AN IN VITRO APPROACH TO THE MOLECULAR BASIS OF HERPES SIMPLEX VIRUS TYPE-1 LATENCY

By

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To my friends and family – for your support, examples, and encouragement

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A burgeoning scientist is never created in a vacuum – there are always faces behind the curtain. This is the case not only during time in the graduate program, the lab, or the classroom, but throughout the life of the scientist; indeed, before he even understands that science is to become his passion. In that spirit, I would like to use this section of my dissertation to acknowledge the educators, friends, and family who have set me on this path, assisted along the way, and are still here to offer congratulations.

First, I would like to thank the educators who are directly responsible for kindling my love of science. The first man in who fits this description was a general science teacher at my middle school, Mr. White. In a write-up of a routine titration experiment, complete with pink phenolphthaline and all, I remarked that this experiment was very cool, and asked if I would have a chance to do more things like it. Mr. White's response, "Stay in science", was aptly prophetic. Then, in high school, while I was excelling at multiple disciplines, it was a biologist named Dr. Barker who taught senior honors biology that focused my efforts in this field. His passion for molecular biology was evident, and infectious, and he certainly instilled me with the work ethic necessary for advancing in this field with his daily pop-quizzes, just to make sure everyone had gone over the topics that we discussed the previous day. And recently, it was my undergraduate advisor, Dr. Craig Tepper, whose enthusiasm and frank discussions with me first allowed me to identify that pursuing molecular biology as a career was a possibility. His advice on graduate programs, design of experiments, and critical thinking during data analysis, will never be forgotten.

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my short rotation through his lab, Dave would discuss ways to optimize ligation and transformation experiments, and offer encouragement when they inevitably failed. His enthusiasm for herpesvirology was immediately evident, and definitely passed on to me, so joining his lab was one of the easiest decisions I have ever made, and I am grateful that he was interested in having me be a part of his research program. Throughout my entire career as a graduate student, Dave offered practical career advice, discussed science concepts with me, and has assisted with the design of experiments. It is plain to me that whatever success I enjoy as a scientist can be traced back to my time in the lab of Dr. David Bloom, and will be in no small part attributable to his guidance.

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LIST OF ABBREVIATIONS

17*syn*+	17 syncitia-positive strain
17ΔPst	17 deletion mutant of Pst fragment (202bp)
APRT	Adenine Phosphoribosyltransferase
Bp	Base Pairs
ChIP	Chromatin Immmuno-Precipitation
CS	Calf Serum
DZNep	3-Deazinoplanocin-A
EZH2	Enhancer of Zeste Homolog 2
FUDR	Floxuridine
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
gC	Glycoprotein C
GDNF	Glial-Derived Neurotrophic Factor
GFP	Green Fluorescent Protein
H3K4,K9ac	Histone 3, Lysine 4,9, acetylation
H3K4me3	Histone 3, Lysine 4, tri-methylation
HSV-1	Herpes Simplex Virus Type-1
ICP0	Infected Cell Protein 0
ICP4	Infected Cell Protein 4
IgG	Immunoglobin G
LacZ	Lactase Z Operon
LAT	Latency-Associated Transcript
lncRNA	Long, non-coding Ribonucleic Acid
miRNA	Micro Ribonucleic Acid
MyoD1	Myogenic Differentiation 1

NBA-B	Neurobasal-A Basic Media
NBA-C	Neurobasal-A Complete Media
NBA-V	Neurobasal-A Virus Media
NGF	Nerve Growth Factor
PCR	Polymerase Chain Reaction
Pen/Strep	Penicillin/Streptomycin antibiotics
Pfu/N	Plaque-Forming Units per Neuron
PRC1	Polycomb Repressive Complex 1
PRC2	Polycomb Repressive Complex 2
qPCR	Quantitative Polymerase Chain Reaction
RCF	Relative Centrifugal Force
RS Cells	Rabbit Skin Cells
RT	Reverse Transcriptase
TG	Trigeminal Ganglia
TK	Thymidine Kinase
UpHoxA5	Upstream of Hox A5 Gene

Abstract of Dissertation Presented to the Graduate School
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AN IN VITRO APPROACH TO THE MOLECULAR BASIS OF HERPES SIMPLEX VIRUS TYPE-1 LATENCY

By

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Herpes Simplex Virus type 1 (HSV-1) initially infects mucosal epithelial cells, where the virus will undergo multiple rounds of lytic infection to produce progeny virus. In addition, HSV-1 genomes will establish a life-long latent infection in sensory neurons of the host. During latency, no viral proteins are translated, and viral transcription is relegated to a single locus, from which the major transcript is the non-coding latency-associated transcript (LAT). The role of the LAT in the establishment of latency is not fully understood, but it may be closely related to epigenetic remodeling of HSV-1 genomes.

Recently, a cell culture model using adult primary sensory neurons harvested from adult mouse trigeminal ganglia (TG) was developed and characterized to effectively model latency *in vitro*. These cultured neurons harbor a quiescent HSV-1 infection which very closely mimics latency *in vivo*. The overall focus of this dissertation was to 1) further characterize the HSV-1 quiescent infection in this culture system and to determine if the epigenetic basis of transcriptional repression mirror that of *in vivo* systems and 2) use this TG culture system to identify the effect of altering the amount of initial infectious virus on the establishment of latency.

We found that infecting cultures with higher viral doses resulted in a number of phenotypes, including differential gene expression, DNA levels, and even epigenetic profiles. Viral transcript levels increased up to 10-fold as a function of initial inoculum, at both time points. Correspondingly, post-translational modifications of chromatin were also dependent on initial inoculum: H3K4me3 levels decreased up to 5 fold at higher MOI, and H3K27me3 levels increased up to 10 fold. Strikingly, when cultures infected with 17+ and LAT promoter deletion 17ΔPst were compared we found that these phenotypes were LAT-dependent, with 17ΔPst exhibiting no significant change in either transcript levels or heterochromatic marks as a function of MOI. This suggests that the LAT plays some role in these dose-dependent phenotypes. These observations elucidate the importance of the initial inoculum of HSV-1 on the establishment and maintenance of latency, and confirm that the TG system provides a suitable model for investigating the mechanisms involved.

CHAPTER 1
INTRODUCTION

Herpesviruses

The *Herpesvirus* family is comprised of large double-stranded DNA viruses which infect a wide range of hosts, including humans. They are characterized by their ability to establish a life-long latent infection in their host, from which periodic reactivation allows for successful propagation of the virus. The latent virus is generally dormant only in a specific cell type, depending on the particular virus, but the genomes do not integrate into the host genome but rather exist as circular episomes.

Human herpesviruses are categorized into three subfamilies: alpha, beta, and gamma. The subfamilies are differentiated based on a number of factors including cellular tropism, site of latency, genome architecture, and length of replication cycle. Members of the alphaherpesvirus subfamily generally infect epithelial cells and establish latency in sensory neurons. Betaherpesviruses establish latency in a number of different cell types, including cells of the myeloid lineage, secretory glands, and lymphoreticular cells. Finally, gammaherpesviruses establish latency primarily in lymphocytes. This dissertation will focus on the latency of human herpes simplex virus type I (HSV-1), a member of the alphaherpesvirus subfamily.

Herpes Simplex Virus Type-1

HSV-1 is a member of the herpesviridae family which causes lesions in mucosal epithelia, commonly known as cold sores or fever blisters, causing the disease Herpes Labialis. Generally, HSV-1 infects the orofacial region, but infection of the genitals can also result in lesions there as well. In addition to infection of the mouth or lips, other mucosal epithelial regions, such as the eye, may also be infected. In fact, recurrent

reactivation of HSV-1 in the eye can result in progressive corneal scarring and eventual blindness, a disease known as Herpes Stromal Keratitis. This disease is the leading cause of infectious blindness in the United States (67). While anti-viral drugs do exist for treatment of the acute infection, these drugs are not capable of eliminating the latent reservoir, so recurrent disease is very likely. Infection with HSV-1 is very common in the population; nearly 70% of Americans older than 12 are seropositive for the virus, and by the age of 70, this percentage increases to 90% (56, 57, 67).

The structure of the virus is fairly well understood and is shown in Figure 1-1. The viral capsid is icosahedral and protects the 152Kbp linear genome. Attached to the capsid is a proteinaceous structure known as the tegument, which is composed of a number of viral proteins important for a productive infection and virion integrity (53). HSV-1 is encapsulated by a membrane-derived envelope which contains a dozen glycoproteins used for binding and entering cells through membrane fusion (10) or endocytosis (33).

Lytic Infection

The acute infection of the virus has been well studied in both cell-culture and animal models. Upon entry into a cell, the viral capsid is transported to the nucleus via the microtubule network using the dynein motor protein (58). Once in the nucleus, viral DNA associates with histone proteins, reorganizes the host cell nuclear structures, and begins a cascade of viral gene expression (31), which can be seen in the left part of Figure 1-2. The viral protein 16 (VP16) is a tegument protein, so it is delivered with the viral capsid, and it is a transactivator of a number of immediate early (IE) HSV-1 transcripts (6). VP16 recruits two cellular proteins, host cell factor (HCF) and the transcription factor Oct-1. This complex of viral and cellular proteins binds to promoters

of immediate early (IE) genes through a conserved TAATGARAT sequence in the promoters of these genes. The VP16 activation domain then stimulates transcription of the IE genes ICP0, ICP4, ICP22, ICP27, and ICP47. ICP4 in particular is required for subsequent viral transcription and replication. These IE genes, some of them transactivators themselves, are translated and enhance the expression of early (E) genes, many of which are involved in viral DNA synthesis. Notable early genes include the viral polymerase (U_L30), the single-stranded DNA binding protein (ICP8), and thymidine kinase (TK), the latter of which is involved in maintaining the pools of nucleotides during DNA replication. The synthesis of viral DNA promotes the transcription of the late (L) genes, which are primarily structural and involved in capsid assembly (32). This gene cascade results in new virus assembly, and the eventual budding of progeny virus from the infected cell.

Latent Infection

Like all herpesviruses, HSV-1 is able to persist in a state of latency. HSV-1 establishes latency in sensory neurons (60). HSV-1 virions can either replicate in epithelial cells, and then spread to the termini of the axons which innervate the site of the initial infection, or can directly infect the nerve termini at the epithelia surface. Through retrograde axonal transport, the capsid of the virion is transported to the neuronal cell body, and the HSV-1 DNA enters into the nucleus (46). Here, the virus infects distinct populations of neurons, producing lytic transcripts in some, and producing only one group of transcripts in other: the latency associated transcripts (LATs), (47, 61), as seen in the right part of Figure 1-2. The mechanisms involved in directing the viral infection towards an acute or a latent infection in sensory neurons is not well-understood, but it is likely due a sensory ganglion being comprised of

populations of disparate neurons—some of which are supportive of a latent infection, and some of which are supportive of an acute infection (47).

The LAT is transcribed from the two repeat regions flanking the Unique Long (U_L) region of the HSV-1 genome (16) and is antisense to ICP0, γ34.5, and also the 3'-most end of the ICP4 sequence. The latency-associated promoter 1 (LAP1) is upstream of the transcription start site, and an enhancer is located just downstream of the transcriptional start site. This enhancer allows for long term expression of the LAT during latency (3, 45). In this region, there are also CTCF binding sites and insulators upstream and downstream of the LAP1, which possibly serve to protect the LAT region from heterochromatic silencing or silence neighboring enhancers (2). The LAT locus is shown in detail in Figure 1-3. In addition to the LAT, there are other viral transcripts which are currently under investigation by our lab in this region, the transcript anti-sense to LAT (TAL), and the antisense transcript antisense to LAT (ATAL). While the functions of these transcripts are still unknown, it is clear that the TAL is transcribed mainly during the acute infection and the ATAL during latency.

The LAT transcriptional unit produces several different transcripts via alternative splicing. The full length transcript is 8.3Kb and accumulates at low levels in latent neurons. In addition, 1.5Kb and 2.0Kb introns processed from the full-length transcript are very stable due to the formation of a lariat structure, and they accumulate in the nucleus at high levels (22). No reproducible studies have found a protein encoded by the LAT transcript (20). Regardless, LAT-deletion mutants show a number of phenotypes, depending on the size of the deletion, animal model, and route of infection. For example, a 348bp deletion in the 5' end of LAT is associated with decreased levels

of reactivation in the mouse (5). Further, it is important to mention that the 17ΔPst mutant, a deletion of 202bp in the LAT core promoter region, reduces transcription levels of the LAT, the TAL, and the ATAL (19 and Giordani and Neeld et al. *in prep*). Other studies have shown that LAT-negative mutants show an increased level of lytic gene expression in sensory neurons during both the acute (25) and latent infection (7). Additionally, the LAT is thought to have anti-apoptotic activities, as evidenced by the observation that transfections with a plasmid with nt 301-2659 of the LAT can protect cells from apoptosis (51). It is clear that the LAT likely has a wide diversity of activities, although these effects have not be fully characterized or investigated.

It is not known whether the LAT transcript plays some direct role in latency, or whether transcription from the LAT locus somehow triggers repression of the lytic genes. There are a number of hypotheses regarding the role that the LAT plays in the establishment of latency. As noted previously, the LAT does not encode a protein, which leaves two roles for the LAT: the mRNA itself plays a role, or the very act of transcription from this locus does. As an example of the former possibility, the LAT may be acting as a pre-miRNA, targeting the ICP4 and ICP0 transcripts which are antisense to LAT. It has been shown that LAT- mutants show similar levels of ICP0 (8), so it is unlikely that LAT negatively regulates ICP0 via miRNA interactions. However, in a closely related system, RNA associating with Argonaute-1 (a component of the RISC complex involved in miRNA) was shown to induce transcriptional silencing in human cells (37). In addition, it is possible that the LAT is acting to help “paint” the lytic genes with heterochromatic marks, as is seen in mammalian X-chromosome inactivation (reviewed in 35). One attractive feature of this hypothesis is that x-chromosome

inactivation is known to be maintained by polycomb group proteins, which a member of our lab recently identified bound to the HSV-1 genome (43).

Animal Models of HSV-1 Infection

Traditionally, HSV-1 infection has been studied in the mouse and the rabbit, both of which are non-native hosts. Both animals can harbor a latent infection in sensory ganglia, but there are distinct phenotypes depending on the animal, the strain of HSV-1, and the route of infection. For example, one system used in our lab is the rabbit eye model. In this model, rabbits are infected by applying virus to the eye following corneal scarification, the virus replicates acutely in the corneal epithelial cells and establishes a latent HSV-1 infection in the trigeminal ganglia. Due to the nature of this infection, relatively high titers of virus must be applied to the eyes, but it is difficult to efficiently deliver the same amount of virus because of the surface of the eye and the blinking of the rabbit. This model is particularly effective for investigating reactivation, as treatment with epinephrine will result in reactivation from latency in nearly 100% of the rabbit eyes, and the rabbit is the only model in which reactivating virus can be detected at the periphery (17).

By contrast, in the mouse footpad model, rear footpads are scarified and infected with various virus titers, the virus replicates within the epithelium of the foot and is transported to the dorsal root ganglia (DRG), where it establishes a latent infection. The viral titer used in this infection is highly dependent on the virus strain. As an example, the wild type strain 17*syn*+ is applied at 500pfu per foot, and this routinely results in an LD_{50} whereas infection with the less-virulent KOS strain can be performed with viral titers upwards of 10^7pfu per foot, and will still not result in death of the animal. Reactivation from the latent infection cannot be stimulated as easily as in the rabbit eye

model, but models do exist for simulating reactivation. One method involves heat-shocking the mice in a 43° water bath, but reactivation from this method is fairly inefficient (54). Other methods rely on excising the latently-infected ganglia and placing them in organ culture (15), in some cases with additional reactivation stimuli, such as α-NGF antibody (21). Reactivation in these organ cultures occurs at a much higher frequency than is observed in the *in vivo* reactivation model, especially when α-NGF antibody is used in the cultures. Ganglion organ cultures are specifically useful for analyzing reactivation of the virus due to the fact that a widely-accepted cell culture model for HSV1 latency has yet to be developed. Hence, in order to test the effect that biochemical compounds may play in the establishment of latency (and reactivation from that latency), these ex-plant models have been the most accepted method.

Cell Culture Models of HSV-1

Historically, cell culture systems for HSV-1 latency have been poorly received due to criticisms regarding how well these models recapitulated the latent state *in vivo*. One of the most common cell culture model systems for HSV-1 latency is the use of normal human diploid fibroblasts (NHDF). A number of studies have shown that NHDF are capable of harboring a “quiescent” infection of HSV-1. This is likely due, in part, to cellular factors in NHDFs that prevent viral replication (28). However, many of these culture systems also employ high temperatures to the infected cells (upwards of 42ºC), as well as chemical viral inhibitors (11, 55). Still other studies used viral mutants deficient in IE genes to force the HSV-1 genomes into latency (34). Aside from infecting a cell type not known to harbor a latent HSV-1 infection, these NHDF systems are quite artificial, using elevated heat levels, serum-starved cells, and viral inhibitors to establish

this state of quiescence, so much of the field has rebuked their usefulness as a faithful model of latency.

A similar model from the 1980s involved infecting a T lymphoblastoid cell line called CEM in the presence of concanavalin A, another inhibitor (27). This cell line was utilized before the LAT was first discovered in sensory neurons and it was thought that lymphoplastoid cells were largely non-permissive for the acute infection. Since this cell type was non-pemissive for the lytic portion of the virus life cycle, it was hypothesized that HSV-1 virions which infected these cells would be shunted towards a latent infection. However, as more was learned about the relevant sites of both HSV-1 acute replication and latency, and the LAT was discovered to accumulate largely in sensory neurons, this particular model was deemed too artificial by the community at large Hence, these cell lines are no longer seen as an appropriate *in vitro* model of HSV-1 latency.

One model that enjoyed some popularity was the rat pheochromocytoma (PC12) cell model (1, 12). PC12 cells are cells derived from rat adrenal gland tumors. They are immortalized and contain various chromosomal abnormalities. However, upon the addition of NGF, they differentiate into neuronal-like cells, send out processes, and present many common cell-surface markers of sensory neurons, likely due to the fact that the adrenal gland is a neural crest-derived tissue, and hence it responds well to nerve growth factors. Once differentiated, the cells can be infected with HSV-1 in the presence of acyclovir, to suppress lytic replication. The acyclovir can then be removed, and the PC12 cells persist–and harbor “quiescent” HSV-1 genomes. In addition to reactivating spontaneously at a fairly low rate, these genomes can be reactivated in

response to a number of different inducers, including heat stress, forskolin or protein kinase A treatment (13, 14). It has been shown that the quiescent infection in PC12 cells share a number of features of latency *in vivo* in sensory neurons: the genomes are maintained as circular episomes and, there are very low levels of lytic viral transcription and high levels of LAT transcription (12, 68). Infection of this cell line shows a remarkable semblance to HSV-1 latency *in vivo*, suggesting that the mechanism of achieving latency in these cells may be similar to the events which happen in the animal. While these cells are still used to study HSV-1 latency, it appears that the nature of the NGF-treated PC12 cells (being essentially growth-factor-treated clonal cancer cells of a non-neuronal cell type) has caused many in the field to look to other, less artificial models.

A major issue that the HSV-1 field has with all of the previous *in vitro* systems is the degree of artificiality associated with them, either through use of attenuated viruses, or infection of non-native cells, so with that in mind, a model using dissociated superior cervical ganglia (SCG) from neonatal rats was developed (39). This system collects SCG from E21 rat pups, dissociates the neurons from connective tissues, and plates the neurons in 96-well plates. After cultures have matured, they can be infected with HSV-1 in the presence of acyclovir (in the case of high MOI), or without acyclovir (if MOI is very low). The use of acyclovir prevents the lytic infection from progressing throughout the culture and directs the infection towards a path of latency. These cultures are therefore able to harbor quiescent or latent viral genomes for short periods (~2-4 weeks), and these genomes can be reactivated by removing acyclovir from the media.

The rat SCG culture system is fairly novel (having been adapted from an older technique only in the last few years), and has been used to elucidate many mechanisms involved in the very early stages of reactivation from latency (66). However, the field still has some issues with the techniques used to produce a quiescent infection in these cultures. First, the fact that these cultures are comprised of neonatal SCG neurons raises two issues: 1) neonatal neurons are not fully developed and as such lack important surface markers of mature neurons and 2) SCG neurons are autonomic neurons, not sensory neurons. It is known that latency only occurs in a sub-population of neurons, and it is very likely that fully mature sensory neurons may be a requirement for latency *in vivo.* The fact that these cultures lack both of these characteristics has raised concerns. Additionally, the use of acyclovir to establish latency is a common target for detractors, and it is no different for this system. It is theorized that the type of latency that results due to the presence of acyclovir is significantly different from the latency that occurs naturally. Additionally, the fact that the reactivation stimulus for this system is simply removal of the acyclovir invites criticisms regarding the nature of the latency and reactivation that is present in these cultures. Nonetheless, to date, this system has been well-received and appears to be promising, but for the studies in this dissertation, I have chosen to pursue a different course.

Another cell culture model which could provide useful insight into HSV latency is the culture of primary adult murine trigeminal ganglia neurons (TG) developed by Todd Margolis and Andrea Bertke (47). These cells are harvested from adult mice, dissociated in a single-cell suspension, and infected with HSV-1 (48). These infections are performed in the absence of acyclovir, and generally rely on the natural

heterogeneity of sensory neurons in the TG to result in latent infections of HSV-1. Unpublished data has shown that the cells which survive the initial infection harbor latent HSV-1 genomes, for up to four weeks post-infection. In addition, the cells which harbor the HSV-1 genomes in this system show the same A5 and SSEA surface markers as Margolis previously reported are present on latently-infected neurons *in vivo*. Also, the KH10-positive neurons are permissive for the acute infection in this culture system, which replicates the observations *in vivo*. Together, these observations make the TG culture an especially encouraging model system (4).

This culture system would allow for the same manipulations as the PC-12 and SCG systems, but is more biologically similar to the cells which are latently infected *in vivo*. Hence, we believe that analyses on these cultures will allow us to establish fundamental parallels between the primary TG cell culture system and the in vivo latent infection and allow more faithful analysis of the signaling processes, some of which may well be cell-type specific that will allow us to study factors involved in the neuronal control of HSV-1 latency and reactivation.

As previously mentioned, the different populations of latently infected cells have been a confounding issue in HSV biology. And within the infected animal models, which are currently the only trusted models for studying HSV latency, there are no current solutions to these problems. Hence, it is of critical importance to the field that cell culture systems be developed that faithfully exhibit the characteristics of the *in vivo* latent infection, especially for studying the more subtle characteristics of HSV-1 latency like epigenetic modifications on chromatin.

Epigenetics and Chromatin

Epigenetics studies the regulation of genes through mechanisms not related to primary DNA sequences, but instead due to modification of the bases, binding of proteins to the DNA and the methods of packaging DNA into the nucleus of a cell. A single human cell is estimated to have 1.8m of DNA packaged into the nucleus, so it is clear that significant condensation of the DNA is required. The first step of this condensation occurs when the naked DNA associates with the core nucleosome, which is an octomer composed the histone proteins H2A, H2B, H3 and H4 (two copies of each histone). This is the standard “beads-on-a-string” structure of chromatin, described decades ago by visualizing partially denatured DNA by electron microscopes (40). The beads-on-a-string structure is further condensed into a 30nm fiber, although the precise organization of this fiber remains controversial (23). There is additional condensation beyond the 30nm fiber, but the precise structure that the chromatin takes at this level and the mechanisms behind its organization are poorly understood at this time.

One important consequence for the packaging of DNA into chromatin is that tightly condensed DNA cannot be transcribed as easily as free DNA. Hence, a critical dogma of epigenetics is that the condensation of the chromatin is an important regulatory element of the cell. In addition, the positioning of the histones on the DNA is an important question, as areas with less histones may be more readily transcribed. The nucleosome organization of HSV-1 has been extensively studied during the lytic infection (36, 41, 42), but not with high resolution during the latent infection, so this is one question that we hope to address.

The histone proteins H3 and H4 each contain a carboxy and amino tail and these tails have amino acids which may be post-translationally modified by various enzymes.

Post-translational modifications confer differing levels of transcriptional permissiveness to the surrounding DNA sequences. For example, some of the residues are readily acetylated (histone 3, lysine 4, or H3K4ac is one example), which results in transcriptionally permissive chromatin, or euchromatin. There are many other marks associated with this permissive euchromatin, such as H3K4me2, H3K4me3, H3K9,14ac, and others. Throughout this study, we will focus primarily on the H3K4me3 mark, which is indicative not only of a less-condensed euchromatin state, but also of recently transcribed DNA. Other residues, such as histone 3 lysine 27 trimethylation (H3k27me3) result in more condensed chromatin, known as heterochromatin, and hence reduce transcription of surrounding genes. A number of other post-transcriptional modifications (PTMs) exist, and specific functions of each are still being elucidated.

Epigenetics of HSV-1

A central question of Herpesvirus biology involves the mechanisms which allow the virus to inhibit lytic gene expression in order to achieve latency, as well as the mechanisms involved in reversing this process during reactivation. Much of the early work on the epigenetics of herpesviruses was performed in systems using gammaherpesviruses latency as a model (reviewed in 44), but this work will focus on the epigenetic control of Herpes Simplex. The literature has focused on the LATs, but no definitive answers yet exist. One hypothesis posits that the LATs act in an anti-sense nature on the ICP0 and ICP4 genes, and regulate their expression in some way, likely akin to small interfering RNAs (siRNAs) or microRNAs (miRNAs) (52) . Recent studies have found a number of miRNA species encoded from the LAT region, leading to the hypothesis that the LATs are primary microRNA species which regulate ICP0,

ICP4, and γ34.5 at the transcript level (65), although study of these miRNAs in the context of the animal model are in their infancy, recent reports have indicated that mutants in some of these miRNAs have an *in-vivo* phenotype (24).

Another model hypothesizes that the LAT is responsible for genome-wide epigenetic changes which result in silencing of lytic genes. One mechanism for effecting these changes is suggested by data from a previous member of the lab who determined that the LAT binds to members of the polycomb repressive complex-2, possibly acting to sequester cellular levels of the complex, and ultimately resulting in a less-repressed HSV-1 genome (Watson et al. *in prep*). In previous studies, our lab has shown that latency is accompanied by a number of epigenetic changes in the HSV-1 genome, and that the deletion of the LAP 1 has definite effects on these changes (2, 41, 43). Taken together, these data suggest that epigenetic mechanisms behind the establishment of latency are critical, and we will focus on these epigenetic phenotypes of HSV-1, and their dynamics throughout the viral life cycle in the present study.

The epigenetic characteristics of the HSV-1 genome have been previously reviewed (38), but several points are worth noting in detail, as seen in Figure 1-4. First, it is important to note that in the productive lytic infection, which takes place in epithelial or other non-neuronal cell types, euchromatic marks are deposited on histones near the lytic genes (36). In fact, previous studies have found that VP16 actively reduces the amount of histone H3 on IE genes (29). Further, ICP0 has been found to block histone deacetylase (HDAC) activity, which prevents the acetylation marks from being removed from lytic promoters (30). These actions result in HSV genomes which are poised for high levels of lytic gene transcription.

In stark contrast, when a neuronal cell is infected, there are two disparate outcomes. Using a LacZ reporter virus, Margolis et al. were able to show that there were two distinct populations of neurons which were infected: those which produced viral proteins at high levels, but LAT at low levels, and those which produced high levels of LAT, but virtually no viral proteins (47). For some neurons, a lytic infection will progress as before, which may spread to other neurons within the ganglion, resulting in long-lasting asynchronous infection, which could take 2-3 weeks to completely clear. However, in neurons which express the A5 and SSEA-3 cell-surface proteins, the lytic genes become associated with heterochromatic marks and the LAT region becomes associated with euchromatin and (9 and 41). In particular, Cliffe et al. demonstrated that a wide array of lytic genes are associated with histones bearing the repressive H3K9me2 mark, whereas Kubat et al. report enrichment of acetylation on histone H3 only at the LAT promoter and enhancer. This chromatin profile clearly reflects the transcriptional profile of a latent viral genome, where the LAT is the only highly-produced transcript. At this time, it is unclear how this chromatin profile is established or maintained in latently infected neurons, although there are some clues.

At the heart of the question is the LAT. The LAT is driven by a promoter with many cellular properties, and is also believed to be expressed at low levels with late gene kinetics following initial lytic transcription in the neuron (22). At this point, the LAT intron accumulates at high levels in neurons destined to harbor a latent infection, and the chromatin profile of a latent cell is established and maintained. Amelio et al. identified chromatin boundary elements, called insulators, which flank the LAT locus (2). It has been proposed that these insulators protect the euchromatin of the LAT locus

from being converted to the heterochromatin of the surrounding genes, and vice-versa. This provides a reasonable hypothesis regarding the maintenance of the chromatin profile, but speaks nothing towards its initial establishment.

Of critical importance to the question regarding the function of the LAT is the observation that not all neurons infected with HSV express the LAT, which leads to the notion that there are at least several distinct populations of neurons which harbor a latent infection, and/or at least several different distinct profiles of latent transcription. Interestingly, recent findings from our lab indicate the LAT locus displays both the latent chromatin mark H3K27me3, as well as the lytic mark H3K4me2. There are two interpretations of this observation. First, there could be two distinct populations of cells – some of which harbor HSV genomes which express LAT, and some of which harbor genomes which do not express LAT. Alternatively, due to the bivalent nature of LAT, one copy of LAT may have condensed chromatin, while the other copy has open chromatin. Unfortunately, it is technically difficult to isolate single-cells in vivo, so the analyses of these populations have been stunted.

Complicating this question is the fact that until recently there were no accepted cell-culture systems which appropriately reproduce the latent state of HSV-1, as previousy mentioned. This makes *in vitro* studies, such as siRNA knock-downs, fluorescence imaging, transfections, and treatment with small-molecule inhibitors very difficult to perform on the latent virus. With an acceptable *in vitro* cell culture system that faithfully reproduces the latent state of the virus, there is opportunity for great insight into the issue of distinct LAT populations and the roles of the LAT. In a cell culture system, it is possible to introduce a LAT reporter virus into the system, and sort

the cells according to levels of LAT expression. Hence, analysis of only the cells with a particular expression pattern of LAT and lytic transcripts is possible, which could give clues towards the earliest stages leading to the establishment of latency.

Finally, it has become increasingly clear that different animal models often lead to confounding results with regards to latency. It appears that route of inoculation, animal model, and viral strain could all play an important role in the establishment of latency. Interestingly, the impact each of these factors may be explained in another way: they often result in different titers of virus being delivered in the system. When discussing the various animal models, we mentioned that footpad infections were more efficient than ocular infections, and that different titers of virus are used to infect these anatomical regions. This is also true for different viral strains, where a more virulent strain must be applied at a much lower titer than an avirulent strain. Hence, it is possible that the differences that have been attributed to viral strain or animal model may be more appropriately explained by different amounts of infecting virus, which could affect the course of the HSV-1 infection.

Aim of Dissertation

When HSV-1 infects sensory neurons, there are two possible outcomes: a productive, lytic infection, and a non-productive, latent infection. Previous models of HSV-1 latency have generally been *in vivo* animal models, and due to the use of different virus strains, routes of inoculation, and animals, the precise mechanisms governing the latent infection are not well understood. In this dissertation, we aim to characterize critical aspects of the TG culture system with regards to the quiescent infection present in these cultures. It is of great importance that the molecular aspects of latency are recapitulated by this *in vitro* system, as we want our model of latency to

be as faithful to the *in vivo* models as possible for our investigation into the hypothesis that the initial inoculum delivered to the neurons plays a critical role in the course of the viral infection. These differences can be indicated by differential levels of viral transcription and chromatin markers depending on the initial inoculum applied to the TG cultures. In addition, since previous experiments have determined that the LAT is involved in these mechanisms, we also aim to investigate whether any differences observed due to altering initial inoculums are LAT-dependent. Therefore, the ultimate goal of this dissertation is to use the TG culture system to carefully investigate the effect of different amounts of virus being delivered to neurons, and whether the LAT is important for any of these effects.

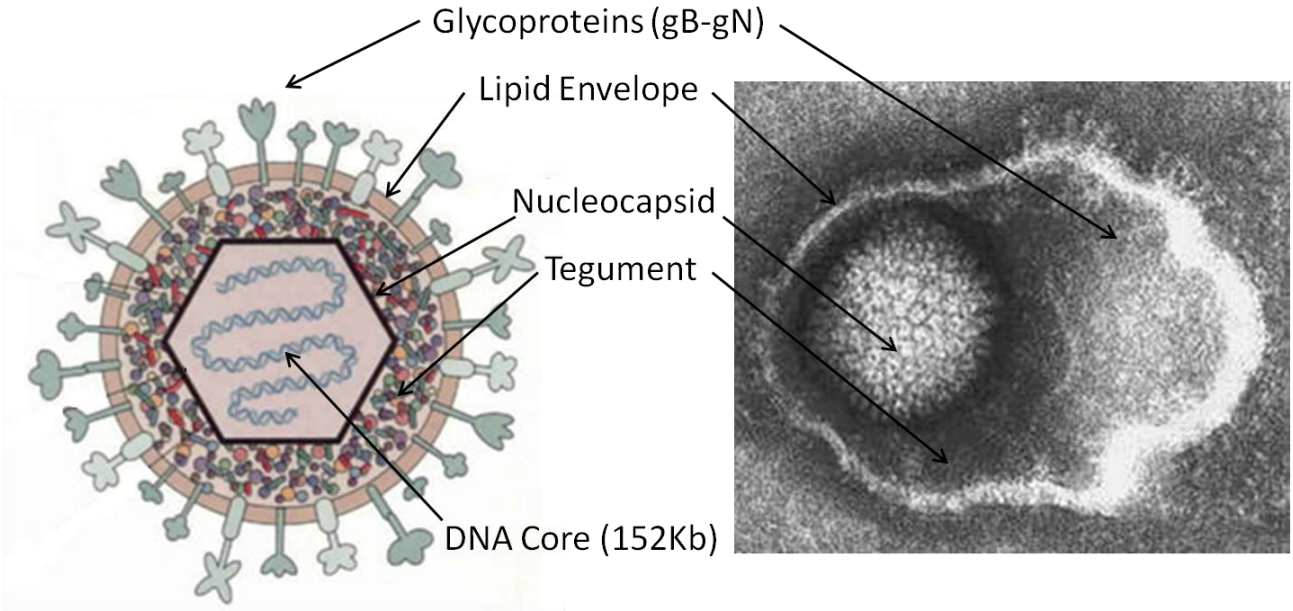


Figure 1-1. Diagram of the HSV-1 virion, both as an illustrative model (left) and electron micrograph (right). Critical components are indicated on each diagram. This figure was adapted from (59).

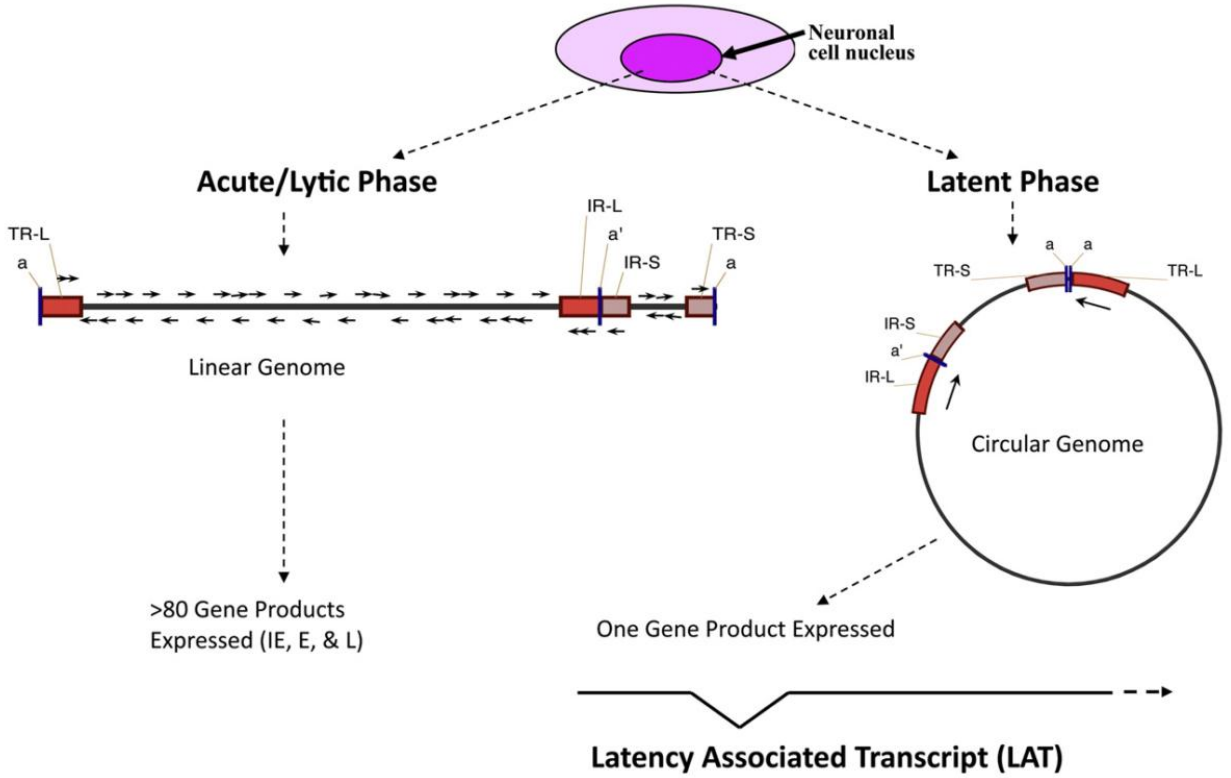


Figure 1-2. Viral lytic and latent phases of infection, with corresponding genome organization and transcriptional profiles. Upon infection of a neuron, HSV-1 can proceed down one of two paths of infection: the acute or lytic phase (left) or the latent phase (right). The acute phase is characterized by a linear genome from which the entirety of the viral transcriptional program is active, eventually producing progeny virus. By contrast, the latent phase is characterized by a circularized genome, from which on the latency associated transcripts are transcribed.

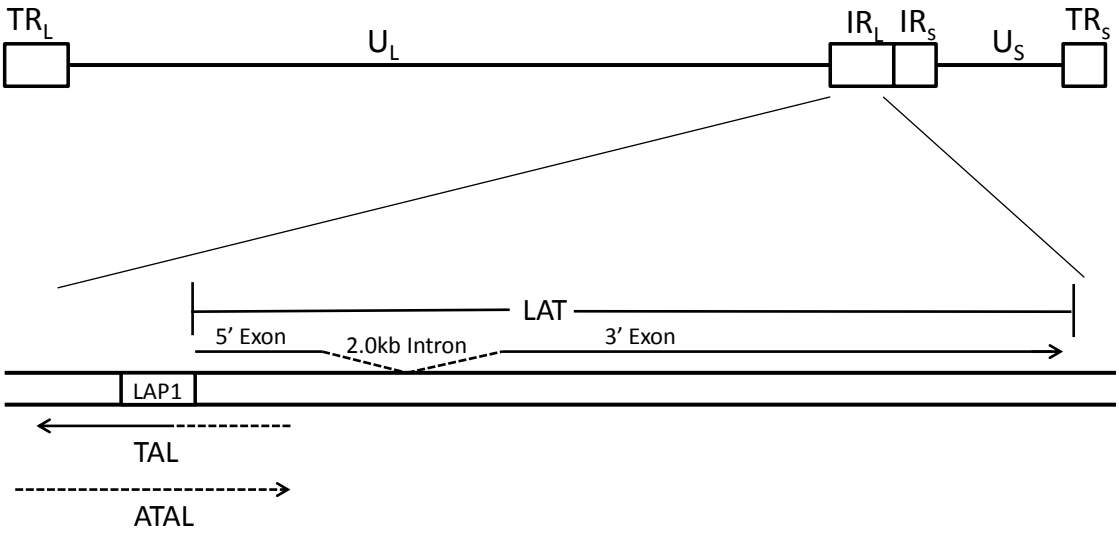


Figure 1-3. Map of the LAT locus including the LAT, TAL and ATAL transcripts. A gross diagram of the HSV-1 genome is shown at the top, with unique and repeat regions (both long and short) shown. The internal repeat long region is magnified to show the specific region from which the latency associated transcripts are transcribed. The LAT is shown with its intron spliced out (dotted line). The precise length of both the TAL and ATAL are not currently known, but dashed lines show likely positions of these transcripts.

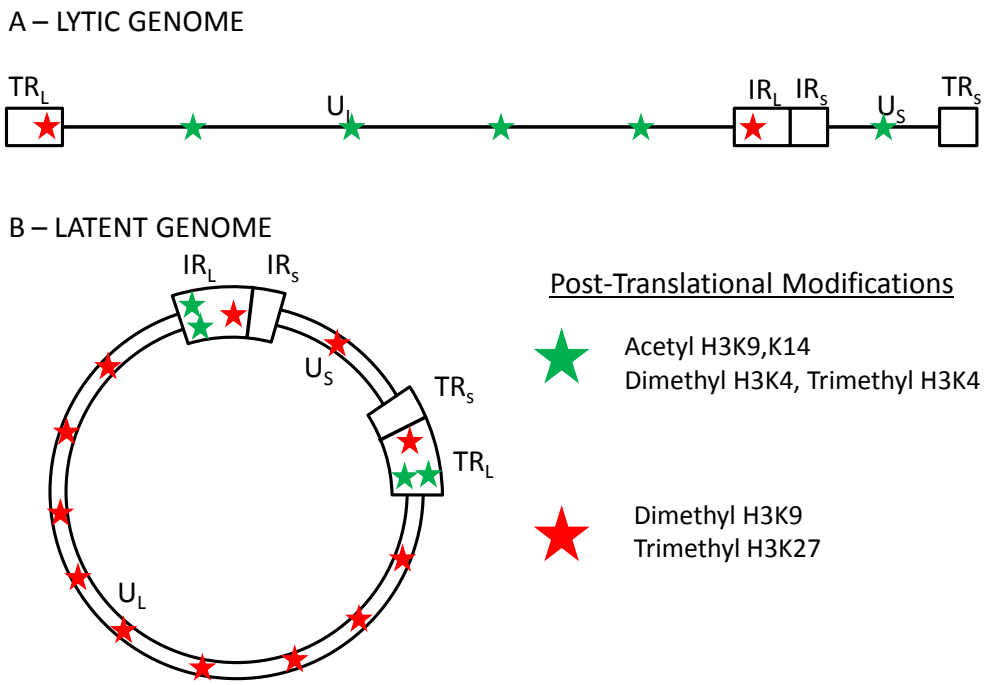


Figure 1-4. Depictions of the chromatin profile of the lytic (A) and latent (B) genome, as assayed by ChIP from animal models of HSV-1 infection. Euchromatic marks are denoted by green stars, and heterochromatic marks are denoted by red stars. During the lytic infection, most HSV-1 genes are in a euchromatic state, and during the latent infection, lytic genes are in heterochromatic state while the LAT locus is found to be euchromatic.

CHAPTER 2
MATERIALS AND METHODS

Viruses and Cells

Experiments will be performed with a variety of viruses. The wild type HSV-1 strain 17*syn*+ and LAT-deletion mutant 17ΔPst were obtained from low-passage stocks; 17syn+ was obtained from J. Stevens and 17ΔPst was constructed, as previously described, with a 202-bp deletion that removes the core LAT promoter and transcriptional start site (16). Reporter viruses were also used in some analyses. 17syn+PGK, has a copy of *E. coli.* LacZ under control of a murine phosphoglycerol kinase (PGK) promoter inserted at the glycoprotein C (gC) in the virus. Mouse PGK is a constitutive promoter, so this virus expresses lacZ with kinetics and strength of a late gene promoter, and hence is a strong indication of lytic gene transcription. After treating these cells with a fluorescent substrate for β-galactosidase, the cells expressing lacZ can be sorted with FACS into populations of cells which express lytic genes, and cells which do not. A second reporter virus, F22, expresses lacZ under control of the LAT promoter in the KOS virus. This virus expresses the lacZ in the context of a latent infection, and hence cells can be sorted into populations expressing the LAT, and those not expressing the LAT.

Low-passage rabbit skin (RS) cells were used for the propagation of virus and for plaque assays. RS cells were maintained in Minimal Essential Media (MEM) supplemented with 5% Calf Serum (CS) and antibiotics (250 U of penicillin/ml, 250µg of streptomycin/ml, and 292µg of L-glutamine/ml). Trigeminal Ganglia (TG) primary cultures were maintained in Neurobasal-A (NBA) media supplemented with 2% B27 (a proprietary serum-free supplement designed specifically for cultured neurons), 1%

Pen/Strep cocktail, 0.5mM L'Glutamine, 40μM FUDR,1.6μg of aphidicolin/50mL media and 10μg/mL of the growth factors NGF, GDNF, and neuturin. This media is referred to as Neurobasal-A Complete (NBA-C) media. A related media, Neurobasal-A Virus (NBA-V) media, is composed similarly, except the FUDR and aphidicolin are replaced by 200μL of pooled human IgG. Finally, a basic Neurobasal-A media eschews all growth factors and contains only 2% B27 and 1% pen/strep, which is referred to as Neurobasal-A Basic (NBA-B).

Preparation of Trigeminal Ganglia Primary Cultures

Adult murine primary neuronal cultures were prepared using a protocol modified from Bertke et al (4). Briefly, ten 6-8 week old ND4 Swiss/Webster mice were euthanized with isofluorane inhalation and perfused with calcium/magnesium free phosphate-buffered saline (CMF PBS). TGs are excised (see Figure 2-1 for details) and placed in NBA-B media on ice, transferred to a 15mL conical tube, and media removed. Pre-warmed (37°C) papain purchased from Worthingtn Biochemical is freshly reconstituted with NBA-B and is added to the conical, and it is incubated on a rotary shaker for 20 minutes. The TGs are then pelleted by centrifugation at 200 RCF, the papain removed, and 3mL Hank's Balanced Salt Solution (HBSS) with 12mg collagenase and 14mg dispase, pre-warmed to 37°C, is added. As before, this is placed on a rotary shaker for 20 minutes, spun down at 400 rcf, and the dispase/collagenase is removed.

Ganglia are then dissociated by adding one mL of warm NBA-B and triturating 5-10 times. The homogenous mixture is then brought to five mL total with warm NBA-B. This mixture is then layered onto two (2.5mL each) 1.15 density Optiprep (iodixanol) gradients (this density is achieved by diluting 25.25mL Optiprep with 24.75 0.8% NaCl),

of the following layers (all gradient layers listed as Optiprep:NBA-B, in mL): Bottom - 0.45:0.55, 2nd layer - 0.35:0.65, 3rd layer -0.25:0.75, Top - 0.15:0.85. The gradients are then centrifuged at 800 RCF for 25 minutes at room temperature. The layers from the topmost dense band, down to the bottom gradient layer, are collected, and the rest is discarded (Figure 2-1B). The topmost layer contains the smallest and lightest cells comprising connective tissue and small support cells, whereas the bottom is largely highly concentrated iodixanol and very heavy non-neuronal cells. The remaining neurons are then washed twice with ~5mL of NBA-B, and pelleted between washes by centrifugation at 600 RCF for 5 minutes.

After final wash, 1mL of warm NBA-C media is added to one tube, the pellet resuspended and transferred to the second tube, and all neurons resuspended well. Neurons are counted via hemocytometer and diluted with NBA-C to a density of 60 neurons/µL. Neurons can plated on BD Matrigel-coated (1mL:4mL, in NBA-Stock) 60mm dishes, 24-well plates, or chamber slides, depending on the application. It is critical to apply the neuron suspension in such a volume as to form a puddle (or bead) on the surface of the dish, without touching the sides of the dish, or the neurons may clump on the periphery of the container. As a guide, 1.5mL is an appropriate volume for 60mm dishes, and a 50µL bead suffices for 8-well chamber slides. The dishes are incubated at 37°C from 1 hour to overnight. During the incubation, neurons will have largely settled to the bottom of the dish (if it appears that many neurons are stuck in the floating debris, the dishes can be lightly shaken halfway through the incubation). After the incubation, dishes can be lightly tapped and tipped over, and a pipette can be used

to aspirate the debris pad. An appropriate amount of NBA-C media is then added, and cultures allowed to develop for 2-3 days before infection with HSV-1.

Infection of TG Cultures with Virus

Infection of TG cultures is fairly straight-forward; infectious titers are calculated on a per-neuron basis, referred to as a plaque-forming unit per neuron (pfu/N) ratio. Briefly, NBA-C media is aspirated from dishes, and NBA-B media is used to dilute the virus to an infectious stock. This stock is placed on the TG cultures for a one hour adsorption time. After the adsorption, media is aspirated, and warm NBA-V media is added (3-5mL). This media contains human total IgG (biological grade) in an effort to limit viral spread.

Gene Expression Analysis by RT-qPCR

In order to determine that the HSV-1 genomes in the cell culture systems in question enter into a quiescent state similar to latency in the animal model, we performed reverse-transcriptase, quantitative Taqman® PCR (RT-qPCR) on TG cells infected with WT and ΔPST HSV-1 viruses. RNA was obtained through Trizol isolation and DNA obtained through back extraction, both according the protocol suggested by Life Technologies, and yield was determined by spectrophotometric analysis. Reverse transcriptase reactions was performed with Qiagen Omniscript RT kits using ~250ng RNA. The effectiveness of RT reactions was checked with spectrophotometric analysis. PCR analysis was performed using the Taqman® PCR primers and probes, with appropriate cellular controls. Levels of transcription of the LAT and lytic genes ICP0, ICP4, tk, and gC were assayed and compared to an endogenous cellular control like APRT or GAPDH, as well as a negative control transcript which should not be present in

infected cell, like MyoD1, a muscle-specific gene. In addition, viral transcript levels were compared to viral DNA, also assayed by Taqman qPCR, for a transcripts/viral genome number. Relative levels were compared to established literature on the animal models, both from our lab and otherwise. The primer/probe set used for each target is listed in Table 2-1. Biological replicates were each generated from a single 60mm culture dish ($6 \leq n \leq 10$, depending on the experiment), and statistical significance determined via a two-tailed student t-test if the variances of experimental groups are equal, and a two-tailed Welch's t-test if the variances are unequal.

ChIP Studies for Confirmation and Population Analysis

Chromatin Immunoprecipitation (ChIP) assays were performed on TG cell cultures, and compared to previous findings from the lab, as well as other established literature. ChIP assays were performed essentially as in (41), with slight modifications due to working with cell culture. Briefly, cell cultures were harvested at a time sufficient for the HSV-1 genomes to enter into a quiescent state (6-7 days post-infection). These cells were fixed with formaldehyde at a final concentration of 1%. Immunoprecipitation is performed on sonicated samples overnight at 4ºC with shaking. Anti-trimethyl H3K27 (Millipore 07- 3 449), Anti-trimethyl H3K4 (Upstate Biotechnology), and Anti-acetyl H3(K9,K14) (Upstate Biotechnology) are used at a concentration of 5 µg per sample. Anti-trimethyl H3K9 (Millipore 07-442) is used at a concentration of 4 µg per sample. Complexes were captured with Salmon sperm DNA-protein A agarose beads and washed in a series of buffers of increasing stringency, according to Millipore's instructions. Samples are decrosslinked, digested with RNAse, and DNA is eluted via Qiagen kits. Bound and unbound fractions were analyzed in triplicate with Taqman® PCR using Applied Biosystems Step-One Plus™ Thermocycler and associated

software. Analysis of cycle thresholds is performed as a relative quantity, comparing to standard quantities of DNA isolated from TG cells. Samples are analyzed as a bound/(unbound+bound) ratio [B/(U+B)] to and normalized to a cellular control B/(U+B) ratio. All ChIPs were validated using cellular targets enriched and depleted in the protein of interest (these controls listed below). Additionally, a negative control ChIP is performed using rabbit Control IgG (Abcam Ab46540) at a concentration of 2 µg per sample. Biological replicates were each generated from a single 60mm culture dish (n = 9), and statistical significance determined via a two-tailed student t-test if the variances of experimental groups are equal, and a two-tailed Welch's t-test if the variances are unequal.

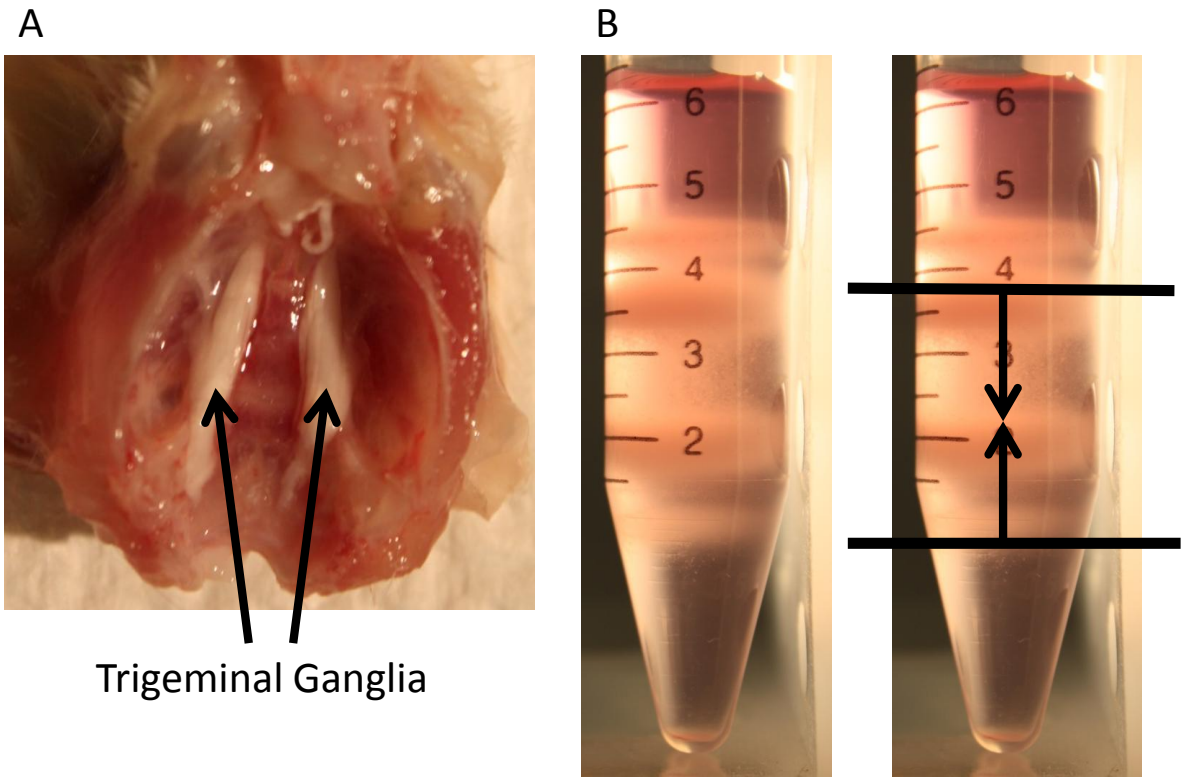


Figure 2-1. TG culture procedure images. A) Location of the trigeminal ganglia (TG) inside the mouse skull after removal of the brain. B) After a low-speed centrifugation on the Optiprep™ gradient, cells band at different layers depending on size and weight. Neurons between the black bars are collected for washing, counting, and plating.

Table 2-1. List of Primer and Probe Sets for Taqman® qPCR Analyses

Primer/Probe Name	Forward Primer	Reverse Primer	Probe (All 5' ~> 3')
LAT Promoter	CAA TAA CAA CCC CAA CGG AAA GC	TCC ACT TCC CGT CCT TCC AT	TCC CCT CGG TTG TTC C
LAT 5' Exon	GGC TCC ATC GCC TTT CCT	AAG GGA GGG AGG AGG GTA CTG	TCT CGC TTC TCC CC
LAT Intron (2.0Kb)	CGC CCC AGA GGC TAA GG	GGG CTG GTG TGC TGT AAC A	CCA CGC CAC TCG CG
ICP0	GGC CGA GGG AGG TTT CC	CCG CTT CCG CCT CCT C	CTC CCA GGG CAC CGA C
ICP4	GTG AAG CGG CCC GTG	GCG ATA GCG CGC GTA GA	AGG TCG CGT CGG C
Thymidine Kinase	GTC TAT ATA AAC CCG CAG TAG CGT G	GGC TCG GGT ACG TAG ACG ATAT	GCA GTT GCG TGG TG
gC	CCT CCA CGC CCA AAA GC	CAA ACC CAA GAA CAA CAC CAC C	CCC CAC GTC CAC CCC
MyoD1	GGC CTT CGA GAC GCT CAA	GCG CAG GAT CTC CAC CTT	CAG CAA CCC GAA CCA G
APRT	CTC AAG AAA TCT AAC CCC TGA CTC A	GCG GGA CAG GCT GAG A	CCC CAC ACA CAC CTC

CHAPTER 3
CHARACTERIZATION OF THE TRIGEMINAL GANGLIA CULTURE SYSTEM

Background

As described by Bertke et al. (4), the adult murine trigeminal ganglia (TG) culture system is a novel method for investigating molecular aspects of HSV-1 biology, especially during the latent infection. It has been reported that these cultures faithfully recreate the heterogeneity of the neurons of the trigeminal ganglia, with subpopulations of neurons in the TG culture existing in proportions similar to those observed directly from the animal. These observations suggested that the TG cultures would be an appropriate surrogate for whole ganglia infections with HSV-1.

Using multiple reporter viruses, Bertke et al. were able to show that TG cultures replicated many aspects of the *in vivo* HSV-1 infection. Namely, in TG cultures, a subpopulation of neurons with the A5 surface marker were non-permissive for the acute infection while neurons with the KH10 surface marker were permissive for the acute infection. In fact, in these cultures, HSV-1 preferentially established a latent infection in these A5-positive neurons, and not KH10-positive neurons, replicating earlier observations from the animal (49). These observations also introduce the importance of using heterogeneous cultures, as the disparate populations of neurons likely play an important role in the biology of the viral infection. At this time, it is also important to note that while the A5 and KH10-positive neurons are relatively well-studied with regards to their role in HSV-1 infection, other neuronal subpopulations exist and are likely involved as well, but these surface markers are poorly understood. It should therefore be clear that a truly faithful *in vitro* system should recapitulate this heterogeneity as much as possible.

While Bertke et al. developed and characterized some basic aspects of these cultures, both with and without HSV-1 infections, there are some aspects of the viral infection which are poorly understood in these cultures. It is important to characterize the biology of this infection in order to determine if these cultures will effectively replicate some of the more nuanced aspects of the latent infection. In particular, while reporter viruses allowed for a general view of viral transcription and replication, it is unclear if there is any aberrant transcription during the quiescent state of the HSV-1 infected TG cultures. Many previous cell culture models show low levels of lytic transcription during this time point, despite efforts to control the acute infection (often referred to as “leaky transcription”), and it is important to determine if the TG culture system replicates this behavior. One possible advantage of the TG culture system is that at a latent time point, the population of neurons permissive for the acute infect has likely died off.

In order to further characterize the HSV-1 infection which results from infection of these TG cultures, we investigated levels of RNA, DNA, and total virus at both lytic and latent time points during HSV-1 infection.

Results

At a Lytic Time Point, Viral RNA Levels in TG Cultures Are Highest for the Late Lytic Transcript, gC.

When infected with HSV-1, the TG culture system effectively harbors an HSV-1 infection for up to two weeks. It was critical to characterize the nature of this infection to discover whether it parallels the *in-vivo* infection using methods more sensitive than reporter viruses and growth curves, as used in the original characterization (4). Therefore, we assayed viral transcription present in these TG cultures 10 hours post-

infection (10 pfu/N) with a wild-type strain of HSV-1 (17*syn*+). At this time point, the cultures do not show clear signs of being infected (no syncitia, dead neurons, or disrupted axons). These data are shown in Figure 3-1. Five viral transcripts were assayed, two LAT species (both the 5' exon and the stable intron) and three lytic genes: one immediate early (ICP4), one early (Thymidine Kinase, TK), and one late (glycoprotein C, gC). These representative RNAs were chosen on the basis that they have widely been used as benchmarks for HSV infection *in vivo*. RNA levels of these transcripts are normalized to a cellular transcript, GAPDH. As shown in Figure 3-1, at 10 hours post-infection, we observe that the late transcript gC is significantly more abundant than all other transcripts assayed at 20 times the level of GAPDH (p-value < 0.05). Interestingly, the second most abundant transcript is the LAT intron, which is significantly more abundant than the LAT exon and both remaining lytic transcripts, as well (p value < 0.05). Finally, we observed that the least abundant transcript in this assay is the immediate early gene ICP4, which is a critical transactivator. This transcript is the only one assayed which was found to be less abundant than the cellular housekeeping gene GAPDH.

The LAT Intron is Expressed at High Levels in Latently-Infected TG Cultures.

In addition to assaying the transcriptional profile of TG cultures infected with HSV-1 at a lytic time point, we also assayed transcript levels at a time point when the HSV-1 infections in these cultures were reported to have established a quiescent or latent infection (4). To that end, we assayed RNA transcript levels of infected TG cultures via RT-qPCR at six days post-infection (10 pfu/N). At this time point, the cultures appear very different than when uninfected, or acutely infected. The axonal network is largely disrupted, the support cells have succumbed to the acute infection,

and only about 10-15% of the neurons remain in the culture. These data are shown in Figure 3-2. Three viral transcripts were assayed: two LATs (the LAT exon and stable intron) and the late gene gC. We observe that the LAT intron is the most abundant transcript, but that the level of LAT transcription is quite variable, with a mean of about 23 times greater than the cellular control. By contrast, the LAT 5' exon is about 10-fold less abundant than the LAT intron. Despite the latent time point, the lytic gene gC is still present at relatively high levels, possibly indicating that this quiescent state of infection still possesses some lytic transcription.

Viral DNA Can Be Detected in Infected TG Cultures at Both Lytic and Latent Time Points.

It has been reported that when TG cultures are infected with HSV-1, the viral infection follows a course similar to that which is seen in animal models, with an active infection followed by latency. We assayed the amount of viral DNA present in the TG cultures at both of these time points. The results are shown in Figure 3-3. These TG cultures were infected with 10 pfu/N and DNA was collected at 10hpi or 6dpi. At the lytic time point (10hpi), viral DNA was present, on average, at about three-fourths the level of cellular DNA. This suggests that for every four cellular copies of the APRT gene, there are about three copies of the viral DNA polymerase gene. By the time latency has occurred, this average has increased to about one copy of the virus gene for every copy of the cellular gene, but this difference is not statistically significant due to the large variance in the data. Regardless, the presence of viral DNA at a latent time point suggests that there are a significant number of viral genomes still present in the culture.

Conclusions

While There Is Evidence of HSV DNA Replication in the TG cultures, the Levels of Replication are Lower than They Are in Non-Neuronal Cell Types In Vitro.

While previous studies had shown that infection of these cultures resulted in persisting expression of reporters driven by the LAT, an analysis of the persistence of viral genomes had not been undertaken. Here we demonstrate that viral DNA was present in the infected TG cultures at both lytic and latent time points. This suggests two important points: 1) that while there is an initial phase of acute infection, this infection does not result in complete killing of the neurons and 2) that the TG cultures harbor HSV genomes beyond the initial acute infection. It is clear that a population of neurons exists which survives the acute viral infection and establishes latency, which then acts as the reservoir for this viral DNA. This must be the case, because if every neuron which becomes infected with HSV-1 dies, then by six days post-infection, all acute infections will have run their course, resulting in no viral DNA being detected at this time point. Further, because the culture media is treated with pooled IgG, there should be minimal secondary infections. Hence, it is very likely that the viral DNA we see at six days post-infection is a latent reservoir from which latent transcription and/or reactivation may occur. These data are consistent with the previous results showing persistence reporter gene expression using a reporter virus expressing a reporter driven by the LAT promoter (4).

This interpretation is further supported by the observation that the average relative amount of DNA is slightly higher during the latent infection. At 10 hours post-infection, there are about three HSV-1 genomes per two cellular genomes, as the cellular control, APRT exists in two copies. From that point, DNA replication,

progression towards a latent infection, and the death of some neurons eventually results in an average of about two HSV genomes per cellular genome. This suggests that by the time of a latent infection, the amount of HSV-1 DNA per neuron has actually increased, as acutely infected neurons have died out.

Transcript Levels in the TG Culture System Closely Parallel *In Vivo* Observations at Both Lytic and Latent Time Points

During an active HSV-1 infection, we can observe two distinct patterns of transcription: one during the active, lytic infection, and one during the latent infection. During a lytic infection, the virus expresses its genes in a temporal cascade to effect production of new virus, while the latency-associated transcripts (LATs), are relatively under-expressed. In *in-vivo* models of infection, such as the rabbit-eye model or mouse footpad model, we have observed high levels of lytic transcripts, especially of structural transcripts like glycoprotein C (gC). In the TG culture model, we observe a similar trend, where the LATs are generally low, and the late, structural lytic genes are expressed at a relatively high level. It is particularly telling that the gC transcript is the most-highly expressed transcript, as gC is an important structural protein. As such, gC is needed in stoichiometric amounts, as each virion is composed of multiple gC proteins. We can therefore infer that the TG cultures infected with HSV-1 are actively producing virus, as transcription of such high amounts of gC would be needed for the production of infectious virions.

By contrast, levels of early and immediate early transcripts are low when compared to gC, which is consistent with *in vivo* data of the later stages of a lytic infection (62). There are two explanations for this observation. First, the earlier viral proteins perform aspects of viral replication which only require small amounts of that

protein and therefore IE and E genes are generally transcribed at much lower levels than L genes. Secondly, at 10 hpi, the time point when we measured lytic transcripts, much of the initial lytic infection has likely run its course, and the earlier transcripts are no longer being actively transcribed at high levels. At this time in the lytic infection, the viral life cycle is beyond the stage of DNA replication, and is beginning to assemble new virions. The viral transcription factor ICP4, which is an immediate-early gene, is an ideal example of this phenomenon. This protein is responsible for trans-activating early viral genes, which are generally associated with viral DNA replication. Since ICP4 is a transcription factor, very low levels of this protein can effect great changes in downstream transcription. Using the TG culture system, we can observe this exact phenomenon: the immediate early gene is expressed at relatively low levels, the early gene at slightly higher levels, and the late gene at the highest levels. This temporal cascade of viral transcription (IE genes first and fewest, E genes second, and L genes last and most abundant) is a well-characterized aspect of the *in vivo* HSV-1 infection, and to see this cascade faithfully replicated during the lytic infection of the TG cultures further validates its use as a model system.

The lytic portion of the viral life cycle has routinely been studied in cell culture, but an *in-vitro* system for studying the latent phase of the virus has remained elusive. This TG culture system has been shown to harbor a latent infection as early as six days post-infection. We assayed our cultures for RNA at this time point, and found that transcription of the LAT intron was the highest of those which we assayed. During latency, the LAT intron is known to accumulate at high levels due to its stability. By contrast, the LAT 5' exon is rapidly degraded after being spliced. In the TG cultures, we

observe that LAT intron levels are appreciably higher than LAT 5' exon levels, which is in agreement with the observations from *in-vivo* data. However, we have observed that levels of the lytic gene gC are slightly higher during latency than we might have hypothesized. Even during latency in animal models, we have observed that there are low levels of lytic transcription which can be detected with sensitive methods, which has been referred to as, "leaky" transcription. Previous investigations into HSV-1 latency have found that some systems exhibit more "leakiness" than do others. For example, the LAT-deletion mutant 17ΔPst shows more transcription of lytic genes during a mouse footpad infection. Since the levels of gC transcription are at levels higher than we would predict, it is likely that the TG culture system is more conducive to higher levels of this leaky transcription, although since the TG culture has a much higher percentage of latently-infected cells than does an infected ganglion (~90% vs ~10%), it is likely that these levels are not significantly greater than those which were observed in the animal.

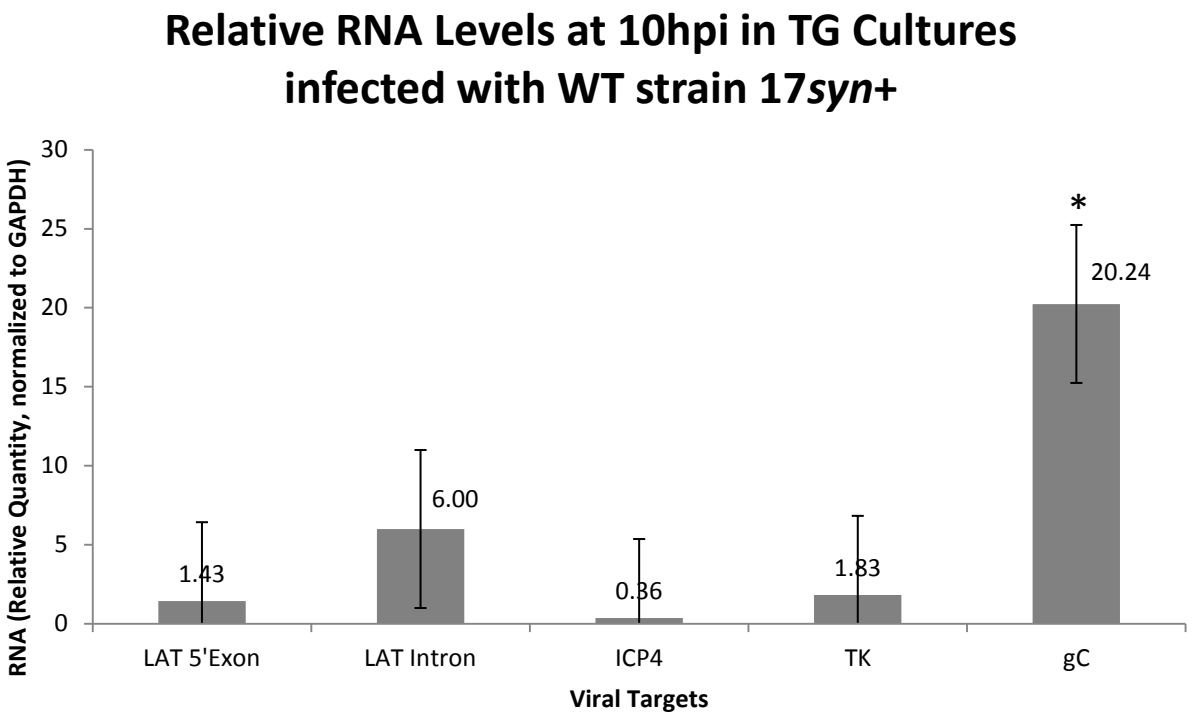


Figure 3-1. At a lytic time point, viral RNA levels in TG cultures are highest for a late lytic transcript. RNA levels of latent and lytic transcripts collected from primary trigeminal ganglia (TG) cultures infected (10 pfu/N) with HSV-1 strain 17syn+ at 10hpi are shown. The most abundant transcript at this time point was that of the late lytic gene, glycoprotein C (gC), akin to observations from animal infections. Protocol for isolating RNA from samples is fully described in Materials and Methods. Briefly, RNA was collected using the Trizol reagent, reverse-transcribed to cDNA with the Qiagen Omniscript Kit and viral targets were quantified through real-time PCR using Applied Biosystems StepOne Plus system. Relative RNA levels are normalized to a cellular control RNA (GAPDH); n = 6 TG cultures, * - p-value < 0.05.

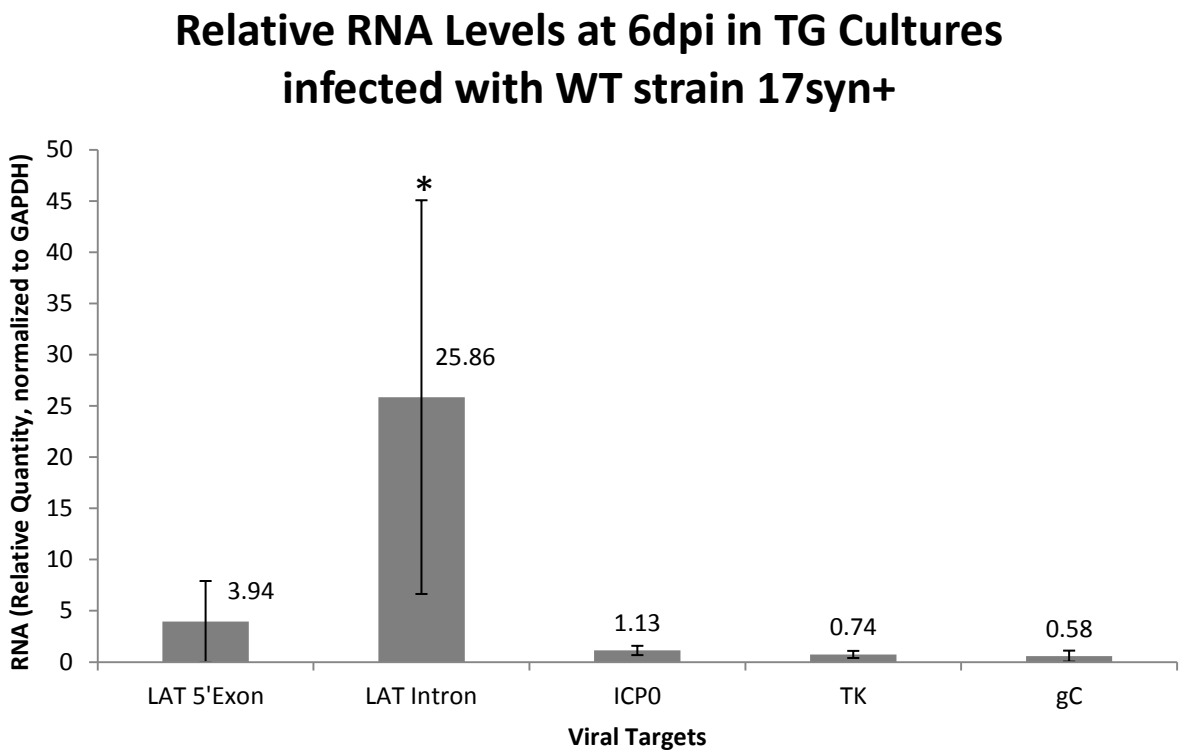


Figure 3-2. The LAT intron is expressed at high levels in latently-infected TG cultures. RNA Levels of latent and lytic transcripts collected from primary TG cultures infected with HSV-1 strain 17*syn*+ at 6dpi (10 pfu/N) are shown. The most abundant transcript at this time point was that of the latency associated transcript (LAT) intron, which is known to accumulate at high levels during latency in animal models. Protocol for isolating RNA from samples is fully described in Materials and Methods. Briefly, RNA was collected using the Trizol reagent, reverse-transcribed to cDNA with the Qiagen Omniscript Kit and viral targets were quantified through real-time PCR using Applied Biosystems StepOne Plus system. Relative RNA levels are normalized to a cellular control RNA (GAPDH); n = 6 TG cultures, * - p-value < 0.05.

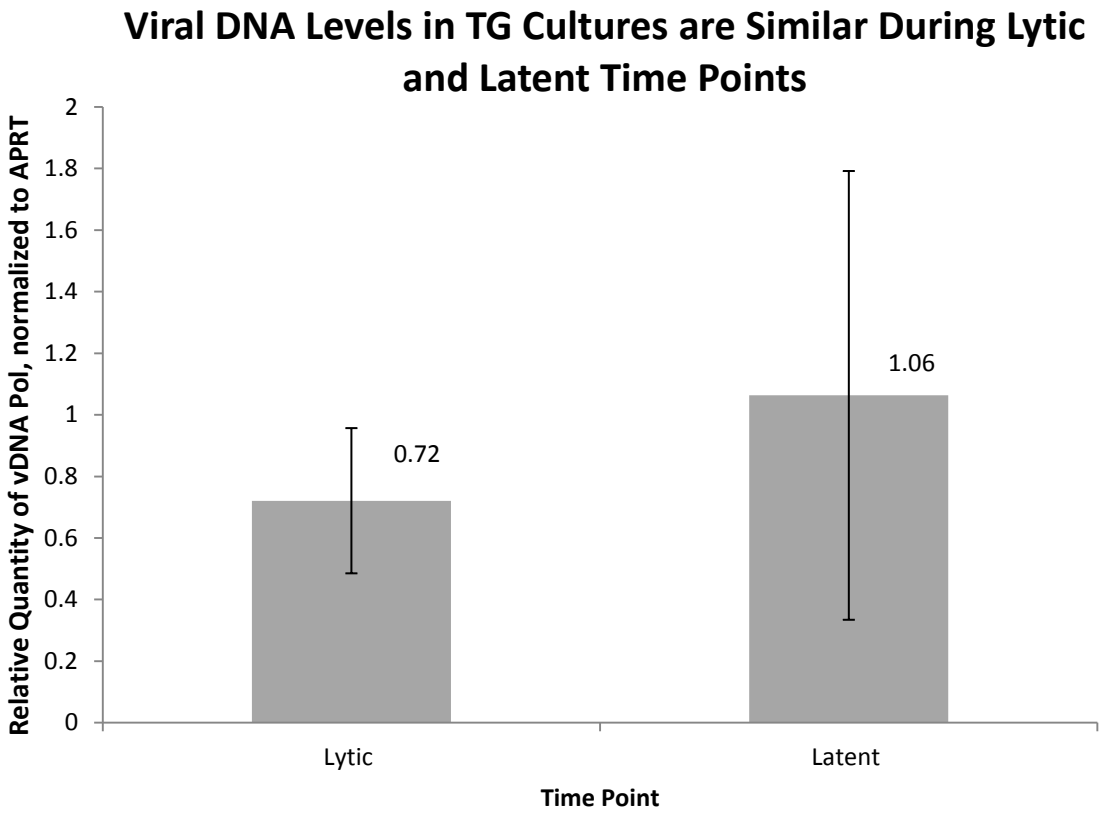


Figure 3-3. Viral DNA levels per cell in TG cultures at lytic and latent time points are similar. DNA levels of primary TG cultures infected with HSV-1 strain 17*syn*+ at lytic (10hpi) and latent (6dpi) time points are shown. The viral DNA polymerase was used as a primer target. Protocol for isolating DNA from samples is fully described in Materials and Methods. Briefly, DNA was back-extracted using the Trizol reagent, and viral DNA was quantified through real-time PCR using Applied Biosystems StepOne Plus system. Relative DNA levels are normalized to a cellular control DNA (APRT); n = 6 TG cultures.

CHAPTER 4
THE INITIAL VIRAL DOSE INFLUENCES VIRAL TRANSCRIPTION *IN VITRO*

Background

The effective dose delivered to neurons may result in different programs of viral infection, resulting in differential levels of establishment of viral latency. For example, it is well-known that ICP0 displays a clear dose-dependent effect, as this viral protein is essential at low MOI, but non-essential at a high MOI. Previous experiments by another member in the lab established that there was a relationship between the initial viral inoculums and the silencing of HSV genomes during latency *in vivo* (43). These data suggested a moderate dependency on inoculum dose on transcription of the LAT 5' exon. Additionally, levels of the LAT intron display a much more robust dependence on initial dose of virus, where we observed the highest levels of LAT intron present at the higher multiplicities of infection. However, in these *in vivo* experiments, the highest initial inoculums resulted in a reduction of transcription, suggesting that there is a possible saturation point where any increases in inoculum does not increase viral transcription. Also of note, these data were generated using the avirulent HSV-1 strain KOS because the virulent strain 17*syn*+ results in a LD_{50} at fairly low titers. In addition, assaying the effect that an initial inoculum delivered at the periphery (in this case, via rear footpad infection) may have on the subsequent latent infection is problematic. Since multiple rounds of replication in both the epithelial and neuronal cell types likely precede the establishment of latency, it may be more effective to investigate any dose-dependent effect *in vitro* using a primary neuronal cell culture.

To that end, we chose to infect TG cultures with different viral inoculums, calculated on a per-neuron basis. We chose to infect with a variety of doses in order to

simulate low, moderate, and high multiplicities of infection. At both lytic at latent time points, we assayed DNA and RNA levels in these cultures to determine if these aspects of HSV-1 infection are affected by the initial inoculum. We chose to look at the all classes of viral transcripts (IE, E, L) in order to assay transcription of lytic transcripts at these time points, and also looked at total viral DNA. We hypothesize that, as seen in the *in vivo* experiments, increased levels of initial virus inoculums will result in differential levels of RNA transcription at a latent time point, possibly suggesting that the initial dose plays a role in the establishment of latency.

Results

The Relative Abundance of HSV-1 Transcripts in Infected TG Cultures at a Lytic Time Point Are Dependent on the Initial Infective Dose for Wild-Type Virus.

To understand the effect that the initial dose of infectious virus has on viral transcription, TG cultures were infected with three different doses of wild-type HSV-1, strain 17*syn*+. Dishes were infected on a plaque-forming unit per neuron (pfu/N) basis (10pfu/N, 1 pfu/N, or 0.1 pfu/N). We assayed viral transcription in these cultures at 10 hpi, and the results are collected in Figure 4-1. Five viral transcripts were assayed: the two LAT species, LAT 5' exon and LAT intron, and a lytic gene corresponding to each temporal class: the immediate early gene, ICP4; the early gene thymidine kinase (TK); and the late gene, glycoprotein C (gC). All viral transcripts were normalized to the cellular housekeeping gene, GAPDH.

For all transcripts assayed, there is a clear dose-dependence wherein the greater the initial infectious virus, the greater is the viral transcript level. This is most pronounced with the late gene, gC. At 10 pfu/N, gC transcript levels are about twenty times more abundant than the cellular gene GAPDH, whereas at 1 pfu/N, transcript

levels are only about five times more abundant than the cellular gene. Finally, at the lowest dose of virus, 0.1 pfu/N, levels of this viral transcript are nearly the same as the cellular gene. Thus, it is clear that higher doses of infectious virus result in significantly higher levels of viral transcription, at least when a late gene like gC is assayed (p-values < 0.05). A similar trend is evident for the other transcripts assayed, as well. Levels of the early transcript TK are highest at 10 pfu/N, at levels nearly twice the cellular control transcript, which is significantly greater than was found at only 1 pfu/N (p-value < 0.05). The immediate early gene ICP4 is at very low levels regardless of the pfu/N used for infection, but there is still a general trend towards greater transcript levels at the higher pfu/N infections. Finally, the LAT transcripts assayed follow the same general trend that we observe with the lytic transcripts. The LAT intron, especially, is expressed at the highest levels (six times the level of GAPDH) at the highest amount of pfu/N. This level of transcription is significantly lower at the two lower doses of virus, where LAT levels are not even greater than GAPDH levels (p-value < 0.05). The same trend is evident for the LAT 5'exon as well, but since the levels of the exon are altogether much lower than the transcript levels of the intron, the trend is much less pronounced, although still significant (p-value < 0.05). Taken together, each of these trends suggests that the overall level of viral transcription is dependent on the initial number of viral particles infecting the neurons in culture, and that the higher the viral load, the higher is the viral transcription.

In addition, it is important to note the relationships between the transcript levels of the various viral gene classes that occur at a lytic time point. The late viral genes are expressed at high levels, while the immediate early and early genes are both expressed

at significantly lower levels (p-value < 0.05), which does not change if viewed on a per-genome basis. In this sense, transcript levels of the LATs, which are driven by a promoter with late kinetics, more resemble the late transcript gC, than they do the other lytic transcripts. It is important to note that these relationships exist not only at the highest infectious dose, but at the other doses, as well.

The Relative Abundance of HSV-1 Transcripts at a Lytic Time Point in TG Cultures Infected by a LAT-Deletion Virus Are Generally Low, with Very Little Dependence on Viral Dose.

In an effort to understand the role that LAT transcription plays during the course of the viral infection of these TG cultures, and to determine whether this role is dependent on different levels of viral dosage, we performed analyses similar to those found above. TG cultures were infected with different levels of pfu/N (10, 1, and 0.1) of the LAT-deletion mutant 17ΔPst, which produces about 10,000-fold less LAT than the wild-type strain 17*syn*+ (26). Once again, RNA from these cultures was harvested during a time point when the lytic infection was still underway (10hpi). The same viral transcripts, one lytic gene per gene class, and both the LAT intron and LAT 5'exon, were assayed and normalized to the cellular GAPDH transcript levels. The results from this experiment are collected in Figure 4-2.

For the LAT-deletion mutant 17ΔPst, relative levels of the different transcription classes of genes (IE vs E vs L) are generally lower across all viral doses than those that were observed for the wild-type virus. As before, the late gene gC is the most abundant of the transcripts assayed, but only at about two times the level of the cellular control transcript. Transcript levels for the early gene TK and the immediate early gene ICP4 are even lower, regardless of the pfu/N involved with the infection. Together, the

abundance of these transcripts, relative to each other, is quite similar to what was observed with the wild-type virus.

By contrast, any *dose-dependence* on the initial pfu/N levels infecting the cultures is *much less pronounced* than with wild-type virus. Whereas before there were marked statistically significant differences between even the two higher viral pfu/N doses, for the LAT-deletion virus, no such differences are evident, unless one considers the lowest infectious dose as well. As an example, we observed that the most abundant transcript, the late transcript gC, was transcribed at similar levels for both 10pfu/N, and 1pfu/N, about twice the level of cellular GAPDH. It is only at the very lowest level of infection at 0.1pfu/N that we see a markedly lower transcript abundance. A similar trend can be observed with each other viral transcript assayed, as well: both the LATs and the other lytic genes show very similar abundance at the two higher pfu/N infections, and lower abundance at the low pfu/N infection. Unlike infections with the wild-type virus, it appears that the LAT-deletion mutant 17ΔPst displays a much more modest dose-dependence, suggesting that the presence of the LAT promoter is required for this dose-dependence that we have observed.

The Relative Abundance of HSV-1 LAT Transcripts in Wild-Type Infected TG Cultures at a Latent Time Point are Dependent on the Initial Infective Dose.

After characterizing the effect that different amounts of pfu/N have on viral transcript levels in TG cultures at a lytic time point, it was important to investigate the corollary effect during a latent time point, as one strength of the TG system is that it allows for an *in-vitro* model of HSV-1 latency. As such, viral transcript abundance was assayed at 6 dpi for various pfu/N levels as before, and these data are shown in Figure 4-3. Since these assays were carried out when the viral infection is in a quiescent or

latent state, we would expect a different transcriptional profile than we found at earlier time points. Indeed, the LATs are very abundant at this time point, especially the LAT intron which is known to accumulate to high levels during latency *in vivo*. The LAT intron transcript was, on average, about 25 times more abundant than cellular GAPDH at the highest pfu/N infection (recall, this transcript was only about 6 times more abundant than the cellular control during the acute infection). The LAT 5'exon transcript is also present at the highest pfu/N infection, but at levels much lower (about four times greater than GAPDH) than the intron.

Similarly, we see a very different transcriptional profile at a latent time point than we did during the acute infection with regards to the lytic transcripts. Where before it was clear that the late transcript gC, was the most abundantly transcribed transcripts, this is not the case during latency. During this point in the infection, at all pfu/N infection levels, the LAT intron is the most abundant transcript. The lytic transcripts all exist at levels near or below the cellular control gene GAPDH. This transcriptional profile is similar to that which is seen during the in vivo mouse infection: high levels of the LATs, and low, transient levels of the lytic genes.

In addition to this particular transcriptional profile, a dose dependence similar to that which was observed during the acute infection can be observed, during a latent infection. For the LATs, which are the most abundant transcripts at this time point, we see a dose-dependence where the higher the pfu/N infection ratio, the higher is the transcript abundance of both the LAT intron and the LAT 5' exon. Hence, when a larger dose of incoming virus infects the neurons in the TG culture, more latent transcripts are transcribed. This is most clear with the LAT intron, where a pfu/N ratio of 10 results in

more than a 3.5-fold increase in transcript abundance when compared with a pfu/N ratio of 1. A similar 3.5-fold increase is also evident between pfu/N of 1 and of 0.1 for the LAT intron. Hence, it appears that successive increases in initial viral inoculums results in corresponding increases in LAT transcription. This trend is also evident for the LAT 5'exon, but since abundance levels of that transcript are much lower to begin with, the increase in LAT transcription is only clear at the highest pfu/N ratio.

With regards to the lytic transcripts, no general dose-dependent trend is evident. The immediate early transcript ICP0 is significantly more abundant from the lowest (0.1) to the highest (10) pfu/N ratio, but neither are statistically different from the intermediate infection (1 pfu/N). Further, the early transcript TK does not even display this modest dose-dependence: at all levels of HSV-1 infection, TK transcript levels are similar. This is also the case with the late transcript, gC. Hence, we observe that the transcriptional profile of HSV-1-infected TG cultures is one of high LAT transcription and LAT intron accumulation, coupled with low levels of lytic transcripts, and that LAT transcription is likely dependent on the initial infective dose.

The Relative Abundance of HSV-1 Transcript Levels at a Latent Time Point in TG Cultures Infected With a LAT-Deletion Mutant is Near Background and Largely Independent of Initial Dose of Virus.

To determine the effect that the LAT plays in the HSV-1 infection at a latent time point in the TG cultures, and to see if this effect is largely pfu/N dosage independent, as was observed at a lytic time point (Figure 4-2), TG cultures were infected with different amounts of 17ΔPst, as before. The same viral transcripts were assayed via RT-qPCR in order to determine the transcriptional profile of this virus during the quiescent infection of the TG cultures. These data are presented in Figure 4-4. Here, we observe

that the normalized values for this experiment are much lower than those observed for TG cultures infected with a wild-type virus. In fact, none of these transcripts exist at levels above the GAPDH transcript levels. Of note, transcript levels of the late gene gC are so low as not to be above no-RT control background samples. The transcript which is most abundant at this time point is the LAT intron, which is also the most abundant transcript observed at a latent time point in the *in vivo* animal models. ICP4 and TK transcript levels are slightly less abundant than the LAT intron transcript, and therefore may be interpreted as normal "leaky" transcription, which is known to exist in LAT-deletion mutants in *in-vivo* models. In this sense, the transcriptional profile of these quiescent cultures closely mirrors that of animal models.

At this time point, only a single transcript, the early transcript TK, displays any evidence of dose-dependence at the level of statistical significance, *but this dose dependence is the opposite of that which has been seen in all other cases*: the lower the pfu/N infecting the TG cultures, the higher is the relative transcript level of TK (p-value>0.05). These transcript levels are very low; around one-fifth of the level of the GAPDH transcript, so it remains unclear whether the observed dependence has any effect on the overall quiescent infection of the TG cultures, as viral transcripts in all other gene classes do not display any kind of dose-dependence. As before, with the lytic time point, *we are left to conclude that the absence of LAT results in a marked removal of the dose-dependent effect that is seen in these cultures when infected with wild-type virus.*

DNA Levels in TG Cultures Infected with WT Virus Are Dose-Dependent Only during the Lytic Infection

In order to determine if the amount of infecting virus affects the total DNA in the TG cultures, we infected the cultures with different pfu/N ratios of wild-type HSV, and back-extracted the DNA from TRIzol extractions. DNA were collected at both a lytic time point (10 hpi) and at a latent time point (6dpi) and quantified by Q-PCR and the results are shown in Figure 4-5. During the lytic infection, we observe that higher initial pfu/N ratio results in more DNA per cell. At the highest pfu/N ratio, the average amount of DNA in the cultures is about 0.7 times the level of cellular DNA. This falls to only about one-quarter the level of cellular DNA at a pfu/N ratio of 1, a statistically significant reduction (p-value ~ 0.001). At the lowest dose, the levels of DNA are even lower, not even one-twentieth of cellular values (p-value < 10^{-4}). As expected, these data indicate that the amount of incoming virus greatly affects the amount of viral DNA present in the cultures at 10hpi in a dose-dependent manner.

By contrast, at a latent time point, there appears to be no direct dose-dependence on DNA levels. Even at the lowest dose (0.1 pfu/N), DNA levels are not significantly lower than at the higher infective doses, although the difference between 1 and 0.1 pfu/N (p-value = 0.058) is barely above statistical significance. However, despite the fact that the different doses do not result in significantly different levels of DNA in the cultures, there are differences between DNA levels during the acute infection and the latent infection. For example, at both pfu/N ratios of 1 and 0.1, DNA levels are significantly higher at a latent time point than they are during the acute infection (p-values < 0.05).

DNA Levels in TG Cultures Infected with 17ΔPst Virus Are Dependent on Initial Viral Dose

We previously determined that the initial dose of WT virus does have an effect on the amount of HSV-1 DNA in TG cultures, so we wanted to investigate whether a LAT-deletion mutant showed the same pattern. To that end, we infected TG cultures with different pfu/N ratios of 17ΔPst, and assayed DNA levels at both lytic and latent time points. The results are shown in Figure 4-6. Just as we observed with the WT virus, we see that during the lytic time point, the initial pfu/N ratio affects the amount of viral DNA present in the cultures at 10hpi: the higher the pfu/N ratio, the more viral DNA can be found in the cultures. However, unlike the WT virus, this is also the case during the latent infection. Each of these differences(0.1 vs 1, and 1 vs 10 pfu/N) is statistically significant (p-values < 0.05). In addition, at a pfu/N ratio of 1, there is significantly more DNA at a latent time point than there is during the acute infection (p-value < 0.001), which is not the case at the highest and lowest pfu/N ratios. Finally, it is important to note that the WT and mutant virus DNA levels during the lytic infection are similar for all pfu/N ratios, but are significantly different during a latent infection at the two lowest infective doses.

Conclusions

Initial Viral Inoculum Affects Viral Transcription Levels of the Most Abundant Transcripts During Both Acute and Latent Infections

The TG culture system allows for precise manipulation of viral dosages, which may play a critical role in the resulting infection of neurons. We found that by altering the pfu/N ratio of the infection, we could increase levels of viral transcription during the acute infection. In Figure 1-1, for example, we observed increased levels of viral transcription during the acute infection as we increased the initial viral dose. Since we

see higher levels of transcripts during higher pfu/N infections, this suggests that by 10hpi, the higher number of input viruses has resulted in more templates from which to transcribe. Furthermore, we see a similar transcription pattern at all doses: that of high gC transcription, lower transcription of earlier gene classes, and moderate LAT transcription. This transcriptional pattern is very similar to that which we see during an *in-vivo* infection. Essentially, *the various doses serve to alter the magnitude of the transcription of each gene class, but do not disturb the overall pattern.* The late gene gC is necessary for production of new virions at this time point, which explains the need for such high levels of its transcription, regardless of the initial pfu/N ratio. Thus, the transcription that we observe from the WT virus during this lytic time point is both dependent on the initial infection, and largely in agreement with previous observations from animal model infections, regardless of the initial infection.

A dose-dependent effect is similarly noticeable during a latent infection with WT virus. The difference in this case is that the dose-dependence is seen with regards to the most abundant latency transcripts – the LAT 5' exon and LAT intron. Figure 4-3 shows that at all pfu/N ratios, the LAT intron is the most abundant transcript. This is expected, as the LAT intron is spliced from the primary LAT transcript and due to its peculiar lariat structure, it accumulates at high levels during latency. By contrast, the LAT 5' exon is rapidly degraded and is seen at lower levels during latency in the animal infection model, an observation which is also supported in the TG culture system. And, the lytic transcripts are seen at very low levels during this time point, which is also supported by data from *in vivo* experiments.

It is interesting to note that viral DNA levels are largely the same at this time point, irrespective of dose (Figure 4-5). *Coupled with the previous data, this suggests that the increased transcription levels during latency are not merely due to more viral genomes acting as templates for transcription.* Hence, at the 10 pfu/N infection, the same amount of HSV DNA produces nearly 3.5-fold more LAT intron than is observed at a pfu/N ratio of 1. The HSV genomes which result from the higher inoculums therefore must be producing more LAT on a per-genome basis. Taken together, these data show that by altering the initial infective dose of HSV-1, the status of the latent genomes can be changed, as well. This may provide an explanation for the disparate phenotypes observed depending on viral strain, route of inoculation, or model system: *higher effective initial doses result in higher LAT transcription, which may drastically alter the latency phenotype that is observed.*

The Dose-Dependent Effect of Initial pfu/N Ratios on Viral Transcription Is LAT-Dependent

The LAT-deletion mutant 17ΔPst does not display the same pattern of dose-dependence with regards to viral transcription at either a lytic or latent time point. At 10 hpi, RNA levels are generally low and are not significantly different, regardless of the initial viral dose. The same general pattern, with high gC levels and lower levels of other lytic transcripts, is evident, but the magnitude of the RNA levels are much lower than were observed with a wild-type infection. But, for all viral transcripts assayed at this time point, different pfu/N ratio infections do not result in statistically significant changes in RNA transcription. Since altering initial infections did result in significantly different RNA transcription at this time point with the WT virus, this suggests that in the TG cultures, this pfu/N-dependent effect is LAT-dependent. Generally, absence of LAT

does not have a profound effect on acute transcription, but that appears to be the case in this instance.

Additionally, RNA levels at a latent time point with the mutant virus do not show a significant trend based on pfu/N infections. Generally, RNA levels at this time point are low across all loci examined, suggesting that global transcription is reduced at this time point. Of particular note is the LAT locus. Recall that 17ΔPst is a deletion in the core LAT promoter; in the animal this virus produced about 10000-fold less LAT than does the wild-type (during latency). So, it is not surprising that we see very little transcription of the LATs at this time, and the little transcription that is observed can be attributed to read-through transcription from other transcripts in the region.

The levels of the lytic transcripts are also very low at this time point, suggesting that *the LAT-deletion mutant is much more transcriptionally repressed than is the wild-type virus*. We know from the DNA data in Figures 2.5 and 2.6 that there is ample HSV-1 DNA in the cultures at this time point, regardless of the infecting virus, but the HSV-1 genomes in the cultures without LAT have become more transcriptionally repressed.

Viral DNA Levels during Acute Infection Are LAT-Independent, but DNA Levels during Latency Are LAT-Dependent

During the lytic infection, it is clear that the initial pfu/N infection ratio has a great effect on the presence of viral DNA at this time point, and that this effect is not dependent on the LATs. For both the wild-type and the LAT-deletion mutant virus, the higher the pfu/N ratio, the more viral DNA is detectable in these cultures at 10hpi. LAT is transcribed during the lytic infection with late gene kinetics, and disruption of this locus can result in multiple phenotypes, but by 10hpi there is no apparent difference in DNA levels between the virus, regardless of the initial pfu/N ratio of the infection. *This*

suggests that the LAT is not critical for the early stages of the viral life cycle like cell entry or DNA replication. This has been the case in animal models as well.

However, LAT does appear to have an effect on viral DNA levels at the latent time point in these TG cultures. By six days post-infection, there are marked differences between the wild-type virus and the LAT-deletion mutant. Both the wild-type and mutant viruses experience an increase in the amount of viral DNA in the cultures from 10hpi to 6 dpi, but at the two lower pfu/N ratios especially, we observe that viral DNA levels have increased nearly four-fold (for 1 pfu/N) and greater than 10-fold (for 0.1pfu/N) for the wild-type virus. By contrast, while we still observe a significant increase for the mutant virus, the increase is of less magnitude: about a 50% increase for 1 pfu/N, and only about 3.5-fold for 0.1pfu/N. These data suggest that in the cultures lacking LAT, there is either less DNA replication as a whole, or that more neurons with HSV-1 genomes with multiple HSV-1 genomes exist in the cultures infected with wild-type virus.

Ultimately, the data in this chapter indicate that the initial amount of infecting virus, here examined as a pfu/N ratio, plays a critical role in many aspects of transcription and replication of the virus, both during an acute infection and during latency. Interestingly, it appears as though LAT plays some role in these effects, as a LAT-deficient mutant expresses much less RNA overall, and replicates its viral DNA to levels lower than does a wild-type virus.

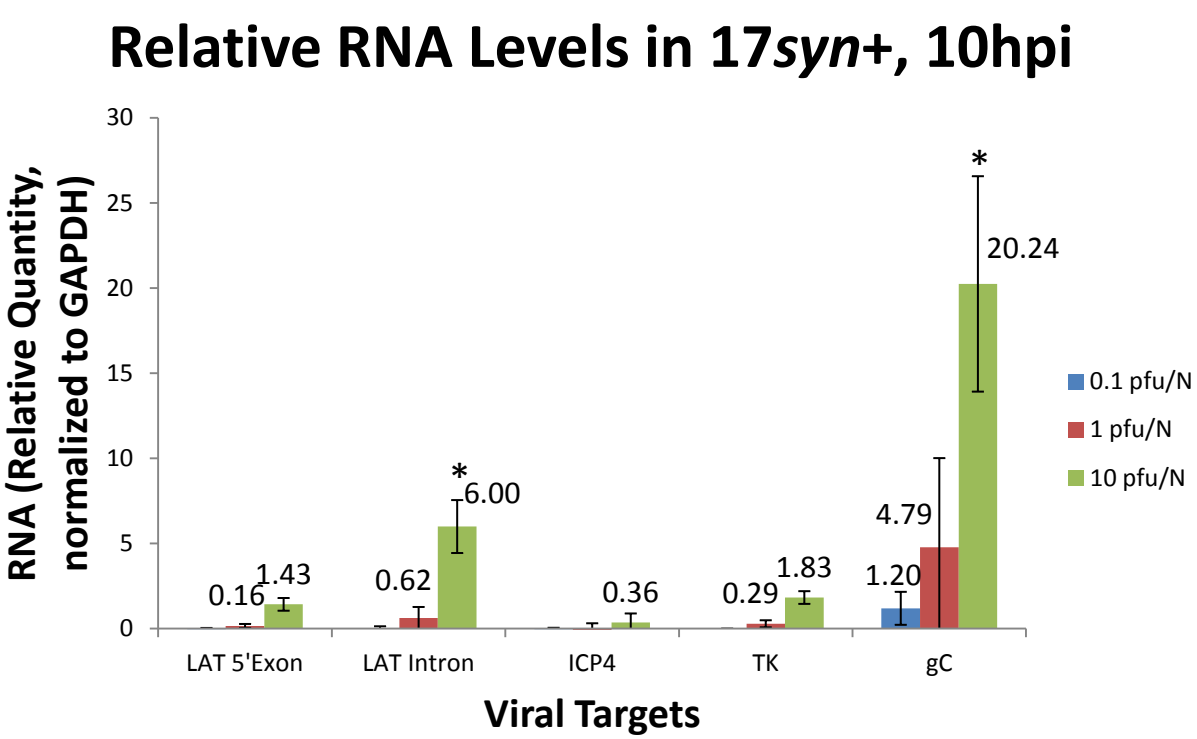


Figure 4-1. The relative abundance of HSV-1 transcripts in infected TG cultures at a lytic time point are dependent on the initial infective dose. Levels of latent and lytic transcripts collected from primary trigeminal ganglia (TG) cultures infected with HSV-1 strain 17*syn*+ at 10hpi with various doses (measured by pfu/neurons) are shown. Protocol for isolating RNA from samples is fully described in Materials and Methods. Briefly, RNA was collected using the TRIzol reagent, reverse-transcribed to cDNA with the Qiagen Omniscript Kit and viral targets were quantified through real-time PCR using Applied Biosystems StepOne Plus system. Relative RNA levels are normalized to a cellular control RNA (GAPDH); n = 6 TG cultures per viral dose.

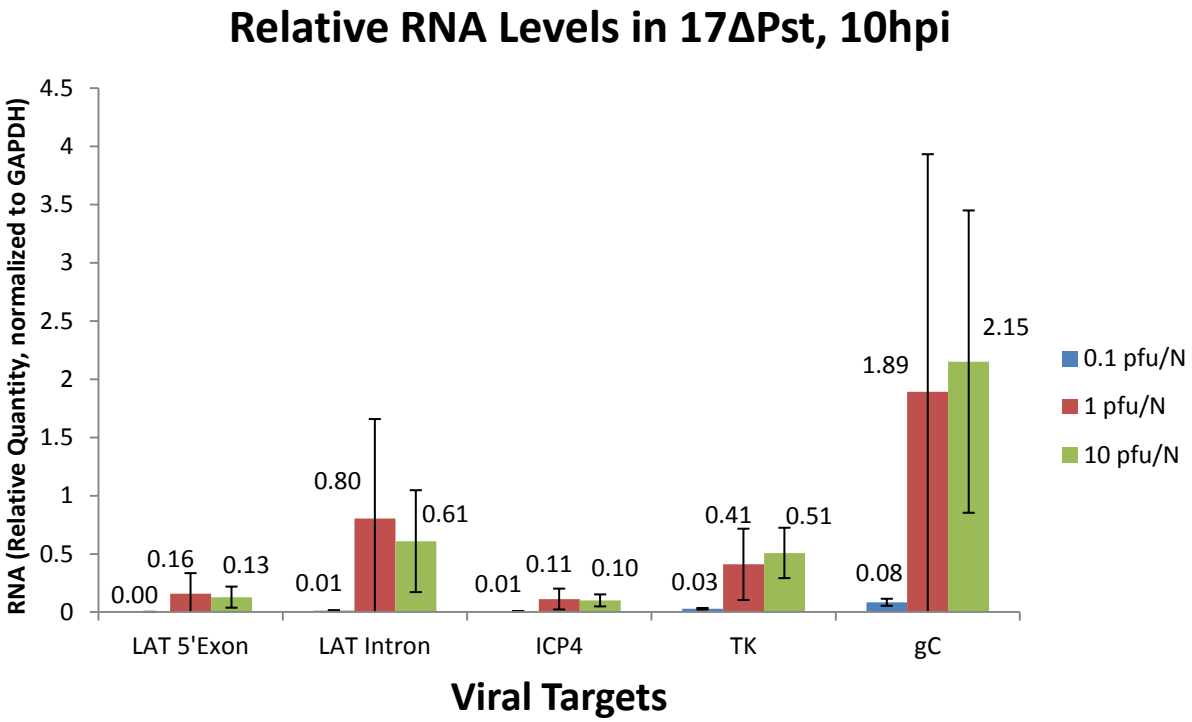


Figure 4-2. The relative abundance of HSV-1 transcripts at a lytic time point in TG cultures infected by a LAT-deletion virus are generally low, with very little dependence on viral dose. Levels of latent and lytic transcripts collected from primary trigeminal ganglia (TG) cultures infected with HSV-1 strain 17ΔPst at 10hpi with various doses (measured by pfu/neurons) are shown. Protocol for isolating RNA from samples is fully described in Materials and Methods. Briefly, RNA was collected using the TRIzol reagent, reverse-transcribed to cDNA with the Qiagen Omniscript Kit and viral targets were quantified through real-time PCR using Applied Biosystems StepOne Plus system. Relative RNA levels are normalized to a cellular control RNA (GAPDH); n = 6 TG cultures per viral dose..

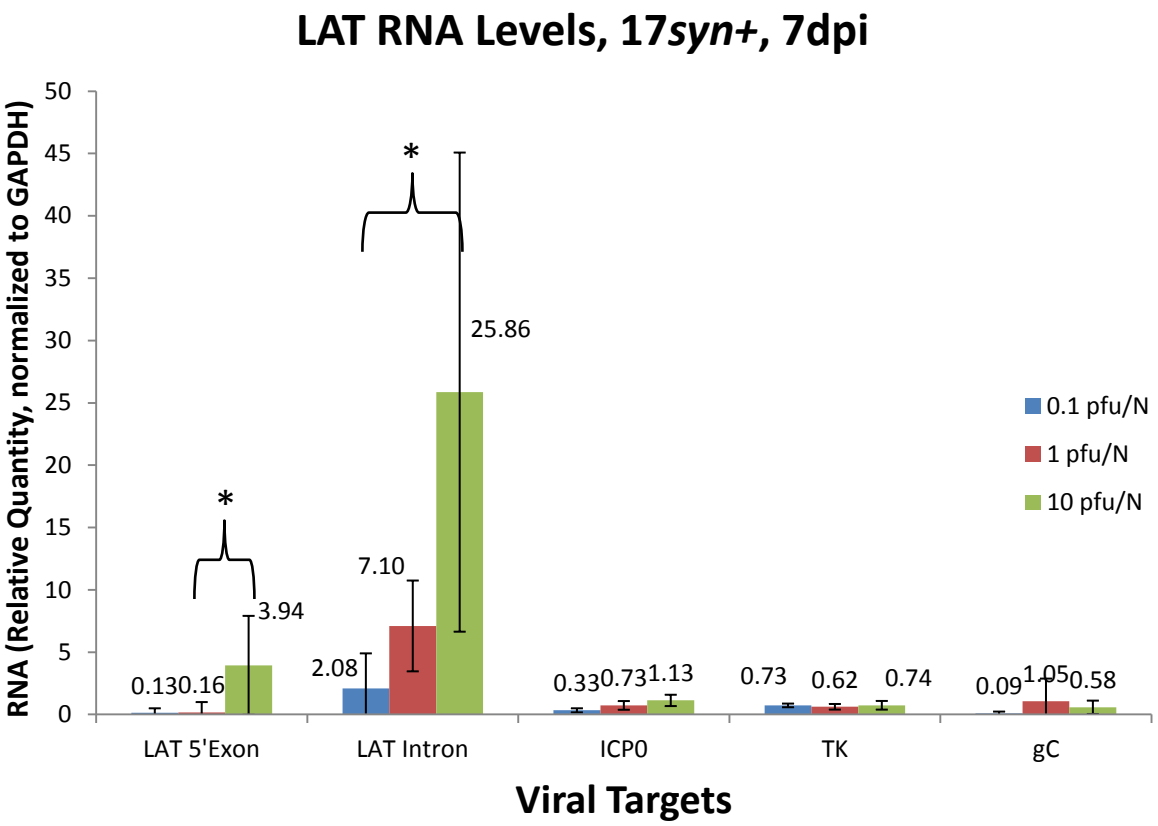


Figure 4-3. The relative abundance of HSV-1 LAT transcripts in infected TG cultures at a latent time point are dependent on the initial infective dose. Levels of latent transcripts collected from primary trigeminal ganglia (TG) cultures infected with HSV-1 strain 17*syn*+ at 6dpi with various doses (measured by pfu/neurons) are shown. Protocol for isolating RNA from samples is fully described in Materials and Methods. Briefly, RNA was collected using the TRIzol reagent, reverse-transcribed to cDNA with the Qiagen Omniscript Kit and viral targets were quantified through real-time PCR using Applied Biosystems StepOne Plus system. Relative RNA levels are normalized to a cellular control RNA (GAPDH); n = 6 TG cultures per viral dose.

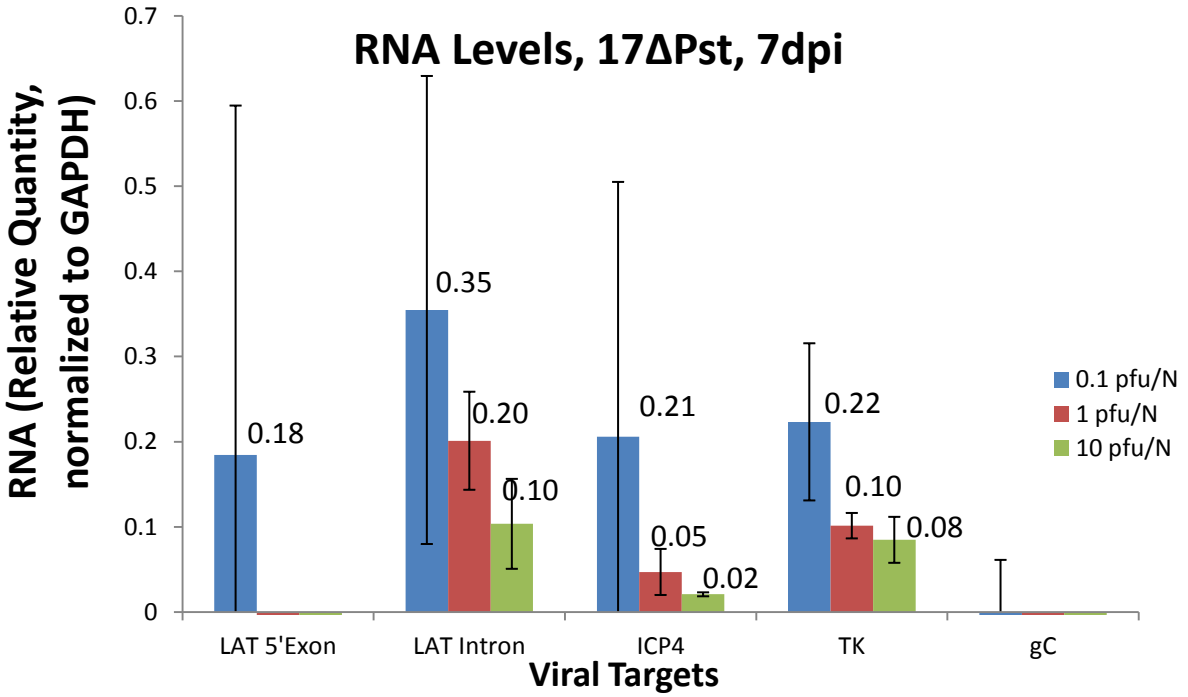


Figure 4-4. The relative abundance of HSV-1 transcript levels at a latent time point in TG cultures infected with a LAT-deletion mutant is largely independent of initial dose of virus. Levels of lytic and latent transcripts collected from primary trigeminal ganglia (TG) cultures infected with HSV-1 strain 17ΔPst at 6dpi with various doses (measured by pfu/neurons) are shown. Protocol for isolating RNA from samples is fully described in Materials and Methods. Briefly, RNA was collected using the TRIzol reagent, reverse-transcribed to cDNA with the Qiagen Omniscript Kit and viral targets were quantified through real-time PCR using Applied Biosystems StepOne Plus system. Relative RNA levels are normalized to a cellular control RNA (GAPDH); n = 6 TG cultures per viral dose.

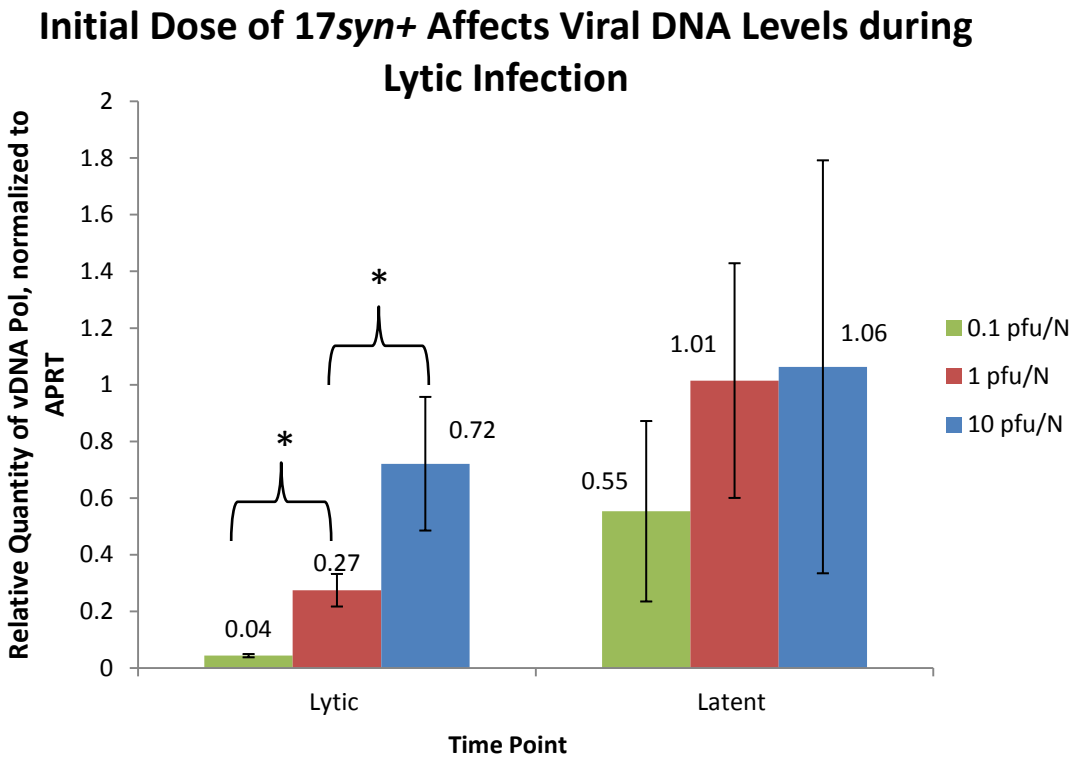


Figure 4-5. The initial dose of 17syn+ affects the viral DNA levels at a lytic time point. DNA levels of primary TG cultures infected with HSV-1 strain 17*syn*+ at lytic (10hpi) and latent (6dpi) time points are shown. The viral DNA polymerase was used as a primer target. Protocol for isolating DNA from samples is fully described in Materials and Methods. Briefly, DNA was back-extracted using the TRIzol reagent, and viral DNA was quantified through real-time PCR using Applied Biosystems StepOne Plus system. Relative DNA levels are normalized to a cellular control DNA (APRT); n = 6 TG cultures.

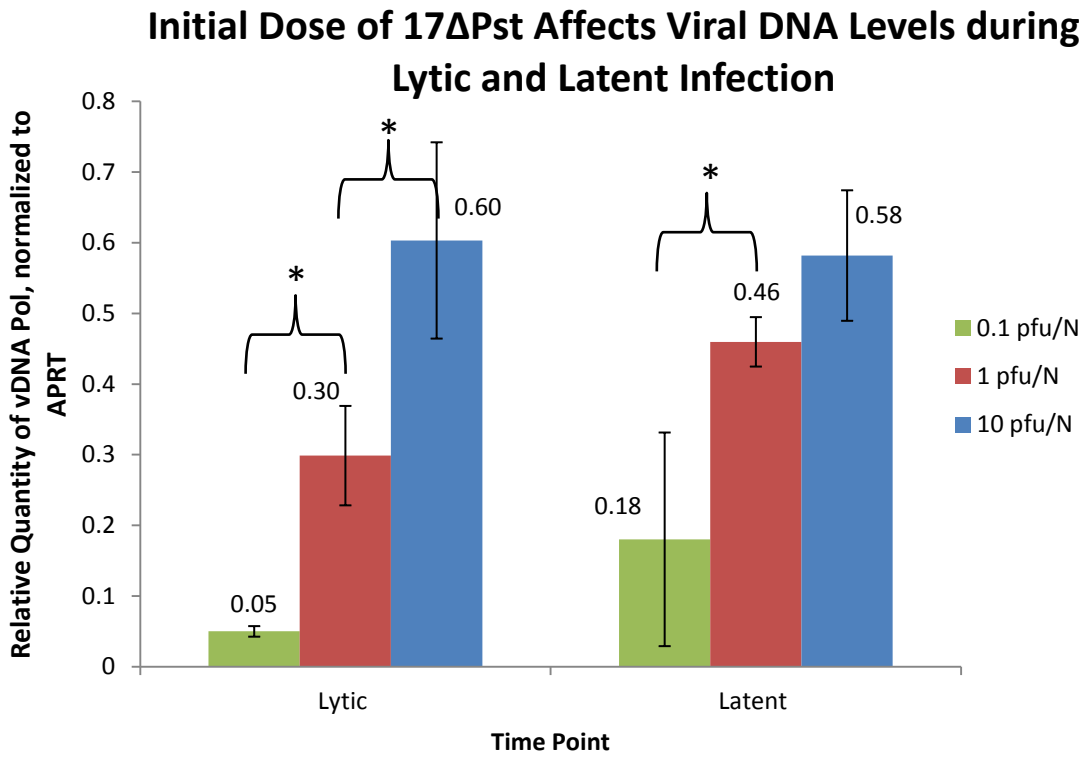


Figure 4-6. The initial dose of 17ΔPst affects the viral DNA levels at both lytic and latent time points. DNA levels of primary TG cultures infected with different pfu/N ratios of HSV-1 strain 17ΔPst at lytic (10hpi) and latent (6dpi) time points are shown. The viral DNA polymerase was used as a primer target. Protocol for isolating DNA from samples is fully described in Materials and Methods. Briefly, DNA was back-extracted using the TRIzol reagent, and viral DNA was quantified through real-time PCR using Applied Biosystems StepOne Plus system. Relative DNA levels are normalized to a cellular control DNA (APRT); n = 6 TG cultures.

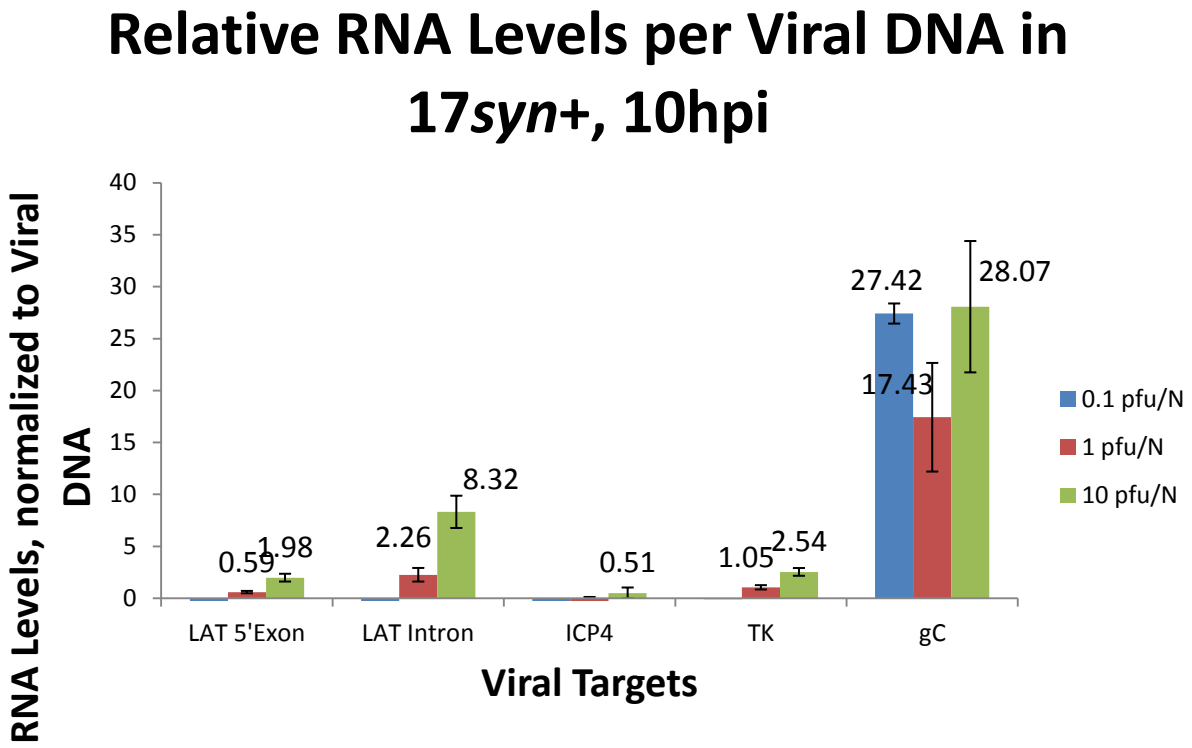


Figure 4-7. The relative abundance of HSV-1 transcripts on a per-viral DNA basis in infected TG cultures at a lytic time point are not dependent on the initial infective dose. Levels of latent and lytic transcripts collected from primary trigeminal ganglia (TG) cultures infected with HSV-1 strain 17*syn*+ at 10hpi with various doses (measured by pfu/neurons) are shown normalized to viral DNA levels. Protocol for isolating RNA and DNA from samples is fully described in Materials and Methods. Briefly, RNA was collected using the TRIzol reagent, reverse-transcribed to cDNA with the Qiagen Omniscript Kit while DNA was back-extracted from the same samples. Viral targets were quantified through real-time PCR using Applied Biosystems StepOne Plus system. Relative RNA levels are normalized to a cellular control RNA (GAPDH); n = 6 TG cultures per viral dose.

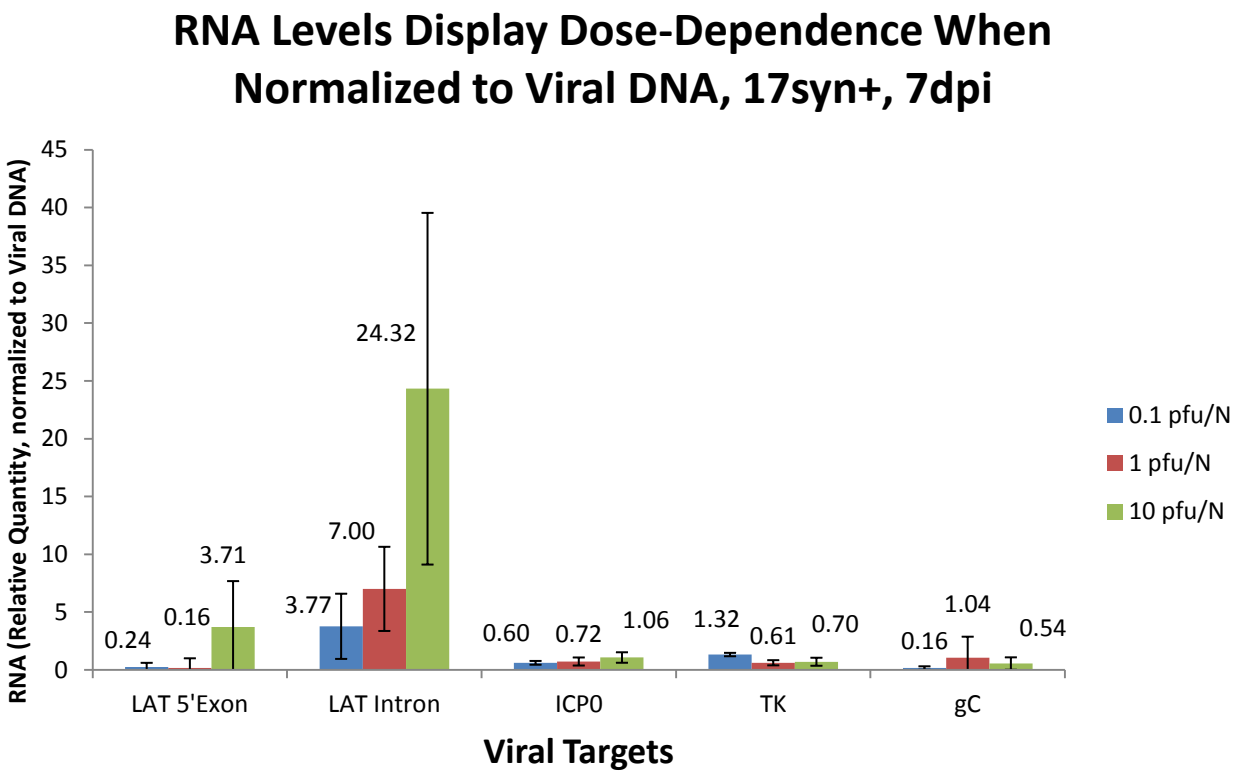


Figure 4-8. The relative abundance of HSV-1 transcripts on a per-viral DNA basis in infected TG cultures at a latent time point are dependent on the initial infective dose. Levels of latent and lytic transcripts collected from primary trigeminal ganglia (TG) cultures infected with HSV-1 strain 17syn+ at 10hpi with various doses (measured by pfu/neurons) are shown normalized to viral DNA levels. Protocol for isolating RNA and DNA from samples is fully described in Materials and Methods. Briefly, RNA was collected using the TRIzol reagent, reverse-transcribed to cDNA with the Qiagen Omniscript Kit while DNA was back-extracted from the same samples. Viral targets were quantified through real-time PCR using Applied Biosystems StepOne Plus system. Relative RNA levels are normalized to a cellular control RNA (GAPDH); n = 6 TG cultures per viral dose.

CHAPTER 5
EPIGENETICS OF HSV-1 INFECTING TG CULTURES

Background

In the previous chapter, we concluded that the initial infective viral dose had a profound effect on subsequent DNA levels and transcription of viral genes during both the acute and latent infections – and that this effect displayed some dependence on the LAT locus, as delineated using a recombinant virus containing a 202bp deletion in the core LAT promoter. With these observations, we hypothesized that changes in the differences in the total number of genomes entering the cell during the initial viral infection may result in differential levels of repression of the viral genome during latency. In this chapter, we aim to investigate a possible mechanism to explain this differential repression by assaying the epigenetic profile of the viral genomes latently infecting these TG cultures, for both the wild type virus, and the LATpromoter-deletion virus.

Previously, it has been shown that HSV-1 is associated with chromatin upon entering the host nucleus, and that differential post-transcriptional modifications to chromatin tails are associated with the virus (18, 42). For example, it has been shown that at a latent time point, the LAT locus is specifically enriched for active chromatin modifications like acetylation of the histone 3 at the positions of lysine 9 and 14 (H3K9,14ac) (41). At this time point, the LAT locus is the only portion of the HSV-1 genome which is heavily transcribed, as the lytic program of the virus is heavily repressed. Due to the presence of these active epigenetic marks, the LAT locus is said to be “euchromatic”, or of having “active” chromatin. Interestingly, the LAT locus is also found to be enriched for repressive chromatin marks characteristic of “heterochromatin”.

Marks associated with this type of chromatin are tri-methylation of the lysine 9 or 27 on histone H3 (H3K9me3 or H3K27me3). These marks are particularly enriched on the lytic genes at a latent time point, but the LAT locus appears to be enriched in them, as well. This is likely due to the fact that only about 1/3 of the latent HSV-1 genomes produce LAT, while the other population of HSV-1 genomes is largely completely repressed.

Since the epigenetic profiles of the HSV-1 genomes infecting the TG cultures was not known, a goal of this study was to determine the determine the specific histone modifications present on the HSV-1 genomes in these cultures during quiescence, and compare these with histone modification profiles known to be present during latency in sensory ganglia *in vivo*. As we have reported clear differences in the viral transcriptional activity of the cultures, we chose to assay two post-translational modifications of chromatin in these cultures: one euchromatic (active) mark—H3K4me3, and one heterochromatic (repressive) mark—H3K27me3. Further, since previous experiments have suggested that the LAT plays a role in the dose-dependent effects observed in viral transcription in the TG cultures, we assayed chromatin in cultures infected with both wild type HSV-1 and 17ΔPst, at different pfu/N ratios.

Results

Trigeminal Ganglia Cultures Latently Infected with Wild-Type HSV-1 Are More Enriched for the Euchromatic H3K4me3 when Infected with a Low pfu/N Ratio.

In order to determine the epigenetic profiles of the HSV-1 genomes during a latent infection of the TG cultures, and to identify the effect that different initial infective doses would play in the establishment of these profiles, we assayed the presence of a mark of transcriptionally active chromatin – the H3K4me3 mark. Cultures were infected

with both a low (0.1pfu/N) and high (10 pfu/N) levels of wild-type virus and processed for ChIP at seven days post-infection; the results are shown in Figure 5-1a and 5-1b, respectively. When TG cultures are infected with a low pfu/N ratio, as in Figure 5-1a, we see that there is moderate enrichment of this mark across all loci assayed, with the exception of the late lytic gene, gC. The other loci are all very similar to each other, with enrichments near the level of the cellular control, but are significantly more enriched than the gC locus. The observation that the levels of enrichment for this mark are globally uniform may suggest that these levels are all around background, but infection with a pfu/N ratio of 10 offers confirmation that these values are not merely background. Figure 5-1b shows that enrichment for this mark is significantly lower following a higher infective dose. This is true for every locus except for the gC locus, which has no enrichment difference for this mark, regardless of the pfu/N ratio.

When Latently Infected with the LAT-Deletion Mutant 17ΔPst, the Initial Dose of Virus Does Not Affect the Enrichment Levels of the H3K4me3 Euchromatic Mark.

We were also interested in the role that the LAT might play in regards to the deposition of H3K4me3 euchromatic marks on TG latently infected with HSV-1. To that end, we infected TG cultures with a LAT-deletion virus (17ΔPst) at both high and low levels of initial viral dose (pfu/N of 10 and 0.1, respectively), and assayed the HSV-1 genomes in the TG cultures for enrichment of this mark during latency. These data are shown in Figure 5-2. At a pfu/N ratio of 0.1, we observe that levels of H3K4me3 are very similar to those seen for the wild-type virus: all loci are enriched at levels near a cellular control, with the exception of the late gene gC, which is significantly lower. However, when we perform the same experiment at 10 pfu/N, as in part b of the figure,

we see similar levels of enrichment for the LAT enhancer, ICP0, and TK loci. This is in contrast to the wild-type virus, where the higher pfu/N infection resulted in a lower enrichment for the H3K4me3 mark.

Latently-Infected TG cultures Infected with a Higher Initial Dose of Wild-Type Virus are More Enriched for the H3K27me3 Chromatic Marks than Those Infected with a Lower Dose.

We were also interested in the effect that different pfu/N infections would have on the establishment of a repressive chromatin mark, in this case the heterochromatic H3K27me3. As before, cultures were infected with both low (0.1 pfu/N) and high (10 pfu/N) levels of wild-type virus, and processed for ChIP at 6dpi; the results are show in Figure 5-3a and 5-3b, respectively. For the lower pfu/N infection, there is generally a low level of enrichment for the H3K27me3 mark, at levels ranging from about one-third the levels of a cellular control (APRT) up to two-thirds of that. The loci which are enriched at the lower level are ICP4 (0.36) and the LAT promoter (0.37). Interestingly, even the locus which is most-enriched for this mark, the gC locus, only shows about double the enrichment (0.69), an increase which is not of statistical significance. Therefore, we can conclude that levels of this mark are relatively similarly low across the wild-type HSV-1 genome when TG cultures are infected at a low pfu/N level.

By contrast, when the TG cultures are infected with a high pfu/N ratio, as in part b of Figure 3.3, there are not only notable differences between the various loci assayed, but there is greater enrichment across the genome when compared to the low pfu/N infection as well. For every locus assayed, enrichment of H3K27me3 is higher with the infection of 10 pfu/N. This is most evident with the ICP4 locus, where a low pfu/N infection resulted in enrichment levels of 0.36, whereas a high pfu/N infection resulted in

enrichment of 2.3, over six-fold higher. Even the loci with the most enrichment at a low pfu/N infection, ICP0 and gC, are more enriched when TG cultures are infected with a higher dose of virus. Therefore, these data suggest that increased pfu/N infections may result in greater deposition of repressive, heterochromatic marks. In addition, while enrichment for H3K27me3 is fairly uniform across the HSV-1 genome infected with a low pfu/N, when infected with a higher dose, there are marked differences between loci. For example, the ICP4 locus is the most enriched locus, particularly in comparison to the LAT promoter and the TK locus. Using the mouse model, we have previously observed that the ICP4 locus has the highest enrichment for this mark *in-vivo*, as well, thereby corroborating previous observations in an analogous system.

Latently-Infected TG Cultures Infected with a LAT Promoter-Deletion Mutant, 17ΔPst, Display Similar Levels of Enrichment for the Heterochromatic H3K27me3 Mark Regardless of the Initial Infective Dose.

We have previously determined that the absence of LAT transcription results in an abrogation of the pfu/N dose-dependent effect on viral transcription. This dose-dependent effect seems to be present at the epigenetic level as well (see Figures 5-1 and 5-3). To investigate the role of the LAT on repressive epigenetic modifications to the HSV-1 latent genomes, we infected TG cultures with a LAT promoter-deletion mutant with both high and low infective doses (10 and 0.1 pfu/N, respectively), and assayed enrichment for the heterochromatic mark H3K27me3 in these cultures during a latent infection. These data are shown in Figure 5-4. Part A shows the enrichment for H3K27me3 at the low pfu/N infection across five viral targets: the LAT 5' exon, the immediate early transactivator genes ICP0 and ICP4, the early gene TK, and the late gene gC. Across these loci, we report enrichment lower than that that seen for the cellular control, and we cannot observe any locus which has significantly different

enrichment from the others. This is similar to what we have observed before with low pfu/N infections. In addition, when we infect with a high pfu/N ratio, we see a similar pattern of enrichment. In fact, for all five loci assayed, none appear to have significantly different levels of enrichment between the low and high infective doses. Taken together, these data suggest that while the initial amount of infecting wild-type virus may result in increased H327me3 enrichment, this is not the case in the absence of the LAT. Thus, it is possible that this dose-dependent effect is closely related to the LAT and its functions.

At a Higher Inoculum (10 pfu/N), the Wild-Type Virus is More-Enriched for the Repressive Mark H3K27me3 than the LAT Promoter-Deletion Mutant 17ΔPst

We have observed that LAT-positive viruses (like the wild-type 17*syn*+) behave differently than LAT-negative viruses *vis-a-vis* the effect of different pfu/N infections; namely, only the LAT-positive virus displays a clear dose-dependence. We think that it is also important to note that at the highest pfu/N ratios, there are significant differences in the enrichment levels for H3K27me3 between these viruses. For example, in Figures 5-3B and 5-4B, we see that while the wild-type TK locus is enriched for this mark at levels 2.3 times that of the cellular control, the LAT-deletion mutant is only 0.89 times that of the same cellular control. This is merely the most clear example: the other loci are all more enriched for this mark in the wild-type virus than they are for the LAT-deletion mutant. This suggests that the wild-type virus is more sensitive to the effect of infective dose such that higher doses result in significantly greater levels of enrichment for the H3K27me3 mark.

The 202bp Deletion in the 17ΔPst Virus Results in Robust Increases in Enrichment for Both Active and Repressive Chromatin Marks at the LAT Promoter Locus.

In addition to the global analyses in previous sections, there is one particular observation at a specific locus that requires specific attention: the LAT promoter locus in 17ΔPst virus infections. These data have been collected and are shown in Figure 5-5. In parts A and B of this figure, we observe that the enrichment for the H3K4me3 mark on the LAT promoter locus is higher than we see at any other locus, over 50-fold higher than the cellular control at low pfu/N infection, and over 80-fold higher with the high pfu/N infection. Additionally, we observe that this mark is clearly more enriched than we see in the wild-type infection as well, over 50-fold higher than wild type LAT promoter at the lower pfu/N infection, and nearly 400-fold higher than wild type LAT promoter at the higher pfu/N infection. Furthermore, we see a similar extreme increase in enrichment for the repressive mark H3K27me3, as seen in parts C and D of the figure, with enrichment 40-fold greater than the cellular control, and 100-fold greater than the wild-type LAT promoter at a low pfu/N infection, and 100-fold greater enrichment than the wild-type LAT promoter at a high pfu/N infection. These observations are remarkable not only due to the fact that these levels of enrichment are the greatest observed in these ChIP experiments using TG cultures, but because we found both an active (H3K4me3) mark and a repressive (H3K27me3) mark enriched at the same locus. Since this locus is located very close to the 202bp deletion of the LAT core promoter, we believe that this DNA element likely has some critical regulatory effect.

Conclusions

The Effect of Initial pfu/N Infection Ratio on Epigenetic Profiles of HSV-1 is LAT-Dependent

In both experiments assaying the chromatin of the HSV-1 genomes latently infecting TG cultures, one assaying the euchromatic H3K4me3 and one assaying the heterochromatic H3K27me3, enrichment levels of the respective mark displayed a dose-dependent effect with the wild-type virus only. With regards to the euchromatic mark (H3K4me3), we observed that at a low pfu/N ratio, enrichment levels were much higher than at a high pfu/N ratio. The converse was true for the repressive mark – a low pfu/N ratio resulted in lower enrichment levels for H3K27me3 than did a high pfu/N ratio. These data suggest that the wild-type virus is sensitive to the initial dose of the virus infecting the TG cultures. However, neither of these effects were observed with the LAT promoter-deletion mutant 17ΔPst. For this virus, enrichment levels of both H3K4me3 and H3K27me3 remained unchanged regardless of the initial viral inoculum delivered to the TG cultures. Taken together, we can conclude that the LAT likely plays a role in the dose-dependence effect that we observe in the wild-type virus.

This effect could have one of two broad explanations. First, it is possible that the LAT RNA, particularly the stable LAT Intron, could be acting as a long non-coding RNA (lncRNA). Unpublished data from our lab indicates that one function of the LAT intron is to act as a sponge or decoy for the Polycomb Repressive Complex 1 (PRC1). This complex is responsible for maintaining heterochromatic marks on chromatin, leading to localized repression of transcription. The LAT may be directing this complex away from the HSV-1 genomes, in an effort to prevent the viral genome from becoming repressed to the point where reactivation becomes less efficient. This model is consistent with the

long-standing observation that one of the most striking phenotypes of LAT mutants is their inability to reactivate efficiently in the rabbit ocular model.

Another possibility is that the 202bp deletion in the 17ΔPst contains important DNA elements, without which a critical regulatory mechanism is disrupted. For example, if elements exist in this region which are responsible for recruitment of chromatin-remodeling complexes, deletion of the core LAT promoter could abolish or alter the control of the chromatin state by the virus. In such a case, the wild-type virus could remain sensitive to dose-dependent effects, as increased amounts of viral genomes compete for cellular chromatin remodeling complexes. The mutant virus would not direct such complexes at all, and would be remodeled passively, a process which may not be dissimilar between high and low infective doses.

At a High pfu/N Ratio, Wild-Type Viral Genomes Appear More Repressed than a LAT-Deletion Mutant

In latently-infected TG cultures, the wild-type strain 17*syn*+ displays a chromatin architecture which suggests that it is in a more repressed state than is the LAT-deletion mutant 17ΔPst. When infected with a pfu/N ratio of 10, wild-type virus is significantly less-enriched for the active euchromatic mark H3K4me3, across the genome, when compared to the mutant. In addition, the wild-type virus is significantly *more* enriched for the repressive heterochromatic mark H327me3. Taken together, we can conclude that the wild-type genomes latently infecting the TG cultures are in a more-repressed epigenetic state than are their mutant counterparts.

During a latent time point, it is advantageous for the virus to be in a repressed state, and the LAT likely plays a critical role in establishing or maintaining this repression. Thus, it is possible that the mutant virus is mis-regulated with regard to LAT

function, and this results in viral genomes which are altogether less-repressed in this system. This would have other consequences as well. For example, if the mutant viruses were truly less-repressed, then they may be more prone to spontaneous reactivation, and reactivate in response to stimuli at increased efficiency, as well. This could result in additional rounds of the acute viral infection, and likely in addition neuronal death in the dishes.

Wild-Type Viral Genomes are Less Repressed When Initially Infected with a Low pfu/N Ratio.

Though wild-type genomes appear to be more repressed than LAT mutants, it is important to note that this is not the case at a lower pfu/N infection. In fact, we have observed that at a lower initial viral dose (0.1 pfu/N), the latent wild-type genomes are significantly less repressed than at a higher viral dose. At this lower initial dose, we observe that H3K4me3 levels are significantly more enriched on the viral genomes than they are at a pfu/N of 10. Concordantly, we also observe that the repressive H3K27me3 mark is less enriched on HSV-1 genomes resulting from a 1 pfu/N infection than on genomes produced from a 10 pfu/N infection. These two observations suggest that at a lower initial infective dose, the wild-type virus exhibits a less-repressed epigenetic phenotype.

The LAT Promoter Locus Is Highly Enriched for Both Euchromatic and Heterochromatic Marks During a Latent 17ΔPst Infection

The LAT promoter locus of 17ΔPst is the most highly-enriched locus which we assayed, for both chromatin marks. This LAT deletion mutant has a 202bp deletion in the core LAT promoter, but the LAT locus is otherwise intact. We were very surprised to observe such high levels of enrichment on this locus during a latent infection with the mutant virus, especially considering that this robust enrichment occurs during both low

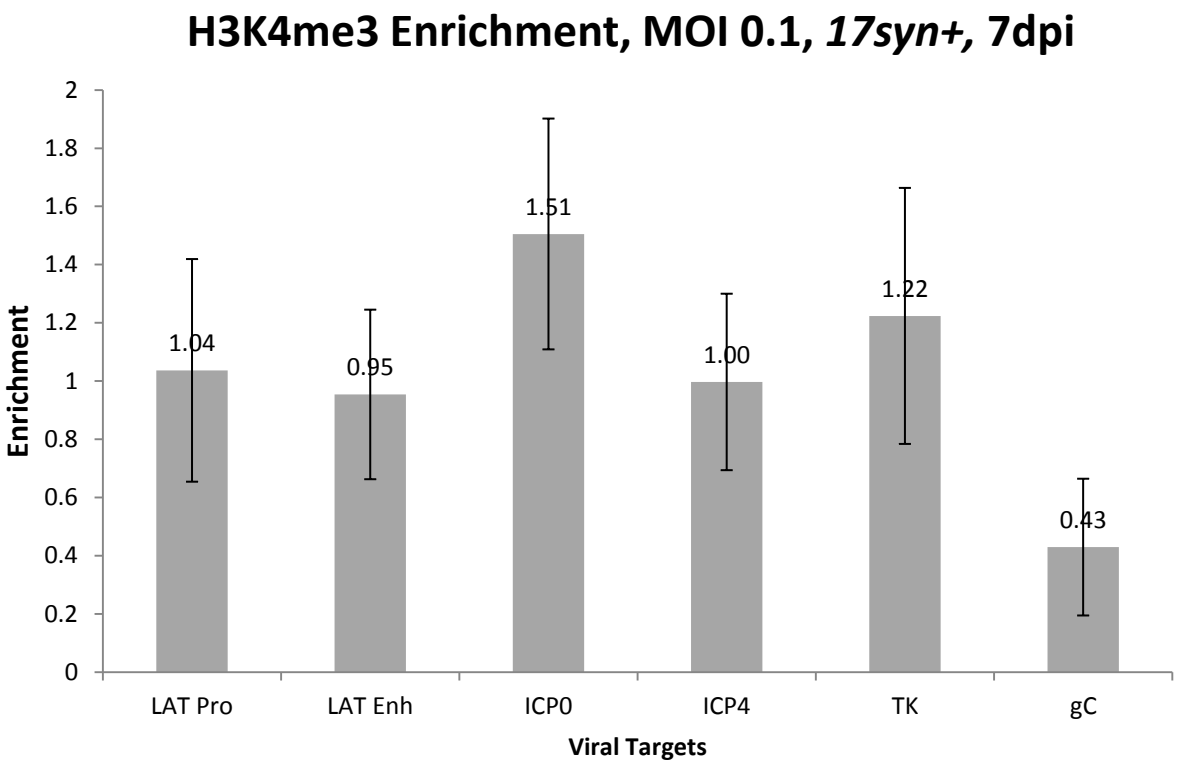
and high pfu/N infections, and involves both euchromatic and heterochromatic modifications. There are two issues with this observation: first, why is the enrichment of these marks so much higher than we have previously observed; and second, why are *both* marks present at the same locus. It is worth mentioning that the heterochromatic mark H3K27me3 was observed to be highly enriched at this locus in mouse footpad infections with the same virus (Kwiatkowski, unpublished data), but this is the first observation of such a robust H3K4me3 enrichment.

One explanation for the presence of both of these marks is that there are two distinct populations in 17ΔPst-infected TG cultures: one which is highly enriched for H3K4me3, and one which is highly enriched for H3K27me3. *In-vivo* experiments in animal models have reported that there are distinct populations of neurons during an HSV-1 infection, and that these populations can have different epigenetic profiles, resulting in the appearance of this bi-valent chromatin (i.e., both eu- and heterochromatin at the same locus). At this time, we are not aware of what mechanism would result in these distinct populations, especially considering that both population would be LAT-deficient, which is not the case with the *in-vivo* observations (they were always with LAT-positive infections).

Another possible explanation for this high level of enrichment was mentioned earlier in relation to the possible effect of the LAT with regards to a pfu/N-dependent effect. In essence, it is possible that the deletion of a 202bp DNA fragment results in the removal of one – or many – DNA elements responsible for the recruitment and regulation of chromatin remodeling complexes. In the absence of these DNA elements, it is possible that whichever chromatin remodeling complex first associates with this

locus permanently remains there, catalyzing the deposition of its post-translational modification. This could result in HSV-1 genomes where very high levels of many disparate chromatin modifications are present across the entire HSV-1 genome pool, all due to massive misregulation of the chromatin remodeling machinery at this particular locus.

A


H3K4me3 Enrichment, MOI 0.1, 17syn+, 7dpi
Enrichment
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.04
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1.00
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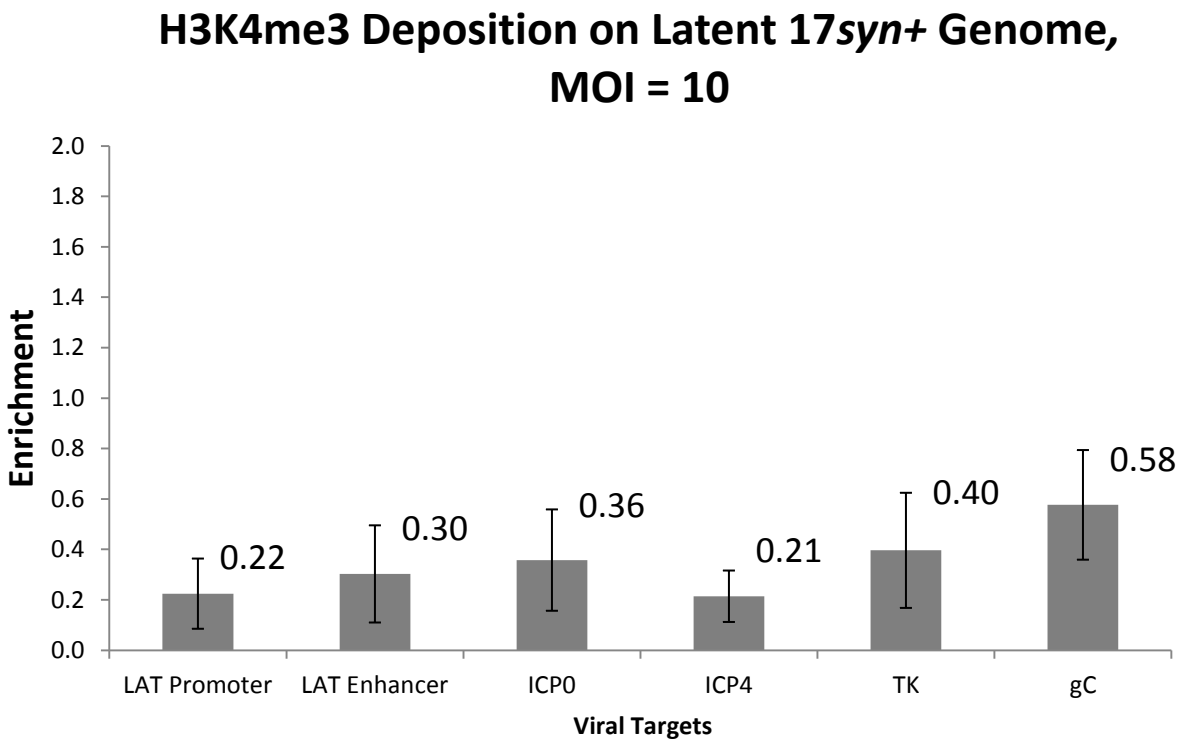
LAT Pro
LAT Enh
ICP0
ICP4
TK
gC
Viral Targets

B


H3K4me3 Deposition on Latent 17syn+ Genome, MOI = 10
Enrichment
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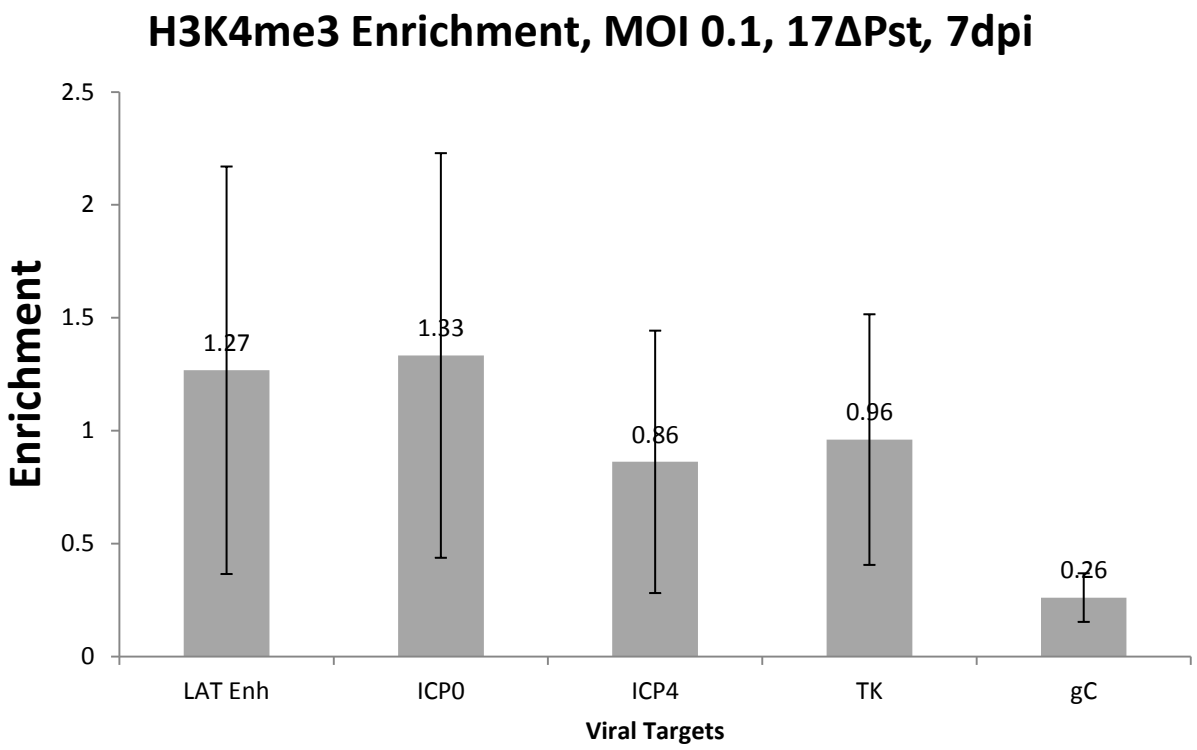
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.22
0.30
0.36
0.21
0.40
0.58
LAT Promoter
LAT Enhancer
ICP0
ICP4
TK
gC
Viral Targets

Figure 5-1. Latently-infected trigeminal ganglia (TG) cultures infected with a higher initial dose are less enriched for the H3K4me3 chromatic marks than those infected with a lower dose. A) Deposition of the euchromatic mark H3K4me3 on TG cultures latently infected with *17syn+* (pfu/N: 0.1), as assayed by chromatin immuno-precipitation (ChIP). B) As in A, except TG cultures were infected with pfu/N ratio of 10. It is evident that at the higher infective dose, there is less enrichment for the active H3K4me3 mark. ChIPs were performed at 7dpi, using a previously described protocol (4). Briefly, TG cultures were fixed with % formaldehyde, sonicated, and immunoprecipitated with α-trimethyl-H3K4 antibody, collecting both bound and unbound samples. DNA was isolated via Qiagen Nucleotide Removal columns and viral genes from each gene class were assayed by Taqman® qPCR via Applied Biosystems StepOne Plus system. Data are shown as relative quantities (Bound/(Unbound + Bound)) compared to an endogenous cellular control, UpHoxA5, shown here as “Enrichment”. Error bars are ± one standard deviation from the mean, n = 6.

A


H3K4me3 Enrichment, MOI 0.1, 17ΔPst, 7dpi
Enrichment
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2
1.5
1
0.5
0
1.27
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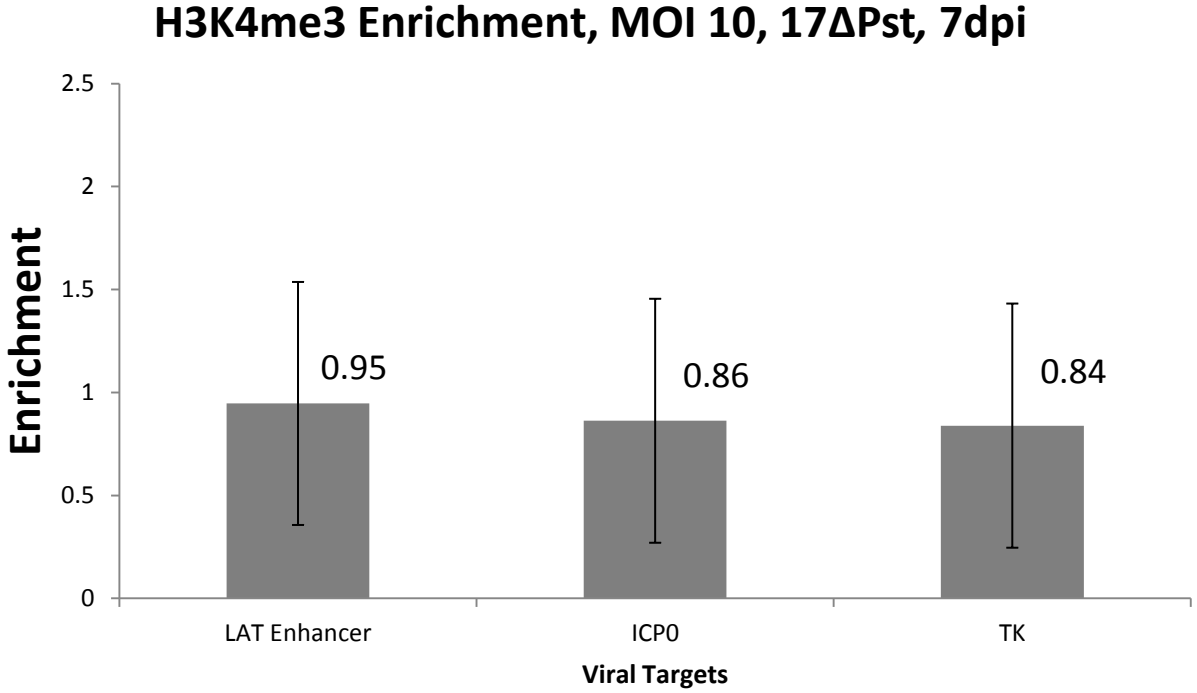
0.86
0.96
0.26
LAT Enh
ICP0
ICP4
TK
gC
Viral Targets

B


H3K4me3 Enrichment, MOI 10, 17ΔPst, 7dpi
Enrichment
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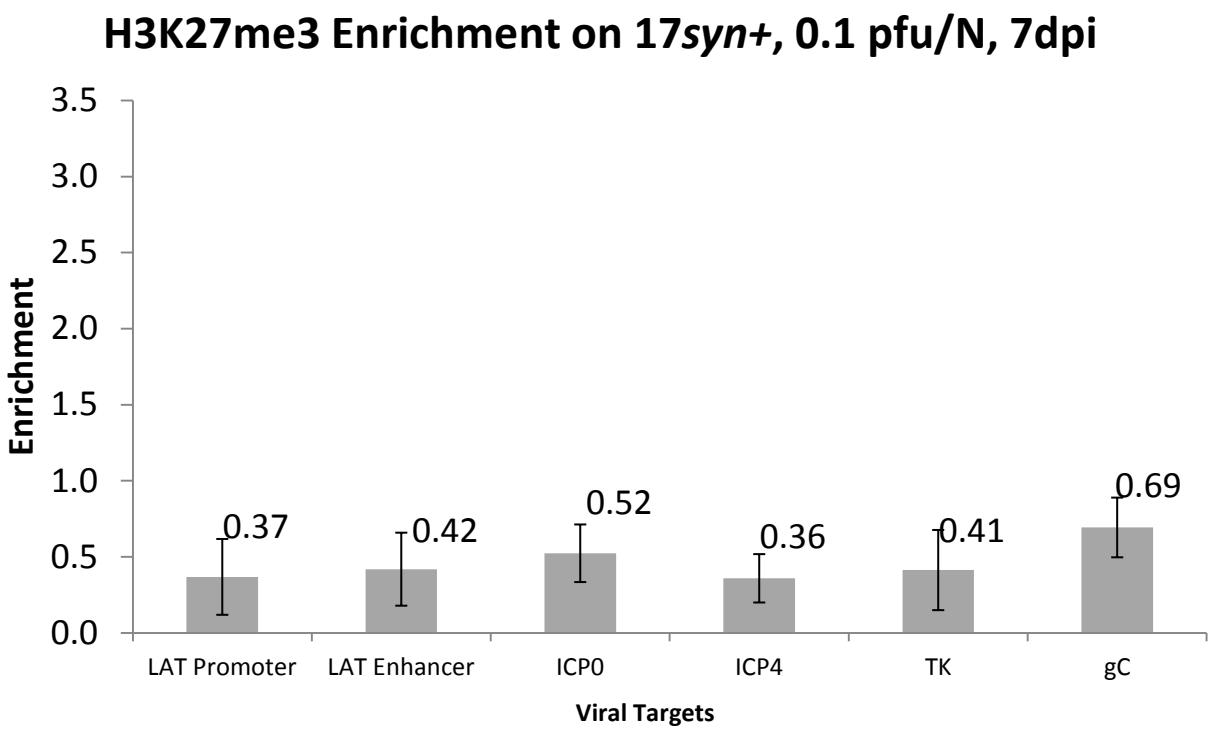
2
1.5
1
0.5
0
0.95
0.86
0.84
LAT Enhancer
ICP0
TK
Viral Targets

Figure 5-2. Latently-infected trigeminal ganglia (TG) cultures infected with 17ΔPst, a LAT-deletion virus, are enriched for the H3K4me3 chromatic marks at equal levels regardless of initial infective dose. A) Deposition of the euchromatic mark H3K4me3 on TG cultures latently infected with 17ΔPst (pfu/N: 0.1), as assayed by chromatin immuno-precipitation (ChIP). B) As in A, except TG cultures were infected with pfu/N ratio of 10. ChIPs were performed at 7dpi, using a previously described protocol (4). Briefly, TG cultures were fixed with % formaldehyde, sonicated, and immunoprecipitated with α-trimethyl-H3K4 antibody, collecting both bound and unbound samples. DNA was isolated via Qiagen Nucleotide Removal columns and viral genes from each gene class were assayed by Taqman® qPCR via Applied Biosystems StepOne Plus system. Data are shown as relative quantities (Bound/(Unbound + Bound)) compared to an endogenous cellular control, UpHoxA5.

A


H3K27me3 Enrichment on 17syn+, 0.1 pfu/N, 7dpi
Enrichment
3.5
3.0
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2.0
1.5
1.0
0.5
0.0
0.37
0.42
0.52
0.36
0.41
0.69
LAT Promoter
LAT Enhancer
ICP0
ICP4
TK
gC
Viral Targets

B

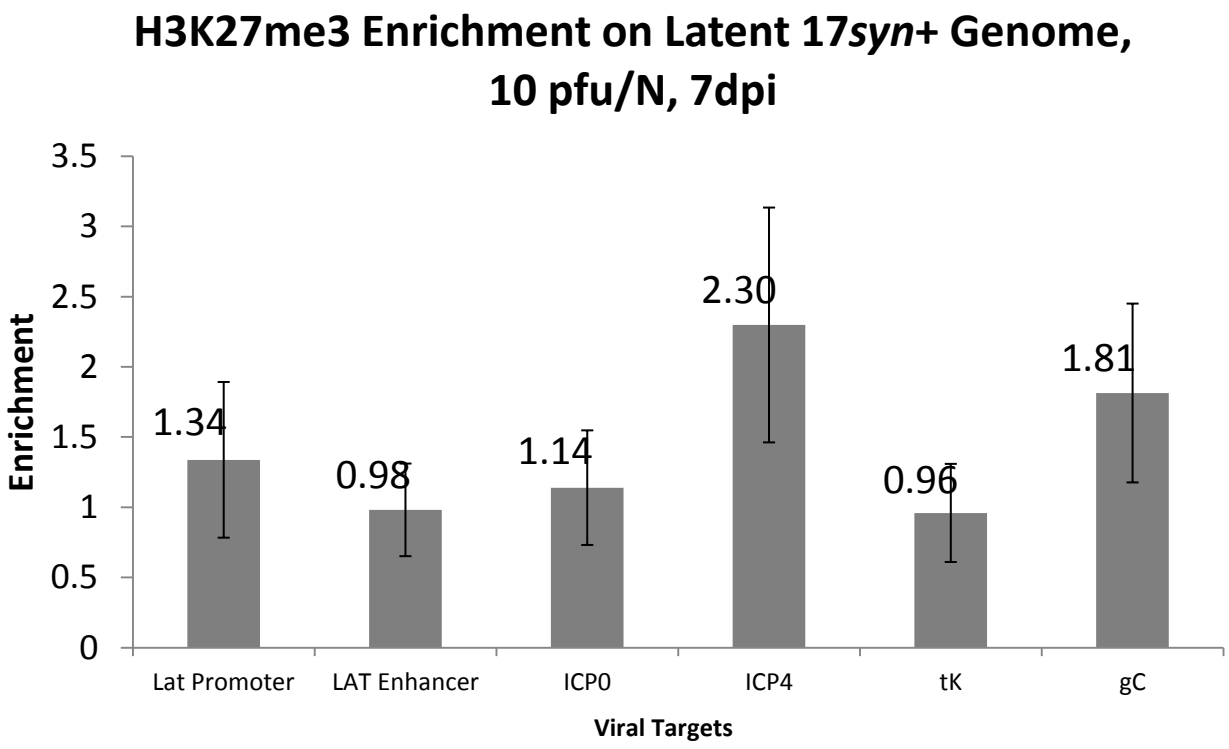
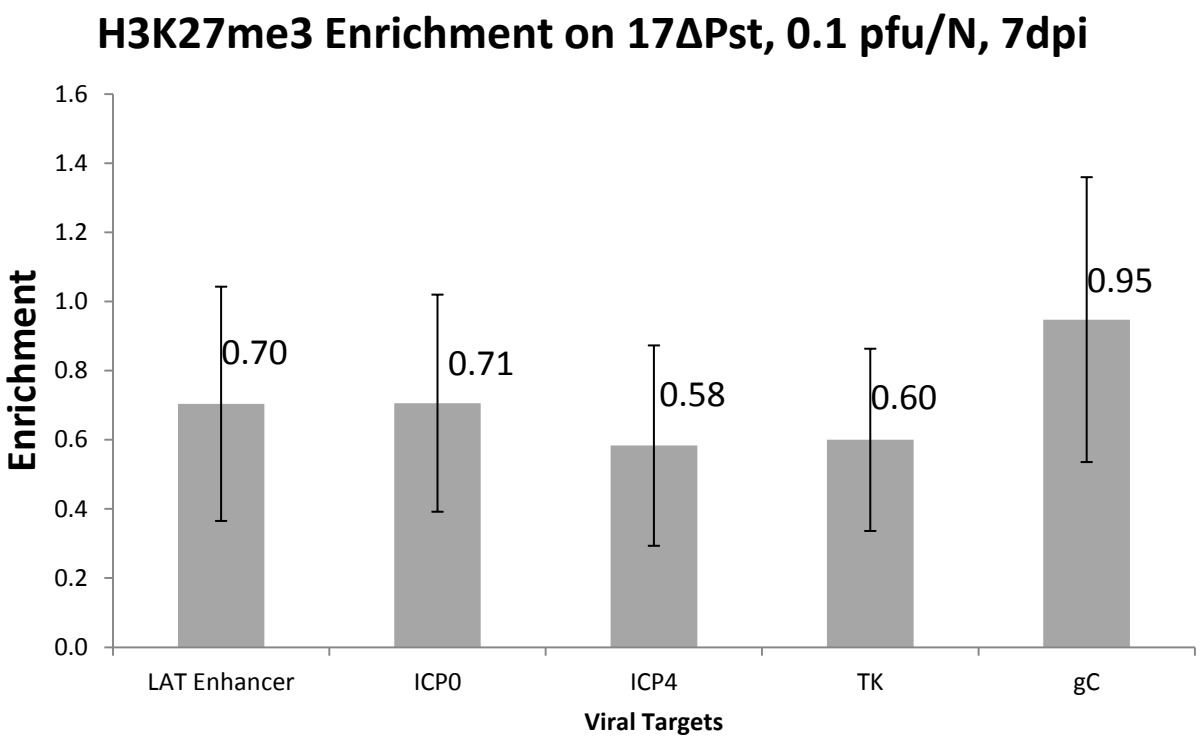

H3K27me3 Enrichment on Latent 17syn+ Genome, 10 pfu/N, 7dpi
Enrichment
3.5
3
2.5
2
1.5
1
0.5
0
1.34
0.98
1.14
2.30
0.96
1.81
Lat Promoter
LAT Enhancer
ICP0
ICP4
tK
gC
Viral Targets

Figure 5-3. Latently-infected trigeminal ganglia (TG) cultures infected with a higher initial dose are more enriched for the H3K27me3 chromatic marks than those infected with a lower dose. A) Deposition of the heterochromatic mark H3K27me3 on TG cultures latently infected with *17syn+* (pfu/N: 0.1), as assayed by chromatin immuno-precipitation (ChIP). B) As in A, except TG cultures were infected with MOI of 10. It is evident that at the higher infective dose, there is greater enrichment for the repressive H3K27me3 mark. ChIPs were performed at 6dpi, using a previously described protocol (4). Briefly, TG cultures were fixed with % formaldehyde, sonicated, and immunoprecipitated with α-trimethyl-H3K27 antibody, collecting both bound and unbound samples. DNA was isolated via Qiagen Nucleotide Removal columns and viral genes from each gene class were assayed by Taqman® qPCR via Applied Biosystems StepOne Plus system. Data are shown as relative quantities (Bound/(Unbound + Bound)) compared to an endogenous cellular control, APRT.

A


H3K27me3 Enrichment on 17ΔPst, 0.1 pfu/N, 7dpi
Enrichment
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.70
0.71
0.58
0.60
0.95
LAT Enhancer
ICP0
ICP4
TK
gC
Viral Targets

B

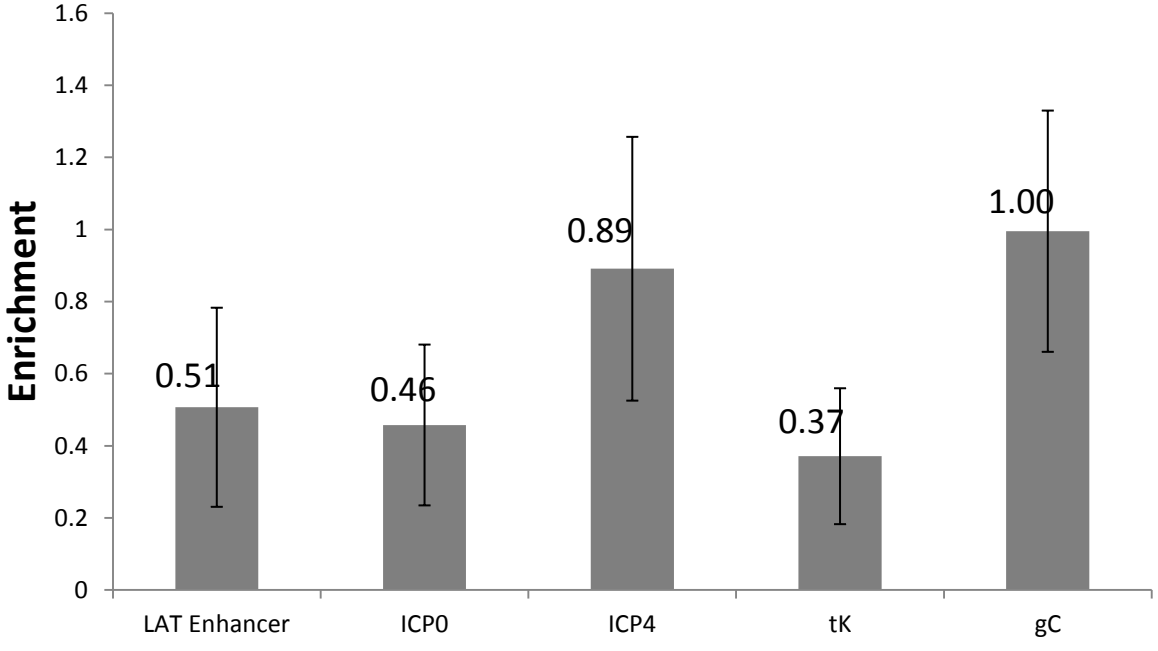
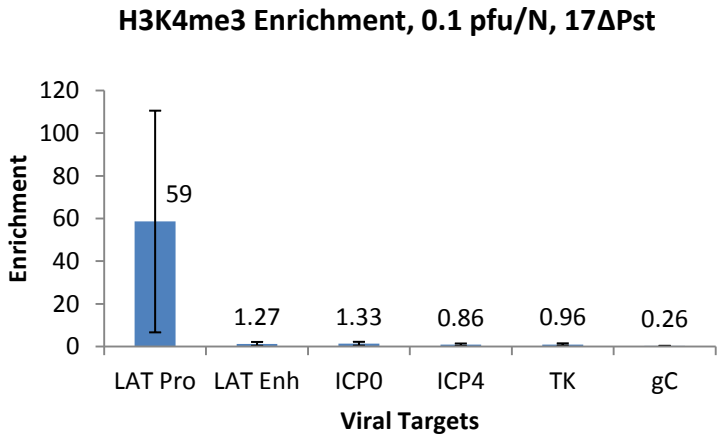
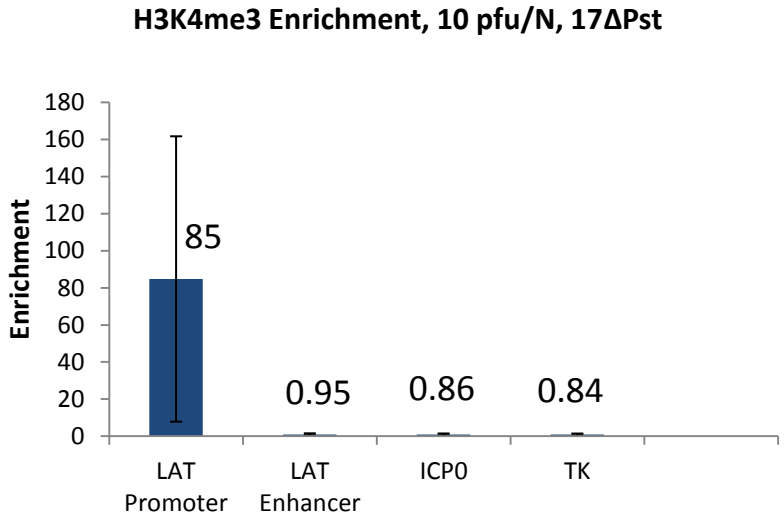

H3K27me3 Enrichment on 17ΔPst, 10pfu/N, 7dpi
Enrichment
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.51
0.46
0.89
0.37
1.00
LAT Enhancer
ICP0
ICP4
tK
gC

Figure 5-4. Latently-infected trigeminal ganglia (TG) cultures infected with a LAT-deletion mutant, 17ΔPst, display similar levels of enrichment in the heterochromatic H3K27me3 mark regardless of the initial infective dose. A) Deposition of the heterochromatic mark H3K27me3 on TG cultures latently infected with 17ΔPst (MOI: 0.1), as assayed by chromatin immuno-precipitation (ChIP). B) As in A, except TG cultures were infected with an initial MOI of 10. Unlike the infection with the WT virus, enrichment for the repressive H3K27me3 mark does not appear to be dependent on the initial MOI. ChIPs were performed at 6dpi, using a previously described protocol (4). Briefly, TG cultures were fixed with % formaldehyde, sonicated, and immunoprecipitated with α-trimethyl-H3K27 antibody, collecting both bound and unbound samples. DNA was isolated via Qiagen Nucleotide Removal columns and viral genes from each gene class were assayed by Taqman® qPCR via Applied Biosystems StepOne Plus system. Data are shown as relative quantities (Bound/(Unbound + Bound)) compared to an endogenous cellular control, APRT.

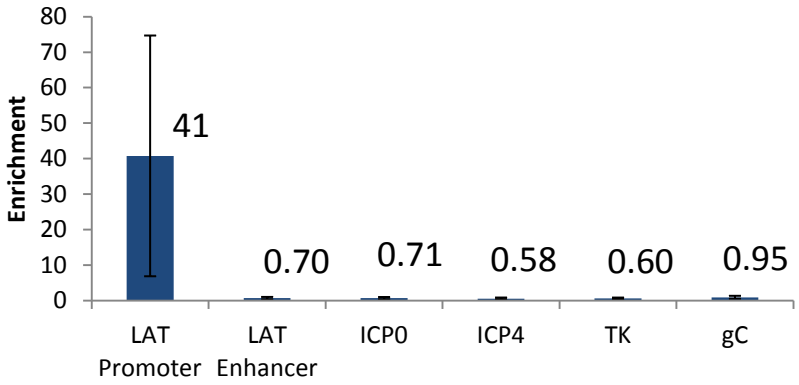
A


H3K4me3 Enrichment, 0.1 pfu/N, 17ΔPst
Enrichment
120
100
80
60
40
20
0
59
1.27
1.33
0.86
0.96
0.26
LAT Pro
LAT Enh
ICP0
ICP4
TK
gC
Viral Targets

B


H3K4me3 Enrichment, 10 pfu/N, 17ΔPst
Enrichment
180
160
140
120
100
80
60
40
20
0
85
0.95
0.86
0.84
LAT Promoter
LAT Enhancer
ICP0
TK

C


H3K27me3 Enrichment, 0.1 pfu/N, 17ΔPst
Enrichment
80
70
60
50
40
30
20
10
0
41
0.70
0.71
0.58
0.60
0.95
LAT Promoter
LAT Enhancer
ICP0
ICP4
TK
gC

D

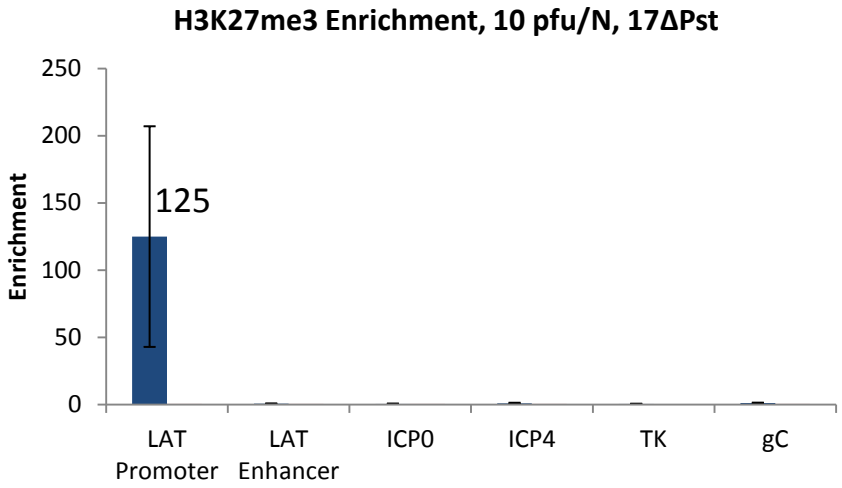


Figure 5-5. Latently-infected trigeminal ganglia (TG) cultures infected with a LAT-deletion mutant, 17ΔPst, display robust enrichment for both a repressive chromatin mark, H3K27me3, and an active chromatin mark, H3K4me3, at the LAT promoter locus. A) Deposition of the euchromatic mark H3K4me3 in TG cultures latently infected with 17ΔPst (0.1 pfu/N), as assayed by chromatin immuno-precipitation (ChIP). B) As in A, except pfu/N = 10, instead. C and D) As in A and B, except the heterochromatic mark H3K27me3 is assayed instead. There is a marked, significant increase in enrichment for both of these marks at the LAT promoter locus, regardless of pfu/N ratio. ChIPs were performed at 6dpi, using a previously described protocol (4). Briefly, TG cultures were fixed with % formaldehyde, sonicated, and immunoprecipitated with α-trimethyl-H3K27 antibody, collecting both bound and unbound samples. DNA was isolated via Qiagen Nucleotide Removal columns and viral genes from each gene class were assayed by Taqman® qPCR via Applied Biosystems StepOne Plus system. Data are shown as relative quantities (Bound/(Unbound + Bound)) compared to an endogenous cellular control, APRT, which is described here as, “Enrichment”. Error bars represent ± one standard deviation, 6 ≤ N ≤9.

CHAPTER 6
REACTIVATION OF LATENT VIRUS FROM TG CULTURES

Background

In previous chapters we found that the initial virus inoculum delivered to the TG cultures affected both transcript levels and the chromatin profile of HSV-1 genomes in the cultures, but only with a LAT-positive virus. In each case, the higher pfu/N ratio infections resulted in a more repressive state: decreased H3K4me3, increased H3K27me3, and increased LAT transcription. If these genomes are more repressed, relative to those resulting from lower pfu/N infection ratios, then we hypothesize that they will reactivate at a greater frequency.

In addition, since the cultures infected with high pfu/N ratios are enriched for H3K27me3, we were interested in a small-molecule inhibitor which could reduce levels of this mark, DZNep (63). This inhibitor has been shown to reduce EZH2 activity and thereby reduce levels of H3K27me3. In addition to its initial use as an inducer of apoptosis in cancer cells (63), this inhibitor was also shown to reduce H3K27me3 levels in two gammaherpesviruses, Epstein-Barr Virus (50), and KSHV (64). These previous findings suggest that the EZH2 inhibitor DZNep can affect H3K27me3 levels on herpesvirus genomes, which we would like to recapitulate in HSV-1.

Results

TG Cultures Infected with the Highest pfu/N Ratio Reactivate More Efficiently than Those Infected with the Lowest pfu/N Ratio

Since previous experiments suggested that the TG cultures infected with higher pfu/N ratios were overall more repressed than their counterparts infected at low pfu/N ratios, we assayed both cultures for their ability to reactivate from latency. After these cultures were infected for seven days, the media was removed, the cells washed, and a

new media lacking growth factors and containing α-NGF antibody was applied for 24 hours. At this point, both media and cells were collected for plaque assays, although the cellular portion did not produce any plaques in this assay. Reactivation media was titrated on rabbit skin cells, with the results shown in Table 6-1. We observed that while the total pfu/mL of the reactivation media was generally low, with the highest levels being around 600 pfu/mL in the 0.1 pfu/N infections, reactivation was much less frequent in the cultures infected with a pfu/N ratio of 10 (average of 188 pfu/mL, compared with 425 pfu/mL for 0.1pfu/N). This difference was nearly statistically significant (p-value of 0.055), suggesting that there may be a dose-dependent trend with regards to reactivation from latency in these TG cultures, as expected.

HSV-Infected TG Cultures Treated with EZH2 Inhibitor DZNep are Less Enriched for H3K27me3 than Untreated Cultures

In addition to assaying cultures infected with different inoculums of virus for reactivation, we were also interested in altering the chromatin state of the cultures through use of the small molecule DZNep to induce a state of more capable reactivation. First, we needed to confirm that DZNep can reduce H3K27me3 levels in our culture system, so we performed ChIP experiments on HSV-1-infected cultures both treated and untreated with DZNep and assayed enrichment of H3K27me3 levels across the same viral loci previously examined. These data are shown in Figure 6-1.

When cultures infected with the wild type strain 17*syn*+ are treated with DZNep (part A), we see a general reduction of H3K27me3 enrichment across all loci, and a significant reduction at both the ICP0 and ICP4 loci. This reduction is generally modest, but it is nearly five-fold at the ICP4 locus. This locus is also the most highly enriched for H3K27me3 in untreated samples, but after treatment with DZNep, enrichment for

H3K27me3 is similar across all loci, suggesting that after treatment with DZNep, H3K27me3 enrichment is reduced to near-background levels, as hypothesized.

Additionally, in cultures infected with the LAT promoter-deletion 17ΔPst, we have observed the same trend, shown in Figure 6-1 B. Here, we observe that when treated with DZNep, there is generally less enrichment for H3K27me3 on these LAT-deletion mutant genomes, especially the ICP4 and gC loci, where the difference is statistically significant. Regardless, across the genome, treatment of the LAT deletion-mutant with the EZH2 inhibitor DZNep reduces enrichment of H3K27me3 to background levels, as was seen with the wild type infection, even though enrichment levels of this mark were lower on the mutant virus than on the wild type virus. Taken together, these data suggest that the EZH2 inhibitor DZNep acts to effectively reduce H3K27me3 levels irrespective of the presence of the LAT, likely producing HSV-1 genomes which are less-repressed and are cpable of ractivating at a greater frequency than untreated samples.

Conclusions

The EZH2 Inhibitor DZNep Reduces Levels of H3K27me3 on the HSV-1 Genome Infecting TG Cultures

The EZH2 inhibitor DZNep has been shown to reduce H3K27me3 levels in cancer cells and gammaherpesviruses, but this work is the first to show that DZNep can be used to reduce the levels of the heterochromatic mark H3K27me3 in HSV-1-infected cultures. This finding is important for two reasons: first, the ability to reduce repressive chromatin marks on the HSV-1 genome may alter the state of latency to the point that reactivation is more efficient. If this were the case, then using DZNep to reduce H3K27me3 levels may be important for studying the mechanism behind the

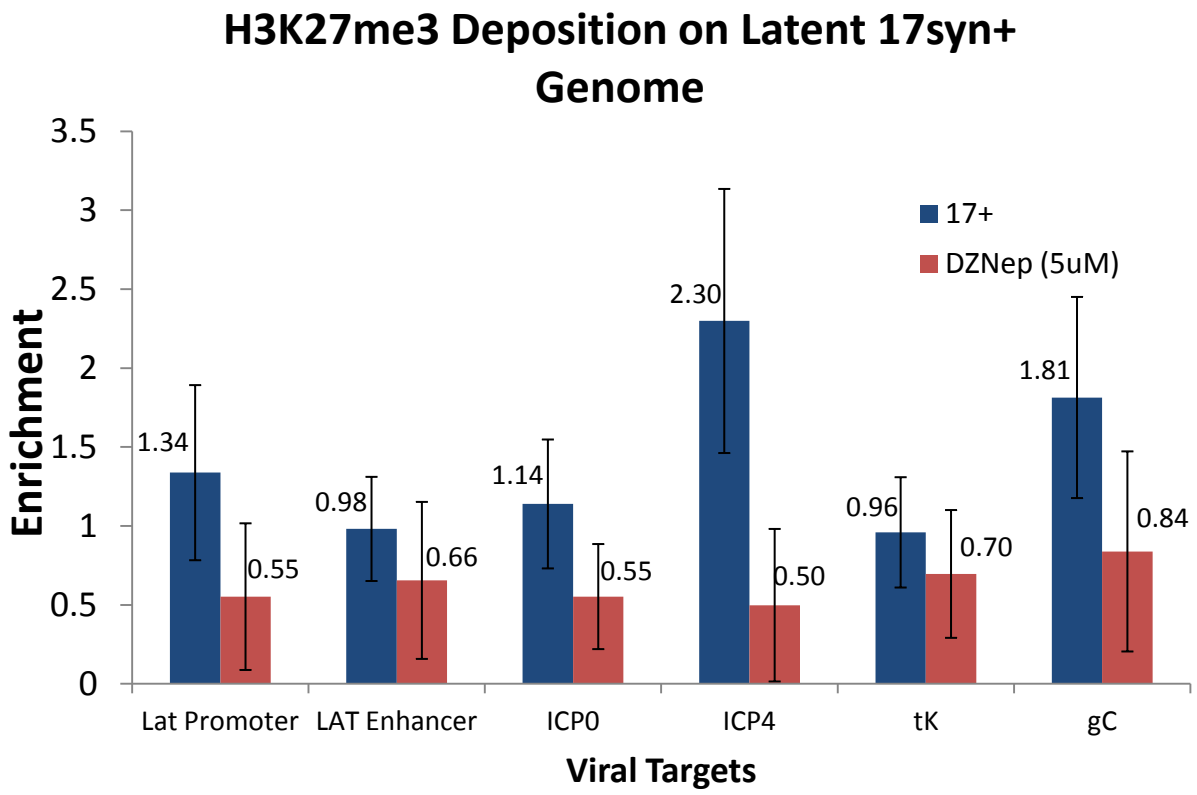
establishment of latency, and having a tool like DZNep to alter these levels could prove vital. In addition, these data show that the TG culture system can be finely manipulated with small-molecule inhibitors, opening up a wide range of possibilities of using other inhibitors to study specific aspects of HSV-1 latency. Finally, the fact that DZNep reduces H3K27me3 levels in both wild-type and LAT promoter-deletion mutants, and across the entire HSV-1 genome suggests that DZNep acts in a global manner, independent of LAT transcription.

Reactivation Efficiency is Greater in TG Cultures Infected with Lower pfu/N Ratios of HSV-1

Previous experiments with HSV-1 transcription and epigenetics suggested that lower pfu/N infections may produce less-repressed HSV-1 genomes, and the data from reactivation experiments supports that conclusion. When TG cultures were treated with a reactivation stimulus, in this case a mono-clonal α-NGF antibody, cultures infected with a lower pfu/N titer resulted in greater titers in a plaque assay than did cultures infected at higher pfu/N titers. These experiments show that the latent HSV-1 genomes resulting from a lower initial infective dose are generally less-repressed than genomes resulting from high initial doses. Further, the fact that the culture media was able to produce virus suggests that the virus being assayed is not from the initial infection, but from new reactivations.

These data provide the final evidence that the initial infective dose of HSV-1 plays an important role in the establishment of latency, and reactivation from latency. And, while previous animal experiments suggested that the initial viral titer played some role, this is the first data showing that the initial amount of infecting virus can affect aspects of latency *in vitro.*

A


H3K27me3 Deposition on Latent 17syn+ Genome
Enrichment
3.5
3
2.5
2
1.5
1
0.5
0
17+
DZNep (5uM)
1.34
0.55
0.98
0.66
1.14
0.55
2.30
0.50
0.96
0.70
1.81
0.84
Lat Promoter
LAT Enhancer
ICP0
ICP4
tK
gC
Viral Targets

B

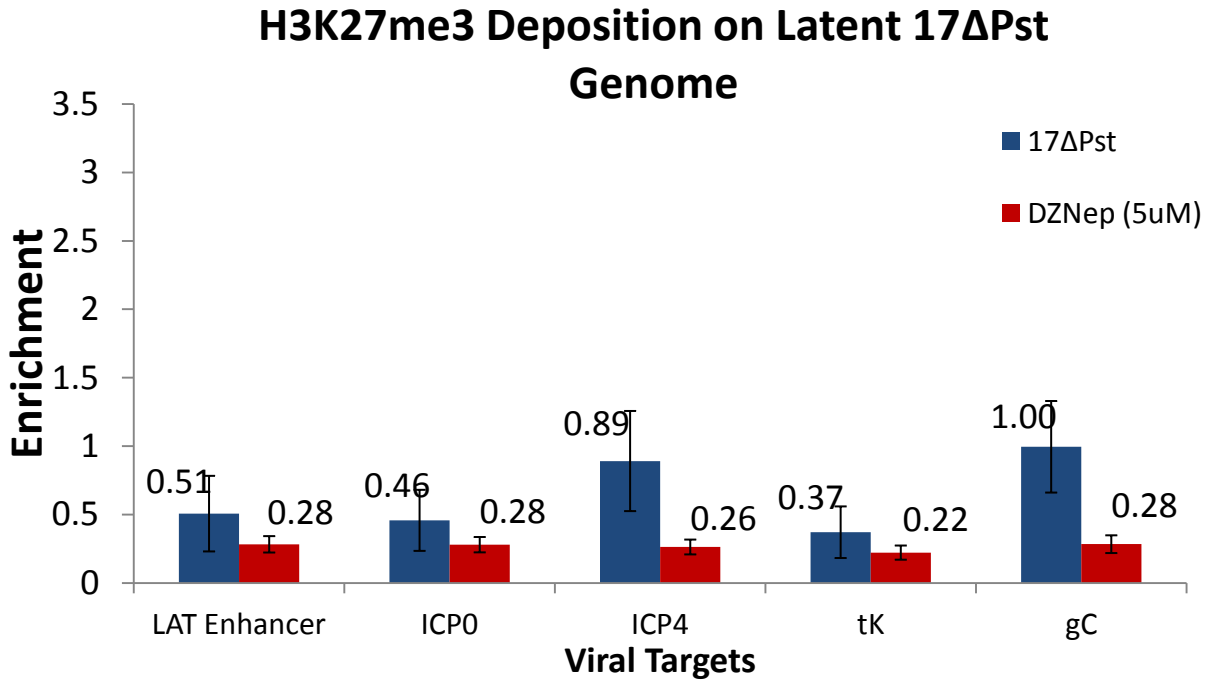

H3K27me3 Deposition on Latent 17ΔPst Genome
Enrichment
3.5
3
2.5
2
1.5
1
0.5
0
17ΔPst
DZNep (5uM)
0.51
0.28
0.46
0.28
0.89
0.26
0.37
0.22
1.00
0.28
LAT Enhancer
ICP0
ICP4
tK
gC
Viral Targets

Figure 6-1. Treatment with the EZH2 inhibitor DZNep reduces H3K27me3 enrichment on both wild type and mutant HSV-1. A) Latently-infected trigeminal ganglia (TG) cultures infected with a wild type HSV-1 at 10pfu/N and treated with DZNep (5μM) or left untreated. B) As in A, except TG cultures are infected with a LAT-deletion mutant 17ΔPst. In both cases, treatment with DZNep reduces H3K27me3 levels across the HSV-1 genome. ChIPs were performed at 7dpi, using a previously described protocol (4). Briefly, TG cultures were fixed with % formaldehyde, sonicated, and immunoprecipitated with α-trimethyl-H3K27 antibody, collecting both bound and unbound samples. DNA was isolated via Qiagen Nucleotide Removal columns and viral genes from each gene class were assayed by Taqman® qPCR via Applied Biosystems StepOne Plus system. Data are shown as relative quantities (Bound/(Unbound + Bound)) compared to an endogenous cellular control, APRT. This is described as, “Enrichment”. Error bars are ± one standard deviation from the mean, n=9.

Table 6-1. TG Cultures Infected at High pfu/N Ratios Reactivate at Lower Levels than Cultures Infected at Low pfu/N Ratios

Multiplicity	Sample 1	Sample 2	Sample 3	Sample 4	Average (pfu/mL)
0.1 pfu/N	675	150	275	600	425
10 pfu/N	200	150	200	200	188

CHAPTER 7
DISCUSSION

The TG Culture System Replicates Many Aspects of *In Vivo* Models of HSV-1 Infection

Viral DNA is Present in the TG Cultures at Lytic and Latent Time Points, but at Levels Lower than Seen in Non-Neuronal Cell Types *In Vitro*

We have demonstrated that viral DNA was present in the infected TG cultures at both lytic and latent time points, suggesting both that: 1) there is an initial phase of acute infection, an infection which some neurons survive and 2) the TG cultures harbor HSV genomes beyond the initial acute infection, up to six days post-infection. Since the TG cultures are not treated with anti-virals, if the neurons in culture were only permissive for the lytic HSV infection, we would expect that an infection would result in nearly total killing of all neurons by six days post-infection, which is not the case. Instead, it is clear that during a latent time point, HSV-1 DNA is still present in these cultures, despite the removal of the initial inoculum nearly a full week earlier. These data are consistent with previous characterization of the culture system which showed persistence of a LAT-promoter-driven reporter gene expressed by a reporter virus (4). Further, at the latent time point, the viral DNA exists at levels slightly higher than at the lytic time point, relative to the cellular control APRT. This suggests that by the time latency is established, HSV infection has resulted in the death of some acutely infected cells. However, the levels of viral DNA in TG cultures appears to be generally lower than levels seen during infection of epithelial cells in culture, such as those used to propagate virus. This is consistent with previous findings suggesting that neurons are generally less efficient at replicating the virus than are epithelial cells.

The Patterns of *In Vivo* HSV-1 Transcription during the Acute and Latent Infection are Recapitulated by the TG Culture System

The general pattern of HSV transcription is well-understood. During the acute infection, the LATs are transcribed at low levels, whereas the lytic transcripts are expressed in a temporal cascade. Then, during the latent infection, lytic transcription is reduced to very low levels of "leaky" transcription, and the LAT is the only abundantly expressed transcript. We have shown that the TG culture system essentially follows these same lytic and latent patterns, with lytic transcription high during the acute infection (10hpi) and LAT transcription high during the latent infection (6dpi). The most-abundantly expressed transcript during the acute infection is the late transcript gC, which is likely due to the fact that as a glycoprotein, gC is needed in stoichiometric amounts in order to produce progeny virus. And, during the latent infection, the most abundantly expressed transcript is the LAT intron, which is known to accumulate to high levels at this time point.

During latency in the TG cultures, we also observed lower levels of lytic gene expression, similar to the "leaky" transcription which has been described in *in vivo* models (26). Levels of gC expression during latency are particularly noteworthy, as they appear to be higher than we would normally observe as a result of "leaky" transcription during an *in vivo* model. However, it is critical to recall that in animal infections, only about 10% of the neurons in infected ganglia harbor latent HSV genomes. In the TG cultures, by the latent time point, much closer to 100% of the remaining neurons harbor latent infections. Hence, it is possible that since many more neurons are infected with HSV, there are greater levels of "leaky" lytic transcription during the latent time point. With more precise immunofluorescence data, these levels

of “leaky” transcription can be properly normalized to the number of latently-infected genomes, possibly providing a more complete explanation of the mechanisms behind this “leaky” lytic transcription.

The Initial Viral Inoculum Affects Viral Transcript and DNA Levels in a LAT-Dependent Manner

Transcript Levels during Both the Lytic and Latent Wild Type Infection Are Affected by the Initial Viral Inoculum

We have shown that by altering the initial viral inoculum, levels of viral transcription during both the acute and the latent infection are affected. At higher inoculums, we see increased levels of transcription across all gene classes, and we observed a similar transcriptional pattern at all doses: that of high gC transcription, lower transcription of earlier gene classes, and moderate LAT transcription. *While different doses do alter the magnitude of the transcription of each gene class, the overall pattern of transcription remains undisturbed, and is very similar to the pattern observed in vivo.* The transcription that we observe at the lytic time point is therefore clearly dependent on the initial inoculum. In addition, a similar dose-dependent effect was observed during the latent infection with wild type virus, but the dose-dependence is seen with the most abundant transcripts at this time point: the LATs. During latency, both the LAT intron and the LAT exon show the same dose dependence as the lytic transcripts displayed during the acute infection. Therefore, it is clear that the transcriptional profile of the wild type virus is closely dependent on the initial viral dose.

Wild Type HSV DNA Levels in TG cultures are Dose-Dependent during the Lytic Infection, but Not during Latency

At 10 hpi, viral DNA levels in TG cultures infected with wild type HSV display a very clear dose-dependence, with the highest levels of viral DNA present in the cultures

infected at the highest pfu/N ratio. However, during latency, this dose-dependent effect is no longer apparent. *Coupled with transcriptional data during latency, these data suggest that increased LAT transcription during latency is not merely due to more viral genomes acting as templates for transcription.* For example, at the 10 pfu/ infection, similar amounts of viral DNA produce 3.5-fold more LAT intron than is observed at a pfu/N ratio of 1, suggesting that the genomes resulting from the higher inoculums are producing more LAT on a per-genome basis, which provides the first clear evidence that TG cultures infected with higher inoculums are more repressed during latency, and that the initial infective dose plays an important role in viral infection. This, in turn, may explain the many disparate phenotypes observed depending on viral strain, route of inoculation, or model system.

The Dose-Dependence of HSV Transcription and DNA Levels are LAT-Dependent

While there was a clear dose-dependence with regards to the level of transcription of wild-type HSV, *the LAT promoter-deletion mutant 17ΔPst does not display this pattern of dose-dependence, suggesting that previously observed dose-dependent effects are LAT-dependent.* In addition to displaying no particular dependence on initial dose, transcript levels for the mutant virus are much lower than were observed for the wild-type infection, which is true for both the lytic time point and latent time point. These observations suggest that in the TG culture system, a mutant virus lacking the LAT not only has reduced levels of overall transcription, especially during latency, but that the amount of initial input virus does not affect the overall transcriptional program of HSV without the presence of the LAT.

While multiple phenotypes have been attributed to the LATs, these data are the first to definitively show that the LAT plays a role in controlling for amount of input virus,

possibly acting in a quorum-sensing role to determine the appropriate level of transcription in the presence of multiple incoming HSV genomes. Precisely how the LAT acts in this fashion is not currently understood, but it is possible that the mechanism of actions is closely related to epigenetic control of the latent virus

The Epigenetic Phenotypes of HSV-Infected TG Cultures are Closely Related to Initial Infective Dose

The Epigenetic Profile of Wild Type HSV-1 is Affected by the Initial Inoculum in a LAT-Dependent Manner

In both experiments assaying the chromatin of the HSV-1 genomes latently infecting TG cultures, enrichment levels of both the repressive and active chromatin mark displayed a dose-dependent effect with the wild-type virus only. At a low pfu/N ratio, we observe high levels of the active mark H3K4me3, and lower levels of the repressive mark H3K27me3. Thus, the epigenetic profile at low inoculums is one of a relatively active chromatin state. The converse is true at high inoculums: H3K4me3 levels are low, and H3K27me3 levels are high, producing a more repressed epigenetic state. Additionally, media titered from reactivated cultures confirms these epigenetic profiles, as a greater reactivation efficiency was observed with a lower initial inoculum. Taken together, these data suggest that the wild type HSV genomes resulting from a low inoculum are less repressed than those resulting from a high inoculum.

As before, this dose-dependence is seen only with wild-type virus, not a LAT promoter deletion. The mutant virus 17ΔPst displays none of the dose-dependent phenotypes with regard to the epigenetic profile, instead displaying a middling phenotype with regards to both chromatin marks, when compared with wild-type virus. This provides a possible explanation for some of the disparate observations in the literature regarding the effect of LAT-deletion mutants on H3K27me3 levels *in vivo*. In

one report, when mice were infected with the virulent wild-type strain 17*syn*+ via the rear footpad route (at generally low titers), it was found that the LAT mutant 17ΔPst resulted in increased levels of H3K27me3 on the viral genome. However, in another report, when mice were infected with the avirulent strain KOS via the ocular route (at much higher titers), the exact opposite was observed: the LAT mutant KOSΔPst resulted in less H3K27me3. The data in the present study (summarized in Table 7-1) provide an explanation for these disparate conclusions. The H3K27me3 entry shows that if an infection initially begins with a low inoculum, removal of the LAT manifests as an increase in heterochromatin (from low repression to intermediate), whereas if you begin with a high inoculum, removal of the LAT produces a decrease in heterochromatin (from high repression to intermediate). These are precisely the observations described in the confounding papers. Together, the findings in this dissertation support the hypothesis that the disparate conclusions drawn from *in vivo* models may not be related to virus strain or route of inoculation – but that these aspects of HSV infection are merely proxies for a more critical factor: the initial viral inoculum.

The LAT Promoter is Highly Enriched for Both Euchromatic and Heterochromatic Marks in LAT-Negative Virus Infections

Interestingly, we observed the most robust enrichment of both chromatin marks at the LAT promoter locus on the LAT-deletion virus 17ΔPst. While the heterochromatic mark H3K27me3 has been observed at similarly high levels *in vivo* (Kwiatkowski, unpublished data), this is the first description of high levels of the euchromatic H3K4me3 mark at this locus, suggesting that the chromatin at this locus may be bivalent in nature. However, in addition to bivalent chromatin, it is also possible that different neuronal subpopulations effect the establishment of one epigenetic mark over

others, irrespective of the presence of the LAT. In this model, one neuronal subpopulation would favor the establishment of heterochromatin at the LAT locus, and another would favor the establishment of euchromatin. In the presence of the LAT, enrichment of these marks would be generally low as the LAT exerts its influence over the epigenetic phenotype. But in the absence of the LAT, one neuronal population defaults to a strong heterochromatic phenotype at this locus, and another defaults to a strong euchromatic phenotype.

Treatment with the EZH2 Inhibitor DZNep Allows Epigenetic Control over TG Cultures

The EZH2 inhibitor DZNep was used in an effort to reduce levels of the H3K27me3 mark on latent HSV genomes, and we observed that both the wild-type and mutant viruses were affected by DZNep treatment. The ability to reduce repressive chromatin marks on the HSV-1 genome may alter the state of latency to the point that reactivation is more efficient, but reactivation studies have yet to be performed on these samples. Using DZNep to reduce H3K27me3 levels in this fashion may be important for studying the mechanism behind the establishment of latency, and having a tool like DZNep to alter these levels could prove vital. Additionally, these data show that the TG culture system can be finely manipulated with small-molecule inhibitors without serious detriment to the neurons, opening up a wide range of possibilities of using other inhibitors to study specific aspects of HSV-1 latency. Finally, the fact that DZNep reduces H3K27me3 levels in both wild-type and LAT promoter-deletion mutants, and across the entire HSV-1 genome suggests that DZNep acts in a global manner, independent of LAT transcription.

The LAT is Critical for the Dose-Dependent Effects Observed in HSV-Infected TG Cultures

Throughout this study, it has become clear that the LAT is essential for any dosage-related effects seen in the *in vitro* infection of primary neuronal cultures. This is the case for both RNA transcript and DNA levels. In addition, epigenetic phenotypes appear to be very dependent on the initial inoculum – but, once again, only while in the presence of the LAT. Taken together, these observations suggest that the LAT acts in a quorum-sensing role, helping to modulate transcription and chromatin formation in response to differential numbers of incoming viral genomes. It remains unclear precisely how the LAT performs this role, but further experiments with the TG culture system, including additional reactivation experiments and infections with different viral strains could provide vital insights into the mechanism of the LAT in regards to quorum sensing.

Finally, it is important to realize that much of this work in would not be possible without an appropriate *in vitro* model of HSV latency. Dose-dependent effects and small-molecular inhibitor studies are profoundly difficult to perform in animal models, and the results can often be confounding due to many unforeseen factors. It is our hope that this work further validates the appropriateness of the primary adult murine TG culture model for investigating questions of HSV latency, as we have shown that the system not only faithfully reproduces many aspects of the *in vivo* infection, but that by using this system, very specific and difficult questions can be addressed in novel ways.

Table 7-1. Summary of Epigenetics and Transcription of HSV-Infected TG Cultures

	17*syn*+		17ΔPst	
	0.1pfu/N	10pfu/N	0.1pfu/N	10pfu/N
H3K4me3 (Active)	+++	+	++	++
H3K27me3 (Repressive)	+	+++	++	++
LAT	+	+++	-	-
Latent Genomes:	More Active	More Repressed	Intermediate	Intermediate

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BIOGRAPHICAL SKETCH

Derek Ryan Jacobs was born in Grand Junction, Colorado in September of 1985. After moving to Salt Lake City, Utah at an early age, he and his family relocated to Westminster, Colorado, a suburb of Denver, where Derek would attend school from elementary school throughout high school at Standley Lake High. He would graduate in May of 2004 and attend Cornell College in Mount Vernon, Iowa for four years, graduating Magna cum Laude with a double-major in biochemistry/molecular biology and mathematics in May of 2008. He began his career in science at the University of Florida in the fall of that same year. He successfully defended his dissertation and received his Ph.D. from the University of Florida in the spring of 2014.