive co-speciation that has occurred between HSV-1 and its host, it seems reasonable for the virus to have acquired tropisms for specific neuronal subsets. Though the lack of a cell culture model for latency makes these issues exceedingly difficult to address, certain neurons have been shown to be more permissive for acute replication while others tend towards latency. In situ analysis of the ophthalmic division of murine trigeminal ganglia reveals that of four classes of neurons examined (Substance P⁺, CGRP⁺, LD2⁺ and SSEA-3⁺), the SSEA-3⁺ population represents the major latent group. Stage-specific embryonic antigen-3 (SSEA-3) is the marker for a major population of neurons and is present on 40% of mature trigeminal ganglia neurons in the ophthalmic division.

Octamer binding proteins

A major determinant of a neurons' response to the presence of viral genomes is dictated by its individual transcription factor profile. Neurons have been shown to
express different isoforms of POU family transcription factor octamer-binding proteins (Oct). This is the transcription factor pivotal in forming complexes with VP16 and HCF during lytic replication to activate IE genes. Octamer binding proteins, like Oct-1 and Oct-2.1, bind a variety of DNA elements (including TAATGARATs) to facilitate transcriptional activation. Neurons are capable of expressing a variety of Oct-isoforms that may render them more or less susceptible to acute replication and reactivation.

While Oct-1 and Oct-2.1 are the predominant isoforms and have been shown to increase transcriptional activity at TAATGARAT elements, they are not the major isoform of all neurons. Some neurons express Oct-2.4 and Oct-2.5 isoforms that lack the strong C-terminal activation domain (AD) of Oct-1 and Oct-2.1. Neurons expressing these Oct-variants have been shown to actually repress IE genes. Co-transfection studies have shown that Oct-2.4 and Oct-2.5 actually repress VP16 transactivation, while BHK cells expressing Oct-2.4 or Oct-2.5 are much less permissive for lytic replication than control BHK cells*. It is possible that these factors determine which fate the virus chooses following entry, and therefore which population serves as the reservoir for reactivation.

Neurons have also been shown to be capable of differentially regulating IE promoters in the absence of viral proteins. Construction of transgenic mice containing viral promoters (ICP0, ICP4, ICP27, or gC) controlling expression of the LacZ gene demonstrate that patterns of β-gal expression vary based on the class of promoter, neuronal phenotype, and even between the same neurons at different ages\(^{38,39}\). While the ICP4 promoter is inactive in many neuronal and non-neuronal tissues, it is very active in the trigeminal ganglia, actually 100-fold more so in newborns than adults. The ICP0 promoter is also negative in non-neuronal cells, but is active in adult rather than newborn
trigeminal ganglia; while the gC (L) promoter is negative in both neuronal and non-neuronal tissues. These results indicate the capacity of latent virus to respond to cellular signals, further emphasizing the importance of maintaining a highly repressive state to prevent untimely reactivations. It also raises the possibility that neuronal factors require only a brief release from HSV-1's repressive state to activate IE promoters; independent of viral accessory factors such as VP16.

**Latency Associated Transcript (LAT)**

The 1987 discovery of a promoter, not involved in lytic replication, capable of subverting global transcriptional repression\(^4\) led many to believe the secrets of latency and reactivation would soon be revealed. Sixteen years, and numerous phenotypic descriptions later, the exact function of LAT remains unresolved.

LAT was originally identified by in situ analysis of latent tissue showing positive signal only within neuronal nuclei, and only with \(R_L\) region probes. This result was in sharp contrast to acutely infected ganglia in which probes for any genomic region provide cytoplasmic and nuclear signals from neuronal and non-neuronal cells. The source of the latent hybridization signal was mapped to a nuclear-localized 8.5-kb primary transcript, containing a 5' cap, intron, poly-A tail, and several putative open reading frames (ORFs) which is partially anti-sense to \(R_L\) genes ICP0 and \(\gamma34.5\). Further examination reveals that the primary LAT does not encode a protein and has a very short half-life, in fact full-length poly-A\(^+\) message is nearly impossible to isolate from latent tissue. The unstable primary transcript undergoes autocatalytic splicing to produce a highly stable 2.0-kb intron. The introns' stability (half-life of approximately 24 hours) explains why in situ analysis yields stronger positive signals with probes to this region, compared to LAT
probes 5' and 3' of the intron. Since the 2.0 kb intron is readily detectable in latent tissue, it is often used as a marker of latent neurons, and is termed the “major” LAT. In certain neurons the major LAT undergoes a second splicing event to produce 1.45 to 1.5-kb introns termed the “minor” LAT. The validity of using the major LAT as a marker of latency is not without problems since, as mentioned previously, only 10-30% of latent neurons produce detectable levels of the 2.0-kb intron⁴¹⁻⁴³.

In vitro transcription of the primary LAT demonstrates that autocatalytic splicing results in a poly-A (-) molecule⁴⁴. S1 nuclease and RNase protection assays of latent tissue have precisely mapped the 5' and 3' ends of this RNA to canonical splice signals, while asymmetric PCR analysis demonstrates that the intron exists as an uncapped lariat⁴⁵. The presence of a non-consensus splice-acceptor site results in an atypically branched intron lariat which is not recognized by the cellular de-branching machinery, thereby accounting for its stability⁴⁶(Figure 1.5).

The size and complexity of the LAT promoter further emphasizes the likelihood that this region is involved in sensing and responding to cellular stimuli. LAT’s 202-bp core promoter is unique compared to even the most complex IE promoters. Numerous canonical RNA pol II like elements render this promoter more typical of cellular rather than HSV-1 promoters. The location of LAT core promoter elements are shown below (Figure 1.6), though it is important to note that factors 5' and 3' of this region have been proposed to also affect its activity⁴⁷⁻⁴⁹.

While the LAT promoter exhibits its greatest activity in neurons; transient and reporter assays, both in vivo and in cell culture, indicate basal activity in most cell types. Considering its basal activity and presence of an ICP4 binding site, the fact that the LAT
promoter does not behave like an IE promoter further emphasizes the likelihood of other factors being involved in promoter regulation during latency.

** Reactivation 

Three observations have led to the general consensus that LAT’s primary function is in reactivation from latency, rather than establishment or maintenance. First, LAT (-) mutants establish latent infections equivalent to, and as readily as, wild type virus. Second, the number of latent genomes in neurons infected with LAT (-) mutants does not progressively diminish over time; eliminating the likelihood that LAT perpetuates maintenance. Finally, multiple animal models demonstrate that LAT (-) mutants exhibit severely restricted reactivation phenotypes compared to their parental strains; making it very likely that LAT primarily functions during reactivation.

It should be made clear that this is a general statement intended to provide a unifying theme that most herpesvirus researchers agree with. A number of caveats exist regarding every aspect of LAT’s “known” role in reactivation. Conclusions vary based on viral strain, animal model, and even animal strain, not to mention variability between research groups. This section concludes with an alternative explanation (to which we do not subscribe) for LAT’s role in latency and reactivation.

Mice and rabbits comprise the two major systems for studying HSV-1 latency and reactivation; while a brief description is given below, they are explained in greater detail later. While murine (eye and footpad) models are the most cost efficient, they do not mimic human reactivation as accurately as rabbits (ocular) models, which exhibit both spontaneous and induced clinical reactivation. Animal models have allowed the portion of LAT responsible for reactivation to be mapped to an 800-bp region termed the reactivation critical region (RCR)\textsuperscript{50-54}. The RCR includes the 202bp LAT core promoter
and entire (603-bp) 5’ exon. The fact that reactivation maps upstream of the intron is fortuitous since it allows mutational analysis to exclude the anti-sense ICP0 transcript extending approximately half way through the intron. The RCR and the individual constructs involved in mapping the reactivation phenotype are shown below (Figure 1.7). Note that even the most severely restricted viral mutants exhibit basal levels of reactivation, indicating that LAT is not the only factor involved.

**Cyclic-AMP response element binding (CREB) proteins**

The complexity, unique transcriptional status, and involvement in reactivation render the LAT promoter a prime candidate for being the site through which the cell and virus communicate stress-stimuli. Of the numerous elements within the LAT promoter, the most appealing from a regulatory standpoint are its two CRE sites. Located within the core promoter defined by the 17ΔPst construct, eight-bp CRE motifs serve as the target of cyclic-AMP response element binding (CREB) proteins, a major member of the POU (CREM/CREB/ATF) family of transcription factors. CREB is a ubiquitous transactivator present in the nucleus in an inactive form. A variety of signal transduction cascades set in motion a chain of events (Table 1.5) leading to increased intracellular concentrations of the second messenger cyclic-AMP, and ultimately CREB activation. One such pathway initiates when adrenergic molecules (such as the hormone epinephrine) bind β-adrenergic receptors on the cell surface. CREB’s conformational change (described in Table 1.5) results from phosphorylation occurs at a “P box” within a basic-domain leucine zipper (bZIP) that allows CREB homodimers to efficiently bind CRE sites. CREB’s N-terminal region is glutamine rich and provides strong interaction with the basal transcriptional apparatus\(^{55,56}\), specifically CREB binding protein (CBP)
and p300. Activated CREB homodimers can also interact with AP-1 family (bZIP) transcription factors such as Jun and Fos.

The cyclic-AMP response element modulator (CREM) gene contains numerous introns, allowing alternate splicing to produce a variety of CREM/CREB/ATF isoforms. The ubiquitous nature of these factors allows specific and rapid response to external stimuli. It is, therefore, equally important that these transcription factors are able to be quickly down regulated or shut-off. Specific de-phosphorylation of active CREB homodimers is one way to halt its activity. A second interesting mechanism by which down regulation could occur is through the activation of a specific CREB/ATF/CREM inhibitor. This CREB regulator results from alternate splicing of the CREM gene to include an intronic promoter (P2) not present in other isoforms. Unlike other family members that are pre-made and awaiting activation, this protein is inducible and is therefore termed the inducible cyclic-AMP early repressor (ICER). The repressive capacity of ICER derives from the lack of an activation domain (AD) present in its ubiquitous/activator relatives. ICER forms inactive heterodimers with CREM family members to either prevent formation of active homodimers or physically obstruct promoter CRE sites. Since ICER and CREB both require Ras-dependent signaling pathways, the time required to express ICER may allow CREB a window during which to activate specific promoters before ICER is present at high enough levels to reverse the process. CRE sites within ICER’s promoter allow for auto-regulation, indicating that it may turn itself off once CREB has been inactivated.

As previously mentioned, the presence of CRE sites within the LAT promoter suggests that cyclic-AMP modulates LAT promoter activity, and thus, stress-induced
reactivation. Mutating the -43 CRE to a non-consensus binding site results in a partial reduction in reactivation from the rabbit eye model\textsuperscript{57}. Mutation of the other (-83) CRE both individually and in tandem with the -43 CRE was the focus of my research for a number of years. While initial results indicated very interesting reactivation and virulence phenotypes for these constructs, factors outside my control unfortunately put this project on hold. Other researchers have, none-the-less, provided evidence of cyclic-AMP modulation of the LAT promoter. Cell culture analysis shows the LAT promoter to be responsive to cyclic-AMP\textsuperscript{*}; and cyclic-AMP antagonists (such as propranolol) inhibit reactivation of latent murine ganglia following explant co-cultivation and heat-shock\textsuperscript{58}.

It is important to note the similarity between HSV-1’s ability to respond to a variety of environmental stress-stimuli, and the CREM family of transcription factors ability to serve as the final point of action for a variety of signal transduction pathways.

**Alternate Model for LAT**

Herpesvirus researchers do not universally accept the model that LAT acts primarily during reactivation. While we may not fully subscribe to these observations and proposed mechanisms, the work is not without merit and certainly deserves mention.

The model system employed for much of this work comes from the labs of Nancy Sawtell and Rick Thompson (University of Cincinnati). Briefly, murine ocular infections allow establishment of latency in the trigeminal ganglia. Reactivation is induced by hyperthermic stress (3-minute swim in a 43°C H\textsubscript{2}O bath, 24-hours prior to sacrifice), followed by single-cell PCR of dissociated neurons. This PCR process has been coined “contextual analysis of DNA” (CXA-D). LAT (-) mutants analyzed by this method exhibit a decreased ability to reactivate similar to previously described. This restriction, however, is said to result from measurably reduced (2-fold) numbers of latent genomes in
LAT (-) infected ganglia\textsuperscript{59-61}. Their results indicate that when infecting with wild type virus, a greater inoculating dose results in greater numbers of latent genomes, allowing more frequent reactivations. The LAT (-) viruses reduced frequency of reactivation is, therefore, simply the result of a smaller latent reservoir. Our own results indicate that a replication-competent virus reaches a level of saturation in terms of the number of latent genomes, regardless of infecting dose.

The model that LAT’s primary role is in establishment of latency is taken further by the observation that LAT (-) mutants exhibit increased neurovirulence; presumably due to the absence of an anti-apoptotic function that protects infected neurons. This effect is manifest in “rare” neurons that harbor thousands of viral genomes rather than the typical 10-100. Since this high M.O.I. neuronal population is proposed to represent the primary source for reactivation, the loss of these neurons removes the most relevant latent population\textsuperscript{62}. It has also been proposed that high M.O.I. neurons result from both lytic and latent phases occurring simultaneously, and LAT’s job is to repress the productive cycle\textsuperscript{63}. Separate groups have reported that LAT (-) mutants exhibit 70% more apoptotic neurons than their wild type counterparts\textsuperscript{64}, and over-expression of LAT in transformed cell culture inhibits IE gene expression\textsuperscript{65}.

We agree that dissociating establishment and reactivation is difficult, and efficient establishment is a pre-requisite for reactivation. Our analyses have, however, been unable to detect significant differences in levels of latent genomes based on either dose or status of the LAT region. Animal models exhibit inherent fluctuations that render 2-fold differences statistically insignificant. We also fail to observe increased neurovirulence
with LAT (-) mutants and find it difficult to believe that large-scale loss of neurons
would not produce a readily observable defect in our animals.

Animal Models

The broad cell and tissue tropism of HSV-1 permits its study in a variety of animal
models. As might be expected of a virus that has undergone co-speciation with its host
for millennia, finding a model system capable of replicating all aspects of HSV-1’s
biology is simply impractical. Potentially interesting viral strains are sometimes avoided
due to extreme neurovirulence and pathogenesis in non-native hosts; analogous to the
high mortality rate seen in humans infected with simian B virus, an innocuous
herpesvirus of primates. Strain-specific glycoprotein alterations can profoundly affect
viral dissemination and latency in specific models, leading to very different conclusions
about the viruses’ biology. Identical strains of virus, likewise, may replicate and spread
very differently depending on the animal model used, as well as the animal strain and
age. Despite their drawbacks, the lack of reliable cell culture models of latency
emphasizes the value of animal models in our current understanding of in vivo
herpesvirus biology. Our laboratory focuses exclusively on the two the most prevalent
animal models for studying latency and reactivation: mice and rabbits.

Murine Models

Due to their cost-effectiveness, relative ease of handling, and wide availability of
reagents, the mouse represents the most common species for herpesvirus research. The
very fact that HSV-1 establishes latency in neuronal ganglia was first demonstrated in the
murine system. The most practical means of infecting mice is on their rear footpads,
allowing the virus access to the major axons projecting to this area; those of the dorsal
root ganglia (DRG). Pre-treatment of the footpad with an injection of 10% saline four
hours prior to infection allows the epidermis to be efficiently removed with an emery board just prior to infection. While under sedation, this large, moist, surface allows the virus to enter and replicate within basal epithelial cells to provide efficient and uniform access to axonal termini. This in turn results in a reliable and homogeneous population of latently infected DRGs. Failure to saline pre-treat footpads results in a much less uniform, and 100-fold less efficient, infection. Footpad infections with virulent strains of virus will often present clear evidence of CNS involvement. Within five to seven days post-infection (d.p.i.) some mice appear lethargic, develop scruffy coats, exhibit hind limb paralysis, and may eventually die. Individuals that recover from the primary infection appear physiologically normal and are typically allowed at least 28 days for the acute infection to completely subside. DRGs can then be removed for analysis of latent genomes. One of the major benefits of HSV-1’s ability to traffic through the PNS and CNS is that it allows the effect of specific mutations to be examined for alterations in pathogenesis, neuroinvasiveness, and neurovirulence.

Reactivation from latent murine DRG is only possible by a process termed explant co-cultivation, in which ganglia are removed from the animal and maintained in media in a CO₂ incubator. Dissection from the animal and propagation in media is clearly a stressful event that may not fully reflect more subtle signals involved in human reactivations. Though correlation with human reactivation may not be perfect, transcription of acute phase genes is evident within four hours post-explantation, viral replication is detected within approximately 12 hours, and infectious virus may appear by three days (using wt or LAT (-) virus). Explant co-cultivation of LAT (-) mutants (strain 17syn') from both trigeminal and dorsal root ganglia will reactivate to produce viral
progeny with slightly (but measurable) delayed kinetics compared to their wild type parents. A less virulent HSV-1 strain (KOS-M), however, demonstrates no difference in kinetics between LAT (-) and wild type 66, whether this is due to strain or investigator differences is unclear. Unfortunately, only molecular reactivation can be examined in murine systems, neither clinical reactivation (detection of virus at periphery) nor spontaneous reactivation occurs.

An alternative to footpad infections performed in our laboratory, are ocular infections that allow the virus to establish latency in the trigeminal ganglia. While neurons of the trigeminal ganglia provide a more accurate representation of the latent site in humans, infections are inefficient and less uniform than the footpad model. Latent murine trigeminal ganglia also use the explant co-cultivation model for reactivation. Some researchers induce reactivation prior to dissection by hyperthermic stress 24 hours before sacrifice. Priming reactivation in this way supposedly allows more efficient and synchronous reactivation.

Rabbit Models

Animal models of HSV-1 reactivation should re-create three main criteria to be considered analogous to the human condition: 1) the ability to occur spontaneously, 2) the ability to respond to stress, and 3) allow complete recovery. While rabbits are sometimes avoided due to difficulty in handling and expense, they meet all of these requirements. Inoculation of scarified rabbit corneas with HSV-1 results in peripheral replication that allows the virus access to axons of trigeminal ganglia neurons. Latent rabbit trigeminal ganglia can be analyzed at the molecular level following dissection, as in the murine system, but provide the added benefit of exhibiting both spontaneous and induced clinical reactivation. After allowing 21 days for acute replication to subside, tear
film swabs can detect the presence of infectious virus due to spontaneous reactivations\textsuperscript{67}. Efficient reactivation can also be induced by direct iontophoresis of \textbeta-adrenergic reagents (such as epinephrine) into the cornea\textsuperscript{68-72}. This results in a high frequency of virus in the tear ducts within 16 to 96 hours post-induction. Iontophoresed rabbits exhibit no adverse effects from this procedure and recover completely. The ability to recover virus from the TG in this manner provides valuable insight into the course of human infections.

Due to the more subtle nature of reactivation stimuli between rabbit and murine systems (iontophoresis vs. explant co-cultivation) the rabbit eye model represents a more discrete reactivation event, similar to the small percentage of neurons responsible for human reactivation. Large scale DNA replication is, therefore, not evident at post-induction time points (48 hours) as with murine co-cultivations. LAT (-) mutants are much more severely restricted in their reactivation frequency and levels of productive cycle transcripts than in murine systems\textsuperscript{73}.

**Cell Culture Models**

Numerous references have been made throughout this text regarding the lack of viable (or widely accepted) cell culture models of latency and reactivation. As with many of the basic tenants of HSV-1's biology, this convention is not without caveats. Most attempts to establish latent infections in cell culture have been met with partial success; generally more closely mimicking a smoldering/persistent type of infection rather than latency. Genomes, for example, tend not to: form episomes, transcribe LAT, or remain "latent" without the assistance of replication inhibitors.
There is a cell culture system that does not suffer from these restraints. Christine Wilcox (University of Colorado) has developed a primary cell culture system using DRG from 15-day old Sprague-Dawley rats. Briefly, ganglia are dissociated and cultured in 5% newborn bovine serum and 2.5S nerve growth factor (NGF). The presence of fluorodeoxyuridine inhibits non-neuronal (dividing) cells, allowing for a relatively pure (1-3x10^5 cells) neuronal culture. This primary culture is maintained for two weeks before infecting with HSV-1. Twenty-four hours post-infection the cells are treated with acyclovir, followed by inoculation with an M.O.I. of 0.01 pfu/cell. The infection proceeds for seven days in the presence of acyclovir, after which time the acyclovir can be removed without the virus undergoing lytic replication. The virus not only remains “latent” at this point, but it genomes are circular and actively transcribe LAT.

Interestingly, all neurons harboring viral genomes in this system accumulate LAT; there is no LAT (-) population as seen in vivo. The homogeneous, LAT (+), latent virus population can then be induced to reactivate following a number of stimuli; including NGF-withdrawal or addition of the membrane permeable cyclic-AMP analog forskolin. An interesting observation of the post-induction reactivation process is a direct correlation between decreased LAT accumulations concomitant with elevated intracellular levels of the CREB antagonist ICER, mentioned previously and discussed in more detail in chapter two.
Figure 1.1. Schematic of HSV-1 virion.

Figure 1.2. Organization of linear HSV-1 genome.

Figure 1.3. Latent HSV-1 episome.
Figure 1.4. Genomic location of Immediate Early genes and LAT.

Figure 1.5. Splicing of primary LAT to produce a stable 2.0kb intron lariat. A) The 3'-OH group of a non-consensus branch point (guanine) attacks the 5' exon/intron border, B) The free 3'-OH of the 5' exon attacks the 3' exon/intron border to connect exons and C) The intron remains as a lariat (5'-2' linkage), non-consensus branch point inhibits debranching.
Figure 1.6. Schematic representation of core LAT promoter elements.

Figure 1.7. Viral mutants used to map reactivation critical region (RCR).
Table 1.1. The Eight Human Herpes Viruses

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Virus and Associated Disease</th>
<th>Replication and Host Cell Range</th>
<th>Site of Latency</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>HSV-1 (Herpes simplex type-1) Oral-facial infection HSV-2 (Herpes simplex type-2) Genital infection VZV (Varicella-zoster virus) Chicken pox/Shingles</td>
<td>Rapid replication, broad host/cell range</td>
<td>Neurotrpic</td>
</tr>
<tr>
<td>β</td>
<td>CMV (Cytomegalovirus) Infectious mononucleosis HHV-6 (Human herpesvirus-6) Mild childhood disease HHV-7 (Human herpesvirus-7) Mild childhood disease</td>
<td>Slow replication, restricted host/cell range</td>
<td>Glandular or lymphatic cells</td>
</tr>
<tr>
<td>γ</td>
<td>EBV (Epstein Barr Virus) Mononucleosis and cancer HHV-8 Kaposi Sarcoma Cancer</td>
<td>Slow replication, restricted host/cell range</td>
<td>T or B cells</td>
</tr>
</tbody>
</table>

Table 1.2. The five immediate early (IE) genes of HSV-1

<table>
<thead>
<tr>
<th>IE Gene</th>
<th>Replication</th>
<th>Location and Modifications</th>
<th>Proposed Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP4</td>
<td>Essential</td>
<td>Nucleus (phosphorylated)</td>
<td>Master switch for coordinated gene expression</td>
</tr>
<tr>
<td>ICP27</td>
<td>Essential</td>
<td>Nucleus (phosphorylated)</td>
<td>Inhibits export of spliced mRNA</td>
</tr>
<tr>
<td>ICP0</td>
<td>Dispensable</td>
<td>Nucleus (phosphorylated) (spliced)</td>
<td>Promiscuous transactivator Restructures cellular transcriptional machinery</td>
</tr>
<tr>
<td>ICP22</td>
<td>Dispensable</td>
<td>Nucleus (phosphorylated) (spliced)</td>
<td>Maintains broad host-cell range (possibly)</td>
</tr>
<tr>
<td>ICP47</td>
<td>Dispensable</td>
<td>Cytoplasm (spliced)</td>
<td>Interferes with antigen presentation by MHC-1</td>
</tr>
</tbody>
</table>
Table 1.3. Non-essential early genes

<table>
<thead>
<tr>
<th>Non-essential E Gene (examples)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine Kinase (UL23)</td>
<td>Deoxypyrimidine kinase far greater substrate range than its cellular counterpart. Phosphorylates a wide variety of nucleoside analogues (responsible for HSV-1s susceptibility to acyclovir).</td>
</tr>
<tr>
<td>Ribonucelotide Reductase (UL6)</td>
<td>Reduces ribonucleotides to deoxyribonucleotides to create dNTP substrates for replication.</td>
</tr>
<tr>
<td>Uracil-DNA glycosylase (UL2)</td>
<td>DNA repair and proof-reading. Corrects insertion of dUTP and deamination of cytosine residues (important due to HSV-1s high GC content).</td>
</tr>
<tr>
<td>dUTPase (UL50)</td>
<td>Hydrolyzes dUTP to dUMP. Prevents dUTP incorporation and provides dUMP pool for conversion to dTMP by thymidylate synthetase.</td>
</tr>
</tbody>
</table>

Table 1.4. Seven essential early genes

<table>
<thead>
<tr>
<th>Essential Early Gene</th>
<th>Number of Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Polymerase (UL30)</td>
<td>1</td>
</tr>
<tr>
<td>DNA Binding Proteins</td>
<td>2</td>
</tr>
<tr>
<td>Origin Binding Protein</td>
<td>1</td>
</tr>
<tr>
<td>Components of Helicase/Primase Complex</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 1.5. CREB signal transduction pathway

<table>
<thead>
<tr>
<th>Order</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Epinephrine (adrenaline), tyrosine derived “fight for flight” hormone. Made from adrenal glands of CNS and neurons of CNS and PNS.</td>
</tr>
<tr>
<td>2</td>
<td>Binds G-coupled receptors (β-adrenergic receptor) on plasma membrane. 7-transmembrane receptor, associated with GTP binding proteins on inner surface.</td>
</tr>
<tr>
<td>3</td>
<td>RAS (GTP binding protein), composed of α, β and γ subunits. A-subunit binds GDP when inactive. Receptor activation causes α-subunit to bind GTP, α can no longer bind β and γ subunits. Free β and γ subunits are effector molecules.</td>
</tr>
<tr>
<td>4</td>
<td>Effector molecules activate the enzyme adenyly cyclase. Catalyzes the formation of 3’5’-cyclic AMP from ATP.</td>
</tr>
<tr>
<td>5</td>
<td>Second messenger (cyclic-AMP) diffuses through the cytosol to activate protein kinase A (PKA).</td>
</tr>
<tr>
<td>6</td>
<td>PKA (2 inhibitory and 2 catalytic subunits) releases catalytic subunits (kinase A) upon activation. They migrate to nucleus topophosphorylate specific targets.</td>
</tr>
<tr>
<td>7</td>
<td>CREB contains a single kinase A recognition site (Serine 133), phosphorylation causes a conformational change that efficient binding of CRE sites.</td>
</tr>
</tbody>
</table>
CHAPTER 2
NEITHER INOCULUM NOR LAT GENOTYPE DETECTABLY ALTER HSV-1
ESTABLISHMENT OF LATENCY

Introduction

HSV-1 establishes latency in neurons of sensory ganglia innervating the site of initial infection. The virus can reactivate spontaneously, or under conditions of stress, to cause a recurrent infection. During latency the genome forms an episome in neuronal nuclei from which no viral replication occurs. Approximately 1/3 of the latent infected neurons express high levels of a single transcript, termed the latency-associated transcript (LAT). This transcript is important for reactivation, despite the fact that LAT does not encode a protein.

While LAT is clearly required for efficient reactivation in animal models, its mechanism is not well understood. One factor that complicates these analyses is that observations vary depending on the animal model and viral strain used. In the rabbit eye model, for example, latency is established in trigeminal ganglia by corneal inoculation, with reactivation (either spontaneous or induced by iontophoresis of epinephrine) scored by recovery of infectious virus in the tear film. In the mouse model, latency is established in the trigeminal or dorsal root ganglia by inoculation corneas or rear footpads, respectively. Viral reactivation from ganglia can be induced by thermal stress (as demonstrated by expression of reporter genes from neuronal tissue), or by explant co-cultivation of dissected ganglia on cultured cells.
Large LAT deletion mutants exhibit reduced numbers of latent viral genomes in neurons of both mice and rabbits. Smaller LAT deletion mutants (such as the promoter mutant 17ΔPst or 17Δ348, a 348 bp 5' exon deletion) do not, however, demonstrate significant differences in total numbers of latent HSV-1 genomes.

It is possible that our previous studies, which use relatively large inoculating doses (1x10^5 pfu), may mask subtle replication or establishment deficits inherent to these LAT mutants. The course of the acute infection in the rabbit eye model was, therefore, examined using a 1000-fold range of 17ΔPst (and its rescue) inoculation doses. Differences in acute infection kinetics and establishment of latency were not detected by this method. The observation that peak establishment occurs with even low dose inoculums suggests that saturation of latent sites occurs relatively early. To determine the contribution of the initial inoculum to establishment, rabbits were infected with a non-replicating HSV-1 recombinant. While this recombinant is capable of establishing latency, the total number of latent genomes is much lower than wild type virus, indicating that peripheral replication contributes to maximal establishment of latency.

**Materials and Methods**

**Cells and Viruses**

Virus was propagated on cultured rabbit skin (RS) cells. Virus titers were determined on Vero cells grown in minimal essential medium supplemented with 5% fetal calf serum and antibiotics. The following HSV type-1 (HSV-1) genotypes previously described were used in these experiments: Wild-type strain 17syn⁺, 17ΔPst (a 203-bp portion of the LAT promoter deleted), 17Δ348, (a LAT mutant with bases 119,007-119355 deleted) and its rescuant (17Δ348R), RHA-6 (a construct expressing the
5' portion of LAT by virtue of nucleotides 120,290-120,467 removed and replaced with a 442-bp fragment of the SV40 encoding the cleavage/polyadenylation signal site).

**Infections and Reactivation**

Unscarified rabbit eyes are inoculated with the indicated number of pfu in 25 µl aliquots. Animals are sacrificed between 1 and 7 days post-infection for acute studies, and their corneas and ganglia harvested. Latently infected trigeminal ganglia are recovered from rabbits at least 40 days post-infection.

**DNA Extraction**

Dissected corneas or ganglia are incubated with 0.6 ml of extraction buffer (25 mM EDTA, 100 mM NaCl, 1% SDS, 10mM Tris pH 7.5) and 50 µl of proteinase K solution (15 mg/ml) overnight at 48 °C. DNA is extracted three times with phenol-chloroform (1:1) and once with chloroform. DNA is precipitated with ethanol overnight and pelleted by centrifugation. The pellet was washed once with 70% ethanol, air-dried and dissolved in 200 µl of water.

**Analysis of the Relative Amounts of Viral DNA by PCR**

Semi-quantitative PCR analysis incorporating $^{32}$P α-dCTP is able to detect 1 pg of purified HSV-1 DNA compared to a control plasmid containing a sub-cloned fragment of the VP5 gene. When purified viral DNA is mixed with uninfected ganglia it is possible to detect less than 1000 viral genomes. This PCR method is also able to detect the viral DNA and cDNA- from a single infected cell. Actin primer sets are used to amplify DNA corresponding to cellular genomes to normalize product intensities. The ratio of both signals is determined by densitometry.
Amplification by PCR was carried out as previously described, using the following primer sets (sense/anti-sense strand): VP5 (149-bp product), 5'-TGAACCCCAGCCCGAGAAACC-3'/5'-CGAGTAAACCATAATTAAAGGACC-3'; rabbit actin (110-bp product) 5'-AAGATCTGGGACCACACCTT-3'/5'-CGAACATGATCTGAGTCATC-3'. The reactions were carried out in M.J. Research thermal cycler as follows: denaturation, 94 °C for 30 sec; annealing, 55 °C for 30 sec; and extension, 60 s at 72 °C. The final cycle was terminated with a 10-min extension step. The products were made radioactive for autoradiography and image quantitation by addition of 0.2 µC of α-32P-dCTP. For each reaction we used 20 µl (10%) of the DNA sample, and the final volume of the reaction was 100 µl. One fifth of the amplified product (corresponding to 2% of the original material) was fractionated on 6% polyacrylamide gels in Tris-borate-EDTA. The PCR signals were visualized by scanning an appropriately exposed autoradiogram by use of a Deskcan II scanner (Hewlett-Packard). The signals were quantified by densitometry using IP Lab Gel software (Signal Analysis Corporation) in accordance with operational instructions.

**PCR Analysis to Determine Relative Levels of Latent Viral DNA and Wild Type Revertants**

PCR primers specific for the cellular actin gene serve as an internal standard for normalizing levels of latent viral DNA between samples. Actin primers (sense 5' AAGATCCTGGCACCCAACCC-3', and anti-sense 5' CGAACATAACCACCTT-3') yield a 110-bp product. PCR primers specific for the HSV-1 polymerase gene are used to detect both wild type and KD6 viral DNA. Polymerase primers (sense: 5' CATCACCGAGGGGAGGCTGCTGTTC3', and anti-sense 5' GGGCCAAGGCCTGTTAGTGGAGTAT3') yield a 92-bp product. PCR primers specific for the HSV-1 ICP4 gene are
used to confirm that levels of establishment are not due to wild type revertants when analyzing the KD6 viral recombinant. ICP4 primers (sense: 5’ CTG ATC ACG CGG CTG CTG TAC ACC 3’, and anti-sense: 5’ GGT GAT GAA GGA GCT GCT GTT GCG 3’) yield a 144-bp product. PCR reactions are performed in a 50µl final volume, consisting of 40.5µl sterile H2O, 1µl of both forward and reverse primers (600 ng/µl), 1µl dNTPs (1.25 mM each), 5µl 10X AS buffer (Qiagen; Tris-Cl, KCl, (NH4)2SO4, 15mM MgCl2, pH 8.7), 1µl respective DNA/cDNA sample, and 0.5µl HotStar Taq DNA Polymerase (Qiagen; 5 U/µl). The amplification profile consists of 15 minutes at 95°C to activate the Taq, followed by one three-minute cycle of 94°C, 55°C, and 72°C; this is followed by 30 identical cycles of one minute each (Ericomp Twinblock™System, Easy Cycler). PCR products are resolved on 5% polyacrylamide gels, stained with SYBR Green (Molecular Probes), and scanned with a Storm Phosphorimager (Molecular Dynamics) using a 450 nm wavelength laser. Relative levels of latent genomes are determined by establishing the ratio of HSV-1 polymerase product to cellular actin levels within each sample. This accounts for fluctuations in signal intensity due to inconsistent sample processing. Viral polymerase specific PCR products are compared to a plasmid titration containing the sub-cloned target sequence spiked into processed uninfected rabbit trigeminal ganglia tissue. The signal intensity of each sample is compared to this titration to determine the relative number of latent HSV-1 molecules in each sample.

Results

Acute Replication in Rabbit Corneas and Trigeminal Ganglia Following High Titer Infection

The contribution of both LAT expression and inoculation dose is analyzed over the course of acute ocular infection of rabbits with either 500 or 500,000 pfu / eye of 17ΔPst
or 17ΔPstR (rescue). Infectious virus yields during the acute infection were measured in tear swabs, corneas, and trigeminal ganglia (Figure 2.1-2.3). At high viral doses, recovery is greatest from tears and corneas on the first day post-infection (d.p.i.). These levels tend to reach a lower stage plateau by days 2 through 8, after which continual decreases result in undetectable levels of virus by day 14. Viral titers from trigeminal ganglia increase during the first 3 days of infection, followed by 3 days (d.p.i. 4-6) of maximal virus titers, and finally a steady decrease. While dose clearly affects infection kinetics, no significant effect is observed relative to LAT status. PCR analysis to determine the relative amount of viral DNA present in corneas and trigeminal ganglia following high dose (5x10^5 pfu) infection also fail to detect significant differences based on LAT genotype at all time points (Table 2.1).

**Analysis of Viral DNA Levels in Corneas and Trigeminal Ganglia During the Acute Infection**

The course of infection is then examined following a much lower dose infection (500 plaque forming units per eye). As with high titer infections, relative amounts of HSV-1 DNA in corneas are greater than trigeminal ganglia during the entire acute infection course. A variety of LAT mutants, in addition to the prototypical LAT (-) virus (17ΔPst), that differ in LAT expression and reactivation phenotypes are included in this analysis. Mutant 17Δ348 expresses LAT, but exhibits a significant reactivation impairment following epinephrine induction. Mutant RHA-6, which contains an SV40 cleavage/polyadenylation sequence in the middle of the 2.0 kb LAT intron, expresses LAT and reactivates normally.

Rabbits inoculated with 500 pfu of reactivation-impaired viral recombinants (17Δ348 and 17ΔPst) demonstrate significantly decreased levels of virus in trigeminal
ganglia during the acute phase of infection, as compared to wild type and RHA-6 viruses (Table 2.2). At day five post-infection, the average value for the low reactivation mutants (0.35 ±0.19) is a marginally significant difference (p-0.068, t=test) compared to normal reactivators (0.56 ±0.38). Average values at day 7 (0.29± 0.18 for low reactivating viruses, and 0.63± 0.31 for normal reactivating viruses) are again significantly different (p=0.006), but by the time latency is established (21 days) all corneal infections are statistically indistinguishable using any construct.

**The Relative Amount of Latent Viral DNA in Trigeminal Ganglia of Rabbits Infected With Wild Type or LAT Mutants are Similar Regardless of Infecting Dose**

Levels of viral DNA in ganglia following clearance of the acute infection suggests that viral DNA levels in the ganglia are independent of LAT genotype and infecting dose. This is confirmed using semi-quantitative PCR to compare relative amounts of latent viral DNA over a range of infecting doses (Figure 2.4 and Table 2.3). Rabbits corneas inoculated with 500 to 50,000 pfu / eye are sacrificed 30 days post infection to determine levels of latent HSV-1 (Table 2.4). No statistical difference in amount of viral genomes is detected as a function of either LAT genotype or initial virus dose. As with high titer infections, neither dose nor LAT genotype effect DNA levels in latently infected trigeminal ganglia.

**A Non-replicating HSV-1 Recombinant Establishes a Latent Infection in the Trigeminal Ganglia, but at Lower Levels Than Wild-Type Virus**

To assess the contribution of input inoculum on establishment of latency, a non-replicating (ICP4 (-)) HSV-1 recombinant (KD6) is used. The amount of HSV-1 DNA in trigeminal ganglia is determined by PCR from rabbits inoculated with 1x10^5 or 1x10^6 pfu of this virus at 14 d.p.i. (Figure 2.5). While trigeminal ganglia of rabbits inoculated with
KD6 contain detectable HSV genomes, overall levels are lower than those observed using replication-competent LAT (-) or LAT (+) viruses. PCR analysis of these ganglia (using primers specific for the ICP4 gene) indicates that the DNA present is not due ICP4 revertants. These results demonstrate that while non-replicating HSV-1 recombinants can establish a latent infection, replication is required to achieve wild type levels of establishment.

Discussion

It has been previously suggested that LAT plays a role in protecting neurons from death or apoptosis during the initial stages of establishment. These observations have been made with deletion mutants that extend from the entire LAT promoter into the 2.0 kb intron, and often display altered virulence. While we have never observed such effects with the 202 bp LAT promoter mutant (17ΔPst), the statistical power required for discerning 4-fold (or less) establishment or virulence defects are difficult to achieve in the rabbit model. The goal of this study was to determine if subtle deficits in replication or establishment are detectable using 10 to 1000-fold lower than normal inoculi of 17ΔPst in the rabbit eye model. Significant differences in the amount of infectious virus produced during the acute infection in cornea and ganglia, or on the level of latent genomes in trigeminal ganglia of the rabbit, are not detected. These results suggest that the primary defect of 17ΔPst in the rabbit eye model occurs at the level of reactivation. While our levels of sensitivity cannot rule out the possibility that 17ΔPst affects quality of establishment, we were unable to detect any differences during the acute infection even when allowing additional replication cycles to occur.
This study provides the additional opportunity to monitor the course of an ocular infection as a function of dose. Not surprisingly, peak acute titers in the tears, cornea and trigeminal ganglia are delayed by several days when lower inoculi are used. Interestingly, peak levels of viral DNA in the trigeminal ganglia are reached slightly earlier, suggesting that maximum establishment occurs fairly early, and at relatively low inoculation doses. This in turn suggests that corneas provide a limited number of entry sites into the nervous system (or number of available neurons), which become saturated relatively quickly. To address this question more directly a non-replicating virus (KD6) is used. Since this virus cannot undergo additional rounds of replication in the cornea, it allows assessment of the amount of viral DNA delivered to the trigeminal ganglia as a direct function of input. Results indicate that while significant establishment of latency is achieved, even doses of $1 \times 10^6$ pfu yield approximately 10-fold lower than wild type levels of establishment. This indicates that while a non-replicating virus can establish latency in rabbit eyes, replication is required to establish maximal latent infections. This requirement is likely due to mechanical barriers that must be overcome to efficiently gain access to the nerve termini projecting to the trigeminal ganglia. While infecting the corneal surface (even with scarification) provides access to many nerve termini, replication and cell-to-cell spread are much more important factors.

A final conclusion of this study is that lower (and probably more physiologically relevant) doses of viruses are sufficient to efficiently establish latency in the rabbit trigeminal ganglia. It is interesting to note that increasing inoculum does not decrease the scatter in total levels of establishment over a range of doses. This adds additional support
to the notion that efficient establishment of latency requires a significant cellular component, which may be regulated at the individual ganglia level.
Figure 2.1. Viral titers recovered from eye swabs.

Figure 2.2. Viral titers recovered from corneas.
Figure 2.3. Viral titers recovered from trigeminal ganglia.

Figure 2.4. Levels of establishment using high and low does 17ΔPst (and Rescue).
Figure 2.5. Reduced establishment using KD6 versus 17syn+. 

Left and Right Trigeminal Ganglia (Polymerase DNA) 
KD6 (1x10^3 pfu) KD6 (1x10^6 pfu) 
1L 1R 2L 2R 3L 3R 1L 1R 2L 2R 3L 3R 

Left and Right Trigeminal Ganglia (ICP4 DNA) 
KD6 (1x10^6 pfu) 
1L 1R 2L 2R 3L 3R 3R + 

17 (1x10^3 pfu) 
1L 1R 2L 2R 3L 3R 4L 4R 5L 5R 

17^+ (1x10^3 pfu) 
1L 1R 2L 2R 3L 3R 4L 4R 5L 5R
<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Primer pair</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 VP5</td>
<td>5’-TGAACCCCAAGCCCCAGAAACC-3’</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>5’-CGAGTAAACCATGTTAAGGACC-3’</td>
<td></td>
</tr>
<tr>
<td>HSV-1 ICP4</td>
<td>5’-CTGATACGGGCTGCTGTACACC-3’</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>5’-GGTGATAGAAGGAGCTGCTTGGTGC-3’</td>
<td></td>
</tr>
<tr>
<td>HSV-1 DNA Pol</td>
<td>5’-CATCACCACCCGGAGAGC-3’</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>5’-GGGCCAGCCGGCTTGGTTGGTGTA-3’</td>
<td></td>
</tr>
<tr>
<td>Rabbit actin</td>
<td>5’-AAGATCTGGCAACGACCACCTT-3’</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>5’-CGAACATGATCTGGTCATC-3’</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. Relative amount of viral DNA (VP5/actin) at high dose of inoculation (500,000 pfu)*

<table>
<thead>
<tr>
<th>DPI</th>
<th>Cornea</th>
<th>Ganglia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>17ΔPst</td>
<td>17ΔPstR</td>
</tr>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>1</td>
<td>1.51±0.54</td>
<td>1.19±0.99</td>
</tr>
<tr>
<td>2</td>
<td>2.29±0.76</td>
<td>1.40±0.94</td>
</tr>
<tr>
<td>3</td>
<td>2.11±0.32</td>
<td>2.38±0.59</td>
</tr>
<tr>
<td>5</td>
<td>2.31±0.64</td>
<td>1.59±0.18</td>
</tr>
<tr>
<td>7</td>
<td>2.16±1.30</td>
<td>2.01±0.27</td>
</tr>
<tr>
<td>14</td>
<td>0.44±0.14</td>
<td>0.36±0.34</td>
</tr>
</tbody>
</table>

*Rabbits’ eyes were inoculated with 500,000 PFU of 17ΔPst or 17ΔPstR (rescue). At the indicated times post infection (DPI), the rabbits (2 rabbits per virus per time point) were sacrificed and corneas (4 per virus per time point) and TG (4 per virus per time point) dissected. Total DNA was isolated from the tissue and amplified with VP5 and actin primer sets in combination. The relative amounts of viral DNA (VP5/Actin) were determined by densitometry.
Table 2.3. Relative amounts of viral DNA in cornea and trigeminal ganglia during acute infections post low dose inoculation with viruses of different LAT genotypes*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Days p.i.</th>
<th>Cornea Mean±SEM§</th>
<th>Ganglia Mean±SEM§</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 syn+</td>
<td>1</td>
<td>0.21±0.12</td>
<td>0.03±0.01</td>
</tr>
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<td>2</td>
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<td></td>
<td>21</td>
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</tr>
<tr>
<td>17ΔPst</td>
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<tr>
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<tr>
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<td>0.04±0.04</td>
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<td>0.23±0.22</td>
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<tr>
<td></td>
<td>21</td>
<td>0.15±0.11</td>
<td>0.33±0.15</td>
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</table>

*Rabbit eyes were inoculated with 500 PFU of 17syn+, 17ΔPst, 17Δ348, 17Δ348R and RHA-6. At the indicated days post infection, cornea and TG (4 each per virus per time point) were dissected and the relative amounts of viral DNA determined.

§Relative amounts of viral DNA presented as the ratio of the HSV VP5 gene to cellular actin ratio as determined by PCR (see Materials and Methods). Means and standard error of the mean (SEM) are presented as Least Squares Means values and were calculated as described in the Materials and Methods.
Table 2.4. Relative amounts of viral DNA present in TG during latency in rabbits infected with different doses of virus*

<table>
<thead>
<tr>
<th>Virus/dose</th>
<th>Rabbit Tattoo # (left or right TG)</th>
<th>HSV-1 DNA (genome equivalents)§</th>
<th>Mean (±SEM)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>17ΔPst</td>
<td>A3(L)</td>
<td>30,000</td>
<td>18,300 ± 7888</td>
</tr>
<tr>
<td>500 PFU</td>
<td>A3(R)</td>
<td>2,000</td>
<td></td>
</tr>
<tr>
<td>500 PFU</td>
<td>A5(L)</td>
<td>40,000</td>
<td></td>
</tr>
<tr>
<td>50,000 PFU</td>
<td>A5(R)</td>
<td>1,200</td>
<td></td>
</tr>
<tr>
<td>17ΔPstR</td>
<td>A9(L)</td>
<td>800</td>
<td>12,200 ± 7888</td>
</tr>
<tr>
<td>(rescue)</td>
<td>A9(R)</td>
<td>8,000</td>
<td></td>
</tr>
<tr>
<td>500 PFU</td>
<td>A10(L)</td>
<td>30,000</td>
<td></td>
</tr>
<tr>
<td>50,000 PFU</td>
<td>A10(R)</td>
<td>10,000</td>
<td></td>
</tr>
<tr>
<td>17ΔPst</td>
<td>A26(L)</td>
<td>1,200</td>
<td>10,750 ± 7888</td>
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<tr>
<td>50,000 PFU</td>
<td>A26(R)</td>
<td>1,800</td>
<td></td>
</tr>
<tr>
<td>17ΔPstR</td>
<td>A30(L)</td>
<td>3,000</td>
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</tr>
<tr>
<td>(rescue)</td>
<td>A30(R)</td>
<td>11,000</td>
<td></td>
</tr>
<tr>
<td>50,000 PFU</td>
<td>A31(L)</td>
<td>8,000</td>
<td>16,500 ± 7888</td>
</tr>
<tr>
<td>50,000 PFU</td>
<td>A31(R)</td>
<td>3,000</td>
<td></td>
</tr>
<tr>
<td>17ΔPstR</td>
<td>A32(L)</td>
<td>15,000</td>
<td></td>
</tr>
<tr>
<td>PFU</td>
<td>A32(R)</td>
<td>40,000</td>
<td></td>
</tr>
</tbody>
</table>

*Rabbits were inoculated with the indicated doses of 17ΔPstR or 17ΔPst in both eyes. Total DNA was isolated from latent ganglia (40 d.p.i.) and analyzed by PCR amplification with actin and VP5 primer sets. Data are from 4 TGs per dose per virus per time point.

§Relative amounts of viral DNA expressed as genome equivalents of HSV determined following semi-quantitative PCR for the HSV DNA polymerase gene and standardized to the amount of cellular actin present in each sample. Standard curves were generated using known amounts of HSV polymerase target DNA were used in order to calculate the number of genomes present in each sample (see Materials and Methods).

‡Means and standard error of the mean (SEM) were calculated as described in the Materials and Methods section.
CHAPTER 3
A DRAMATIC DECREASE IN LAT ABUNDANCE OCCURS DURING THE EARLY HOURS OF EXPLANT INDUCED REACTIVATION IN BOTH WILD TYPE INFECTED AND LAT-TRANSGENIC MOUSE DORSAL ROOT GANGLIA

Introduction

Examination of the molecular processes involved in HSV-1 latency and reactivation represent a major portion of our laboratory’s resources. Projects consist of defining chromatin boundaries during latency, identifying specific cellular factors that signal reactivation, and examining LAT’s role as a liaison between the cell and the virus. A fundamental issue central to accomplishing these goals is to characterize the very early, if not first, events of the reactivation process. While acute replication kinetics in cell culture are well established, the events of in vivo reactivation are partially understood at best. A complete picture of the molecular processes controlling reactivation is not only interesting in its own right, but can also serve as a template for examining specific viral mutants.

Our models of latency and reactivation center on the theory that the factor(s) responsible for maintaining the delicate balance between the repressive state of latency and the ability to sense and respond to subtle cellular stimuli are located within the LAT region. Despite its unique transcriptional status, location, promoter complexity, and mutational analysis indicating an involvement in reactivation, a clear demonstration of how (or if) LAT responds during reactivation has yet to be shown. Transcription of LAT during latency provides an ideal marker by which to monitor changes in relative abundance during the early events of explant co-cultivation, to determine if (and indeed
how) LAT responds to reactivation stimuli. Considerable effort optimizing reverse transcription and PCR conditions now allows sensitive and quantitative analysis of the early molecular events of explant-induced reactivation. These results will provide valuable insight towards our reactivation models, specifically regarding the degree to which LAT is involved and whether it plays an active or passive role.

Explant co-cultivation of latently infected murine DRG is well suited for this analysis because the stress of explantation permits more extensive transcriptional activity than subtler reactivation models. Using this model, LAT’s abundance is examined over an early range of time points using wild type virus strains 17syn + and KOS. A separate analysis of ganglia from a partially characterized LAT-transgenic mouse is also included as a means of monitoring LAT in a more homogeneous environment independent of exogenous viral factors. These results will indicate the extent to which this locus is able to respond to reactivation stimuli, and also provide insight regarding LAT’s location in the signaling cascade and whether it behaves as an activator or repressor.

The second goal of these experiments is to establish a correlation between changes in LAT abundance and activation of lytic cycle transcripts during reactivation. Reliably detecting such a discrete event will be more difficult than monitoring LAT levels. Since LAT is actively transcribed during latency (up to 30% of latent neurons produce abundant levels) a readily detectable baseline exists for comparison to later time points. Lytic genes must, however, reach minimal thresholds before they can be reliably detected. This sensitivity requirement is further complicated by well-documented basal lytic gene “leakiness” during latency. Reliable determination that a given transcript is
present at significantly higher levels than background is difficult, especially when the goal is to monitor the very first events of reactivation.

Our final goal is to determine which of the key IE genes (ICP0 or ICP4) is the first to respond during reactivation. In addition to wild type (17syn+ and KOS) infected ganglia, viral mutants deleted for either ICP0 or ICP4 (KD6) are also be examined. The experimental design is based on the logic that the first transcript activated during reactivation will not be affected by the others absence. If, for example, the promiscuous transactivator ICP0 is the key IE gene, then its appearance should not be affected by the absence of ICP4. Likewise, if the master regulatory gene ICP4 is the major determinant then it should be detected even in ICP0’s absence. In addition to providing a genetic basis for deciphering the events of reactivation, these mutants provide the additional benefit of being replication-deficient, thereby reducing the transcriptional leakiness associated with latency. Besides the sensitivity requirements, a potential drawback associated with these mutants is that their replication restriction prevents them from establishing nearly as robust of a latent infection as wild type virus. The possibility exists that the resolution required for this analysis will be impossible from less than maximal levels of latent genomes.

In the event that a clear distinction between ICP0 and ICP4 activation can not be established, this work should at least address the issue of whether IE genes are activated during the early hours of explant co-cultivation. Previous reports claim that reactivation does not follow the standard IE, E, L, gene kinetics determined in cell culture. These results state that E transcripts precede IE’s by as much as 24 hours. While we feel these
experiments are not nearly as well controlled or optimized as ours, the observation deserves to be addressed.

**Materials and Methods**

**Cells and Viruses**

Wild type HSV-1 strain 17syn⁺ (syncytia forming) is amplified and titered on rabbit skin (RS) cells. Briefly, RS cells are maintained in minimal essential media (MEM, Gibco Life Technologies, Gaithersburg, MD) with 5% calf serum and antibiotics (250 U penicillin, 250 µg/ml streptomycin, 2.5 µg/ml amphotericin B, and 292 µg/ml L-Glutamine) at 37°C in a humidified 5% carbon dioxide atmosphere. Sub-confluent RS cell monolayers are infected with an M.O.I. of 0.01 pfu/cell and allowed to replicate 3-4 days or until 100% cytopathic effect (C.P.E) is visible. Cells are detached from the flasks by shaking, and the media transferred to 250 ml Sorvall bottles. Virus is concentrated by centrifugation at 10,000K at 4°C for 40 minutes. The virus containing pellet is resuspended in 1 ml of media, freeze thawed twice, and stored at -80°C for titering.

The 17syn⁺-based ICP4 (-) virus (KD6) has the entire ICP4 coding sequence removed from both Rs copies and requires the E5 helper cell line for amplification due to its replication restriction. E5 cells are Vero (African Green Monkey Kidney) cells stably transfected with the HSV-1 ICP4 gene. Cells are maintained as previously described, using media supplemented with 10% fetal bovine serum (FBS) rather than 5% calf serum. Viral infection and harvesting is identical to the method described above. The KD6 virus is capable of spontaneously reacquiring the ICP4 gene during amplification on E5 cells. Revertancy to wild type status occurs with a frequency of approximately one in every ten thousand infectious particles (1x10⁻⁴). To ensure that KD6 viral stocks exhibit as low a
revertant rate as possible, the virus is plaque purified and titered on both E5 and Vero cells. Comparing viral titers on permissive (E5) and non-permissive (Vero) cells allows the percentage of wild type virus present in the stock to be determined.

Since a frank deletion of its coding sequence would disrupt the anti-parallel LAT region, the KOS-based ICP0 (-) mutant (provided by Priscilla Schaffer, Harvard University) contains translational stop codons of all three ORFs. This construct does not require a helper cell line for amplification due to an ability to overcome its replication deficit by infecting cells at a high M.O.I. (5 pfu/cell). Since ICP0 does not require a helper cell line, there is no chance of reverting to wild type status. A Vero based helper cell line is, however, available for titering ICP0 (-) stocks since viral plaques cannot arise from the low M.O.I. infections required (single viral particles) to determine the number of infectious particles/ml.

Wild type HSV-1 strain KOS is a much less virulent isolate than 17syn+ but is fully capable of establishing a latent infection and reactivating. It is included in this analysis to serve as a second wild type strain and because the ICP0 (-) mutant is KOS-based.

**Mouse Infections**

Four to six week old female Swiss-Webster mice are anesthetized with halothane and pre-treated with 0.05 ml of 10% saline solution injected under each rear footpad. Four hours after pre-treatment the mice are anesthetized (ketamine, acepromazine, xylazine), and the foot’s keratinized epithelial layer is lightly abraded with an emery board. The moist surface is infected with 500 pfu of virus (17syn+ or 17ΔPst) in a 50 µl volume applied to the entire surface with the side of the pipet tip.
Since wild type revertants of KD6 are fully capable of replication, the presence of even low-level revertants may overwhelm the replication-defective KD6 virus, making analysis impossible. This requirement is further complicated by the extremely high dose inoculation of KD6 required to establish sufficient levels of latent genomes in the DRG. The replication-deficient status of KD6 requires infecting with the maximal allowable titer (10^5-10^6 pfu/mouse) that contributes the fewest (1-10 pfu/mouse) wild type revertants. To ensure that KD6 infected ganglia do not contain significant levels of revertants, back-extracted DNA is examined with PCR primers for deleted (ICP4) and non-deleted (i.e. polymerase gene) regions. If ICP4 is detectable at levels equivalent to the polymerase specific primers, the tissues cannot be used. In general, mice exhibiting CNS involvement and death following infection strongly indicate the presence of an unacceptable revertant rate. If mice show none of the symptoms of a wild type infection, they are most likely infected with KD6 alone.

KOS infections (wild type and ICP0 (-) mutant) are both performed at high M.O.I.s due to their extremely low neurovirulence. Even though both viruses are replication-competent, the absence of specific viral surface glycoprotein entry mediators renders this HSV-1 strain avirulent. Since wild type revertants of the ICP0 (-) mutant are not a concern, the ability to infect with high doses allows efficient establishment of latency.

Animals are placed on their backs while the anesthetic wears off (30-45 minutes) to allow efficient viral adsorption. Mice infected with replication competent viruses may exhibit lethargy, scruffy coats, and hind limb paralysis associated with CNS involvement within 5-7 days post-infection. Fifty percent of these mice typically succumb to viral dissemination; those that survive exhibit complete recovery and are allowed 28 days for
acute replication to subside. Mice are considered latently infected by 28 days post-infection (d.p.i.) and are suitable for examination of reactivation.

**Generation of LAT Transgenic**

Transgenic mice (c57b6) were constructed (Fox Chase Cancer Center) using a 3.0 kb sub-cloned fragment of the HSV-1 LAT region extending from the core promoter to the first 500 bp of the 3’exon. Though not yet fully characterized, transgenic mice contain an intact promoter and at least the first 2.0 kb of the primary transcript.

**Explant Co-cultivation**

Mice are sacrificed by cervical dislocation and dorsal root ganglia (DRG) L4 and L5 are removed under aseptic conditions. Dissected ganglia are immediately placed in 500 µl of media (MEM plus 10% FBS) and maintained at 37°C in a humidified CO2 incubator. At specific intervals post-explant (0, 1, 2, 3, or 4 hours), ganglia are transferred to 1.5 ml tubes and snap frozen in liquid nitrogen (LN2). Since approximately three minutes are required to remove the DRG from a single mouse, zero hour time points are removed and frozen individually. Co-cultivation experiments of latently infected DRG represent the pooled tissue from three mice (approximately 12 ganglia), while the transgenic mouse experiments require DRG of a single animal per time point (four ganglia).

**RNA Processing**

Frozen DRGs representing various co-cultivation time points are homogenized on ice in 600µl Trizol (Gibco/BRL) using glass dounces. Following complete tissue disruption the dounce is rinsed with an additional 600µl Trizol, the homogenate is transferred to 1.5 ml tubes, followed by incubation at room temperature for 5 minutes. 240µl of chloroform is added, followed by 15 seconds of vigorous vortexing, a 15-minute
incubation at room temperature, and centrifugation at 9,000 x g for 15 minutes at 4°C. The aqueous phase (approximately 600µl) is removed to a separate tube and precipitated with 0.7 volumes isopropanol. While maximal RNA recovery is important, the likelihood of DNA contamination makes it wise to avoid getting too close to the interface. RNA is precipitated by centrifugation at 14,000xG for 15 minutes at 4°C. The resulting pellet is rinsed briefly with 70% ethanol, taking care to remove all excess liquid. Contaminating DNA is removed by resuspending the RNA in 90µl DEPC-treated water (Qiagen), 9µl 10X DNase buffer (Ambion), 2µl DNasel (Ambion), and 1µl RNasin (Ambion), followed by incubation in a 37°C H2O bath for one hour. The reaction is stopped by addition of 0.2 volumes of DNase Inactivation Solution (Ambion) for 2-3 minutes, mixing periodically. Following a 15 second centrifugation at 9,000 x g, the aqueous RNA supernatant is removed to a sterile tube.

**Reverse Transcription**

Reverse transcription reactions are performed using the Omniscript-RT kit provided by Qiagen. The range of total RNA for optimal reverse transcription efficiency using the enzyme provided in this kit is 50ng-2µg. Since the pooled ganglia from three mice (12-16 ganglia) yields approximately 15µg of total RNA, RNA aliquots must be divided into 15-18 separate reactions of 20µl for optimal cDNA synthesis. An individual 20µl reverse transcription reaction includes 2µl 10X buffer, 2µl 5mM dNTPs, 2µl random decamer primers (2.5mM each)(Ambion), 0.25µl RNasin (Ambion), 6.75µl DEPC H2O, and 1µl (4U/µl) Omniscript-RT. Reverse transcription reactions are incubated for 1 hour at 37°C, followed by heat inactivation at 93°C for 5 minutes and rapid cooling on ice. Individual reactions are combined into a single tube and
precipitated with 0.1 volumes 3M-sodium acetate (NaAc), 0.02 volumes linear acrylamide, and 2.5 volumes 100% ethanol. Following centrifugation at 14,000 x g for 15 minutes, the pellet is dried and resuspended in 20µl sterile H20. A minus reverse transcription control (-RT), to account for DNA carry-over, is essential since very few HSV-1 genes contain introns for cDNA specific analysis. An approximately 20µl RNA aliquot not used in the reverse transcription reaction is put through the same regimen as the other samples to ensure that it is concentrated in a manner equivalent to the cDNA samples. To avoid wasting reagents, reaction components are replaced with sterile water.

LAT is not nearly as difficult to detect from the transgenic mice since the DRGs contain many more copies of target DNA than even the most efficiently infected ganglia. This allows the DRGs from a single mouse to be used per time point; which is important since they are often in short supply. LAT is abundant enough that only one-third of the total RNA isolated from each mouse is sufficient for analysis. The remaining sample is frozen for future use if necessary.

**DNA Processing**

DNA is purified from the aqueous/organic interface to ensure that tissues are latently infected (or positive transgenics). The interface is back-extracted with 3 successive volumes (150µl each) of 0.1M Tris (pH 8.0) plus 0.1% Sarkosyl. After each back-extraction the sample is vortexed 15 seconds, briefly centrifuged, and the aqueous phase transferred to a fresh tube. The final sample (approximately 450-500µl) is incubated with 20mg/ml proteinase-K at 37°C overnight. DNA is phenol/sevag extracted and ethanol precipitated as before. The resulting pellet is resuspended in 10µl sterile
H20. While recovery of DNA by this method is not as efficient as from a straight DNA isolation, it is sufficient for this application.

**PCR Analysis**

Analysis of cDNA (or back-extracted DNA) requires comparing time points within the experiment as well as results between experiments. Primers to the single copy cellular APRT gene allow samples to be normalized for efficiency of RNA isolation and reverse transcription. LAT specific PCR primers are available for the 5’exon, and 2.0 kb intron. PCR reactions are performed in a 50µl final volume, consisting of 40.5µl sterile H20, 1µl of both forward and reverse primers (600 ng/µl), 1µl dNTPs (1.25 mM each), 5µl 10X AS buffer (Qiagen; Tris-Cl, KCl, (NH4)2SO4, 15mM MgCl2, pH 8.7), 1µl respective DNA/cDNA sample, and 0.5µl HotStar Taq DNA Polymerase (Qiagen; 5 U/µl). The amplification profile consists of 15 minutes at 95°C to activate the Taq, followed by one three-minute cycle of 94°C, 55°C, and 72°C; this is followed by 30 identical cycles of one minute each (Ericomp Twinblock™System, Easy Cycler). PCR products are resolved on 5% polyacrylamide gels, stained with SYBR Green (Molecular Probes), and scanned with a Storm Phosphorimager (Molecular Dynamics) using a 450 nm wavelength laser. LAT 5’exon primers (sense: 5’CGG CGA CAT CCT CCC CCT AAG C 3’, and anti-sense: 5’ GAC AGA CGA ACG AAA CAT TCC G 3’) yield a 149-bp product. LAT intron primers (sense: 5’ GAC ACG GAT TGG CTG GTG TAG TGG G 3’, and anti-sense: 5’ ACG AGG GAA AAC AAT AAG GGA CGC 3’) yield a 102-bp product. APRT primers (sense: ACT CCA GGG GCT TCC TGT TTG 3’, and anti-sense: ATC CAC AAT GAC CAC TCT CTG 3’) yield 373-bp product DNA, and a 186-bp cDNA, amplification products, respectively. ICP0 primers (sense: 5’ GGG CGG
GCG GTA CGT AGT CT 3', and anti-sense: 5' GAC GGG CAA TCA GCG GTT CG 3') yield 275-bp DNA and 138-bp cDNA products respectively. ICP4 primers (sense: 5' CTG ATC ACG CGG CTG CTG TAC ACC 3', and anti-sense: 5' GGT GAT GAA GGA GCT GCT GTT GCG 3') yield a 144-bp product.

**Real-Time PCR Analysis**

To achieve higher resolution and more easily quantifiable data, real-time primer/probe oligos were constructed for the LAT 5’exon as well as cellular APRT and XIST genes. The “Assays-by-Design” program of Applied Biosystems guarantees optimized primer/probe sets for any DNA sequence submitted to them. The primer/probe sequences for the XIST gene are (forward: 5' GCT CTT AAA CTG AGT GGG TGT TCA 3', reverse: 5' GTA TCA CGC AGA AGC CAT AAT GG 3', probe: 5' FAM-ACG CGG GCT CTC CA 3'). The primer/probe sequences for APRT are (forward: 5'CTC AAG AAA TCT AAC CCC TGA CTC A 3', reverse: 5' GCG GGA CAG GCT GAG A 3', probe: 5' FAM-CCC CAC ACA CAC CTC 3'). The primer/probe sequences for the 5’LAT exon are (forward: 5' GGC TCC ATC GCC TTT CCT 3', reverse: 5' AAG GGA GGG AGG AGG GTA CTG 3', probe: 5' FAM-TCT CGC TTC TCC CC 3'). ICPO (cDNA) and ICP4 specific real-time primers and probes are used for detection of lytic genes. The ICP0 prime/probe sets are (sense: 5' CAC CAC GGA CGA GGA TGA C 3', anti-sense: 5' GGC GGG CGG TAC GT 3', and probe: 5' FAM-ACC TGG ACG AAG CAG ACT 3'). The ICP4 primer/probe sets are (sense: 5' GAC GGG CCG CTG ATC ACC 3', anti-sense: 5' GCG ATA GCG CGC GTA GA 3', and probe: 5' FAM- CCG ACG CGA CCT CC 3'). All reactions are run in triplicate in a 20µl final volume consisting of: 1µl of sample, 10µl of Mastermix (Applied Biosystems: TaqMan Universal PCR Master Mix, No AmpErase® UNG), 1µl of Assay Mix (containing primers and probe),
and 8µl of sterile H₂O. Reactions are run on the Applied Biosystems Model 7000 Taqman machine under the following conditions: one two-minute cycle at 50°C, one ten-minute cycle at 95°C, and forty cycles of fifteen seconds at 95°C followed by one minute at 60°C.

Results

The first aim of this project was to monitor the primary LAT’s relative abundance over a range of explant co-cultivation time points, to determine the degree to which the LAT locus responds during the early hours of explant-induced reactivation. The murine explant co-cultivation model was chosen for a number of reasons. First, mice are relatively inexpensive and easy to handle. Since this work requires replicate analysis of multiple time points using different viral strains and mutants, more cumbersome systems (such as the rabbit eye model) are far too inflexible. Second, mice allow uniform and efficient establishment of latency. Comparisons between time points and experiments are impossible if dramatic fluctuations in total numbers of latent genomes occur. Finally, murine dorsal root ganglia respond well to the stress of explantation, thus providing the most likely environment for determining if the LAT promoter responds from a distinguishable percentage of the total latent genome population.

Wild type virus reactivation analyses are based on mice latently infected with HSV-1 strain 17syn+ or KOS. Time points (0, .5, 1, 2, 3, and 4 hour) represent the pooled dorsal root ganglia of three mice (approximately 12 ganglia total). For each time point mice are sacrificed and their ganglia harvested over no more than a fifteen-minute period, thus, actual culture times may vary plus or minus seven minutes. The potential for regulatory events to occur during the three to five minute dissecting process requires
zero-hour ganglia to be individually removed and snap frozen in liquid nitrogen. Pooling ganglia is intended to increase consistency between time points by averaging out variations of infection efficiency, while also maximizing the amount of total RNA for analysis.

To ensure consistent handling, tissues are processed at the same time. Sample preparation is divided into DNA and RNA components. The majority of the isolated RNA (90%) is reverse transcribed into cDNA using random decamer primers, while a minimal amount (approximately 10%) is set aside as a minus reverse transcription (-RT) control. DNA is purified by back-extraction of the aqueous/organic interface following RNA removal. Though done as almost an afterthought this step provides sufficient material for PCR examination. DNA analysis is included to prove that all tissues harbored equivalent levels of latent genomes, while -RT controls show that cDNA PCR products are not due to contaminating DNA. The -RT samples importance is underscored by the lack, in most cases, of spliced HSV-1 message.

Initial sample analysis was to be performed via quantitative (quantitative-competitive) PCR. This technique requires extensive sample dilution and competitor-oligo titrations to ensure PCR reactions reflect a linear range of detection, such that signal intensity reflects the amount of target. For this reason we switched to the much higher resolution technique of Real-Time PCR. While data from both methods are shown, quantitation is based on Real-Time PCR results. Two cellular controls are included to demonstrate consistent RNA isolation and reverse transcription. The single copy APRT (adenine phosphoribosyltransferase) gene is a standard cellular control representing an abundant cytoplasmic mRNA. The second cellular control is the mammalian dosage
compensation gene XIST (X-inactive specific transcript), which, like LAT, is an
alternately spliced, nuclear-localized RNA encoding no known protein that associates
with repressed DNA. Because we are trying to quantitatively assess the relative levels of
LAT over a range of time points, it is important to demonstrate that changes in abundance
are not due to general transcriptional activation or RNA degradation. Cellular controls
from nuclear and cytoplasmic regions should address these concerns.

A Severe Decrease in LAT Abundance Occurs During the First Hours of Explant
Co-cultivation of Latently Infected Dorsal Root Ganglia

Results of Figure 3.1-3.4 demonstrate that cellular APRT and XIST controls exhibit
only minor fluctuations (a maximum of 6-fold) during explant co-cultivation. The results
of multiple experiments demonstrate that, relative to zero hour levels, severe reduction in
LAT abundance occur during explant co-cultivation. Although the timing is variable,
LAT levels decrease 10 to 1000-fold relative to cellular controls. Figure 3.1 represents
Real-Time PCR analysis of DRG latently infected with HSV-1 strain 17syn+. Results
demonstrate a dramatic (500-fold) decrease in LAT abundance at only the one-hour time
point. Cellular controls indicate that the decrease at one hour is LAT specific; rather than
overall RNA degradation during co-cultivation, the total amount of RNA seems to
increase slightly at later time points. Figure 3.2 indicates a more gradual pattern of LAT
reduction, reaching a maximum of 10-fold. Decreased LAT levels are slightly more
dramatic (5-fold) than cellular controls, which also tend to decrease slightly.

Results are similar when DRG latently infected with HSV-1 strain KOS (Figure 3.3
and 2.4) are examined. As shown in Figure 3.3, a dramatic decrease (reaching 1000-fold)
in LAT occurs at the three and four-hour time points. Cellular controls again fluctuate no
greater than 5-fold over the same time points. Figure 3.4 demonstrates steadier and more
gradual LAT reductions of approximately 9-fold, similar to the results of Figure 3.2. Though LAT kinetics are not as dramatic as in the previous experiment, cellular controls indicate that this reduction is LAT-specific and not simply the product of general RNA degradation or promoter repression. Control PCRs (DNA and –RT) determine that tissues are latently infected with equivalent levels of HSV-1, and RNA is devoid of contaminating DNA. Standard PCR analysis of 17syn+ experiments (2.1 and 2.2) is shown in Figure 3.5. Primers specific for both the 5’LAT exon and 2.0 kb intron both show dramatic decreases in LAT abundance at the previously reported time points. Control PCRs to detect viral and cellular DNA (HSV-1 polymerase and APRT, respectively) indicate equivalent infections and sample processing. The –RT control indicates that DNA was successfully removed prior to cDNA synthesis.

**Reduced Abundance of LAT Also Occurs During Co-cultivation of Uninfected Transgenic Mouse Dorsal Root Ganglia**

The variability associated with explant-induced decreases in LAT abundance from wild type infected DRG led to analysis of LAT-transgenic mice to provide a more uniform LAT environment. Transgenic mice express sufficient LAT that only a portion of the RNA (20%) from a single animal (four ganglia) is adequate for monitoring LAT’s abundance. Since these mice are not infected, there is no variation in the amount of LAT within each tissue. The availability of LAT-transgenic mice provide an independent system to validate observations from latently infected ganglia, while also providing insight into LAT’s ability to respond to stress in the absence of other viral factors. Though not yet fully characterized, the inserted transgene is known to contain the portion of LAT (the core promoter extending into the 2.0 kb intron) to which reactivation has
been mapped. Since these experiments employ both male and female animals, only APRT controls are valid for this analysis.

Results of Figure 3.6-3.9 demonstrate that decreased LAT abundance also occurs following explant co-cultivation of uninfected LAT-transgenic mouse DRG. The decreases in LAT levels are, however, as variable as in latent infections. Figure 3.6 demonstrates a dramatic (1000-fold) LAT reduction at 0.5 hours only. Cellular APRT controls fluctuate only 2-3 fold over these same time points. Results of Figure 3.7 exhibit decreased LAT-abundance at all time points, reaching a maximum of 1000-fold by four hours. While cellular APRT controls tend to mirror LAT kinetics, they vary only 4-5 fold. Figure 3.8 displays severely reduced LAT levels (500-fold) at only the three hour time point, while Figure 3.9 exhibits dramatic decreases in LAT abundance (800-fold) at both one and four hour time points relative to the cellular control. The –RT controls (not shown) show the absence of contaminating DNA. Standard PCR analysis using PCR primers to the 5’ LAT exon are again included (Figure 3.10) to demonstrate the LAT decrease, and to emphasize the value of Real-Time PCR for quantitative analysis. As before, APRT and –RT controls indicate that samples were handled consistently.

Detection of Immediate Early Transcripts During Explant Co-cultivation

Given the dramatic LAT kinetics observed, we wished to determine whether decreased LAT levels during co-cultivation correlate with the appearance of immediate early (IE) genes, as reported from the rat primary DRG cell culture model (Wilcox et al). Since Real-Time PCR primer / probe oligos provided unsuitable sensitivity, standard PCR was used to detect the IE gene ICP0 with primers spanning the genes first intron. The ability to detect spliced product allows specific detection of ICP0 cDNA, eliminating possible contributions from both DNA and anti-sense LAT. Select cDNA samples were
also analyzed with PCR primers specific for the IE gene ICP4. Since ICP4 is unspliced, it is not as easily distinguished from background DNA. Preliminary results using Real-Time PCR primer and probe sets specific for ICP4 failed to detect ICP4 cDNA at any co-cultivation time point (not shown). Unfortunately, significant portions of the cDNA samples were used for the Real-Time analysis. Select remaining samples were re-examined by standard PCR using ICP0 and ICP4 specific primers. As shown in Figure 3.11, examination of cDNA from 17syn+ experiment 2.1 demonstrates the presence of both ICP0 and ICP4 during the early hours of explant co-cultivation.

**Does Lytic Gene Activation Require a State of LAT-Repression**

The fact that both lytic (ICP0 and ICP4) and latent (LAT) phase transcripts exhibit variable kinetics during explant co-cultivation emphasizes the complex nature of reactivation, and indicates that numerous factors are involved. Our longstanding model has been that the LAT locus functions (at the DNA or RNA level) to repress lytic gene expression during latency until proper reactivation signal(s) are received. We have proposed a tentative model to explain the decreased LAT abundance during explant co-cultivation. If LAT repression is the first step of reactivation, then the variable nature of its kinetics will make reliable monitoring of lytic gene activation nearly impossible. The optimal environment for monitoring lytic gene activation during explant-induced reactivation is not from wild type virus but rather a LAT mutant that provides a pre-set state of LAT repression. The absence of LAT should, therefore, provide a synchronous population of latent genomes, which already have the “safety” off. A uniformly LAT-negative environment should provide much more reliable detection of lytic cycle transcripts. The LAT promoter mutant 17ΔPst is a 17syn+ -based construct in which the
202-bp core promoter (including the TATA box) has been removed. Though this construct does not make LAT, it establishes latency as readily as its wild type parent. While 17ΔPst is almost completely null for reactivation in the rabbit eye model, it reactivates with only a slight delay in kinetics during co-cultivation. Interestingly, this construct is also known to exhibit greater lytic gene leakiness during latency*, possibly fitting with our model that it lacks a layer of regulation.

The results of two separate 17ΔPst co-cultivation experiments are shown in Figure 3.12. The first experiment demonstrates the presence of both ICP0 and ICP4 at 30 minutes, one, and two hours. The second experiment detects ICP0 by one hour and ICP4 by two hours. The second half of Figure 3.12 examines KOS tissues from experiment 2.3 and 2.4 to determine if time points demonstrating dramatic LAT decreases are positive for ICP0. As before, ICP0’s detection is sporadic and only partially corresponds with decreased LAT abundance.

**Which is the First Immediate Early Gene Activated During Reactivation**

Having proven the ability to detect IE genes within the first four hours of co-cultivation, our final goal was determine which IE gene (ICP0 or ICP4) responds first during reactivation. The experimental design employs viruses deleted for each respective gene (ICP0 and ICP4 / KD6). Since the gene that responds first should not be affected by the others absence, these mutants should allow us to determine the order of their appearance. While the replication impairment of both viral mutants provides much lower transcriptional background noise during latency, it also results in many fewer latent genomes within each dorsal root ganglion. As evident in Figure 3.13, infectious doses as high as $2 \times 10^6$ pfu/mouse fail to achieve levels of establishment equivalent to wild type
virus (KOS). The fact that the ICP0' mutant seems to have established latency more efficiently than its KD6 counterpart (despite infecting with half as many infectious virions) indicates that it is capable of limited replication in vivo. Since detection of ICP0 proved to be less than straightforward in wild type infected tissue, this reduction in total latent genomes only makes our job more difficult. Indeed, these fears proved valid, as lytic transcripts were not reliably detected from multiple experiments. Two separate ICP0 (-) co-cultivation experiments spanning 0, 1, 2, 3, and 4-hour time points failed to detect either ICP4 or ICP0 transcriptional activity. Four separate KD6 experiments spanning similar time courses also failed to produce a consistent result. In some cases ICP0 cDNA signal was detected from KD6 experiments, but it was unclear whether this was due to wild type revertants. The fact that the KD6 virus fails to accumulate enough LAT to be detected by Real-Time PCR underscores the disparity in overall levels of latent genomes. While the ICP0 (-) virus does accumulate enough LAT to be detected by Real-Time PCR, it unfortunately fails to provide any insight regarding acute gene activation. In summary, the level of sensitivity required to reliably detect acute transcripts, coupled with less than maximal establishment of latency, prevents this analysis from being readily accomplished. This result is not wholly surprising considering the difficulty of detecting IE transcripts from wild type infected tissues. The failure to detect lytic genes from either mutant may result from the failure of LAT to respond appropriately, as well as sub-optimal establishment of latency.

Discussion

Final assessment of the goals outlined at the beginning of this chapter can be summarized as a collectively partial success. While changes in LAT abundance were detected during reactivation, they were sporadic; while lytic phase transcripts were
identified, their appearance demonstrated neither reproducible kinetics nor a correlation with decreased LAT abundance. Arguably the most important result of this project has been the thorough demonstration of the inherent variability associated with explant co-cultivations. Rather than viewing the situation as bleak, at a certain level these inconsistencies should not only be accepted, but also expected. Why should detailed examinations of animal models behave perfectly when reactivation in the natural host exhibits extreme variation? Some infected people reactivate frequently over their entire lives, some sporadically, and others not at all. Regulation of reactivation is clearly a complicated event, in which LAT’s involvement is important but not absolute. In the rabbit eye model, which most closely reproduces the strict regulation of human infections, even the prototypical LAT reactivation mutant (17ΔPst) exhibits induced-reactivation frequencies approaching 10%. The intrinsic variability associated with animal models, combined with HSV-1’s penchant for redundant back-up mechanisms do not logically lend themselves to refined molecular analyses. My experience has been that variability compromises reproducibility, and the more carefully controlled and optimized an experiment is, the more apparent these inconsistencies become.

**Decreased LAT Abundance During Explant Co-cultivation**

Real-Time PCR results from both wild type infected and LAT-transgenic mice provide direct evidence that the molecular switch through which cellular signals are relayed to latent HSV-1 genomes resides within the LAT locus. This argument is based primarily on two points: 1) decreased LAT abundance represents (within our limits of sensitivity) the first quantifiable molecular event following the reactivation stimuli, and
2) observations of the LAT-transgenic show this effect to be reproducible in the absence of other viral factors.

The major concern regarding with our observations of LAT’s dramatic decrease in abundance is the erratic nature of its timing and magnitude. One reason latently infected DRG were pooled for each time point was to prevent aberrant infection efficiencies from skewing results. Even in the event that one of the three mice within a given time point was completely uninfected; this should contribute a mere 3-fold change in LAT abundance. Standard PCR analysis of back-extracted DNA clearly demonstrates that all time points harbor roughly equivalent levels of latent genomes. The use of two (very different) cellular controls further strengthens the argument that individual samples are more similar than different. Considering the number of processing steps involved in these experiments, error is certain to be introduced by the investigator. Cellular controls, however, would have indicated if wholesale mishandling occurred in a specific sample. While APRT and XIST levels fluctuate to a certain extent, they tend not to do so over greater than 2-3 fold, indicating that neither RNA degradation nor general promoter activation occur during explant co-cultivation.

The use of lines to connect time points when graphing each experiment may be potentially misleading. Though difficult to prove, I do not believe that LAT-levels necessarily rise and fall hundreds of fold over the four-hour time course. Given LAT’s unreliable nature, tissues of certain time points simply do not respond as dramatically as others. Whether LAT abundance would eventually decrease if given more time is impossible to know. Experiments demonstrating less dramatic (10-fold) decreases in LAT abundance may be due to dilution caused by asynchronous kinetics of individual
The most dramatic time points would, likewise, reflect more perfectly timed LAT kinetics. Finally, if decreased LAT abundance represents a random artifact, then of the eight individual experiments performed, the effect should occasionally occur in time zero samples.

Analysis of the LAT-transgenic was included to eliminate averaging effects of latently infected DRG. The fact that results were as variable when using only 20% of the total RNA from one mouse, indicates that pooling of tissues was not the cause. Despite the availability of a single cellular control, Real-Time PCR results again indicate that LAT abundances are accurately reported. While some of these experiments compare DRG of males and females, previous work has shown that sex does not influence LAT-expression profiles. Since LAT is capable of responding to explant-induced stress independent of exogenous viral factors, it seems logical to state that this represents the first event of sensing cellular stimuli to initiate the reactivation cascade.

**Lytic Gene Activation During Explant Co-cultivation, and the Requirement of LAT-Repression**

The original goal of monitoring lytic gene activation during the early hours of explant co-cultivation proved to be more easily planned than accomplished. The unpredictable nature of molecular reactivation coupled with the extreme sensitivity requirements made textbook completion of our goals nearly impossible. Valuable observations were, none-the-less, obtained from IE transcript analysis. Though sporadic in nature, ICP0 (and to a lesser extent ICP4) was detected during many of the co-cultivation time points examined. The fact that lytic gene activation does not precisely correlate with decreased LAT abundance is understandable given LAT’s inherent variability, and the pooling of ganglia for each time point. The observation that a LAT-
null (17ΔPst) mutant may exhibit more consistent lytic gene activation fits our current model and is encouraging, but requires more detailed analysis.

The major benefit of the acute portion of the reactivation study is that the original question responsible for this entire project is answered. We wanted to examine the validity of observations claiming that E genes preceded IE genes (by up to 24 hours) during explant co-cultivation. Our data clearly show that IE genes can be detected well before twenty-four hours. We were not able, however, to prove or refute claims of E genes preceding IE genes, since our results detect lytic transcripts representative of all gene classes at very early times post-explant (data not shown). While this result may not be earth shattering, we feel well-controlled experiments demonstrating a systems complexity are more valuable than poorly controlled experiments that diagram complex mechanisms. If this work contributes anything to the HSV field, it should be that explant co-cultivations (as with human reactivation) are not static, and should not rely on a clock for molecular analysis.

Proposed Mechanism for LAT’s Role in Reactivation

A unifying model for LAT’s involvement in reactivation has eluded HSV-1 researchers since its discovery. LAT has long been known to play a major role in reactivation; the effect of specific promoter and 5’exon deletions have been carefully examined in a variety of animal models. Cell culture systems capable of re-creating certain aspects of HSV-1 latency and reactivation have, likewise, demonstrated LAT’s capacity to sense and respond to induction stimuli. While previous in situ analysis detected 2-fold decreases in LAT abundance during co-cultivation of murine trigeminal ganglia, this is the first evidence of a dramatic and global decrease in LAT abundance in
vivo. The most exciting extension of this result is the correlation it establishes with Christine Wilcox’s cell culture model of latency and reactivation (see introduction). This is one of the few in vitro systems capable of reproducing the three hallmarks of latency: genome circularization, LAT accumulation, and an ability to respond to induction stimuli. Using this model, it was reported that six hours post-induction and continuing through twenty-four hours, in situ analysis reveals a dramatic (10-fold) decrease in LAT abundance, concomitant with activation of the CREB antagonist ICER. This represents a potentially powerful relationship whereby observations in vivo can be examined in more rigorously controlled settings. Likewise, observations from cell culture can be applied to more biologically relevant animal models. Future work will, for example, certainly include determining whether reduced LAT abundance correlates with increased levels of ICER.

HSV-1 faces the unique problem of having to balance two very different requirements; while it expends considerable effort creating a state of extreme repression that may last forever, it must retain the ability to respond to stressors as subtle as ultraviolet light. The mechanism controlling this process must require layers of regulation, which begin at a general (global) level, but become increasingly stringent. Here is the problem: reactivation cannot be too easy (the viruses’ evolutionary strategy is based on subversion), or so difficult that it requires a lottery of events. With this in mind, the difficulty associated with detecting ICP0 during reactivation should not be terribly surprising. Even in the explant co-cultivation model, less than one percent of latently infected neurons reactivate. The dramatic decreases in LAT abundance reported here clearly occur on a much greater scale than this! I believe that LAT directs the first step of
the reactivation process, and while it is important, it is not absolute. The mechanism controlling decreased LAT abundance serves as a global “safety” to provide specific neurons the opportunity to fire. More stringent downstream factors (neuronal phenotype, transcription factor profile, latent genome copy number etc.) then decide the viruses’ ultimate fate.

A number of interesting models have been invoked to explain the actual mechanism by which LAT functions during latency and reactivation. These include roles as a functional RNA to potentiate genome silencing (similar to XIST’s inactivation of X chromosomes), or as a ribozyme or RNAi to selectively degrade unwelcome lytic transcripts during latency. Others propose that transcription from this region maintains a window of accessibility in an otherwise highly chromatinized molecule to allow select transcription factors a place to initiate reactivation. Evidence has also suggested that LAT’s reactivation critical region (RCR) contributes a bi-modal enhancer that alternately modulates lytic (most likely ICP0) and latent (LAT) promoters. While promoter mutants, and in vitro correlations with ICER induction, indicate transcription level LAT regulation, the sudden and dramatic decreases in LAT abundance cannot be solely explained by promoter activity. The fact that the stable 2.0 kb intron’s (24 hour half-life) abundance also decreases, indicates that specific factors (such as RNAi) are actively facilitating this degradation. The most recent and compelling data from our laboratory indicates that specific histone modifications play an important role in establishing chromatin domains that render certain regions transcriptionally active (euchromatin) and others inactive (heterochromatin). Preliminary analysis of latent genomes indicates that such a boundary resides around the LAT region, with acetylated histones associated with
the LAT promoter, and methylated histones associated with nearby lytic gene promoters. If the RCR acts an enhancer capable of activating ICP0 during reactivation, chromatin boundaries might prevent this from occurring during latency. Our co-cultivation analysis will have, therefore, identified the window during which chromatin boundaries are absolved. Results (from our lab and others) indicate that the acetylation profile of histone tails in the LAT region change during the early hours of reactivation. Nancy Sawtell and Rick Thompson (University of Cincinnati) have recently reported that the LAT promoter becomes more highly associated with acetylated histones following explant co-cultivation of murine trigeminal ganglia. I believe that continued transcription of the LAT locus during latency allows the RNA polymerase to maintain the proper chromatin state. Transcriptional repression of the LAT locus would allow insulator boundaries to restructure so that the RCR enhancer could activate lytic genes. This work has already begun, but requires a more thorough understanding of histone profiles during latency before comparisons to reactivation can be made. A fifth LAT-transgenic co-cultivation experiment was performed to determine if decreased LAT abundance correlated with the promoter’s transition from acetylated to methylated histones. While a significant decrease in LAT cDNA levels was not observed at either time point (2 or 4 hours), an approximate two-fold reduction of acetylated histone-4 (histone 4 acetylated at lysine 9) was detected by two hours. While other results in our laboratory have shown more dramatic effects, two-fold differences determined by Real-Time PCR are considered reliable in the chromatin field.
Figure 3.1. 17syn+ co-cultivation experiment number one.

Figure 3.2. 17syn+ co-cultivation experiment number two.
Figure 3.3. KOS co-cultivation experiment number one.

Figure 3.4. KOS co-cultivation experiment number two.
Figure 3.5. Standard PCR analysis of 17syn+ co-cultivation experiment one and two.

Figure 3.6. LAT-Transgenic co-cultivation experiment number one.
Figure 3.7. LAT-Transgenic co-cultivation experiment number two.

Figure 3.8. LAT-Transgenic co-cultivation experiment number three.
Figure 3.9. LAT-Transgenic co-cultivation experiment number four.

Figure 3.10. Standard PCR analysis of LAT-Transgenic experiments 1-4.
Figure 3.11. Detection of ICP0 and ICP4 from 17syn+ experiment number one.

Figure 3.12. Detection of ICP0 and ICP4 from 17ΔPst and KOS infected mice.

Figure 3.13. Levels of establishment from ICP0(-), ICP4(-) and KOS viruses.
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BIOGRAPHICAL SKETCH

Jerome E. O’Neil was born on January 5, 1972, in Pittsfield, Massachusetts. He attended the University of New Hampshire, graduating with a Bachelor of Science degree in microbiology in 1994. He worked as a technician in the laboratory of Dr. Edmund Gosselin at Albany Medical College from 1995 to 1996. He then pursued his doctoral degree in the laboratory of Dr. David C. Bloom, spending his first 3 years at Arizona State University before transferring with his mentor to the University of Florida. After graduation he plans to attend Albany Law School.
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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