CCCTC-Binding Factor Acts as a Heterochromatin Barrier on Herpes Simplex Viral Latent Chromatin and Contributes to Poised Latent Infection

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ABSTRACT Herpes simplex virus 1 (HSV-1) establishes latent infection in neurons via a variety of epigenetic mechanisms that silence its genome. The cellular CCCTC-binding factor (CTCF) functions as a mediator of transcriptional control and chromatin organization and has binding sites in the HSV-1 genome. We constructed an HSV-1 deletion mutant that lacked a pair of CTCF-binding sites (CTRL2) within the latency-associated transcript (LAT) coding sequences and found that loss of these CTCF-binding sites did not alter lytic replication or levels of establishment of latent infection, but their deletion reduced the ability of the virus to reactivate from latent infection. We also observed increased heterochromatin modifications on viral chromatin over the LAT promoter and intron. We therefore propose that CTCF binding at the CTRL2 sites acts as a chromatin insulator to keep viral chromatin in a form that is poised for reactivation, a state which we call poised latency.

IMPORTANCE Herpes simplex virus 1 (HSV-1) is a human pathogen that persists for the lifetime of the host as a result of its ability to establish latent infection within sensory neurons. The mechanism by which HSV-1 transitions from the lytic to latent infection program is largely unknown; however, HSV-1 is able to co-opt cellular silencing mechanisms to facilitate the suppression of lytic gene expression. Here, we demonstrate that the cellular CCCTC-binding factor (CTCF)-binding site within the latency-associated transcript (LAT) region is critical for the maintenance of a specific local chromatin structure. Additionally, loss of CTCF binding has detrimental effects on the ability to reactivate from latent infection. These results argue that CTCF plays a critical role in epigenetic regulation of viral gene expression to establish and/or maintain a form of latent infection that can reactivate efficiently.

KEYWORDS chromatin, epigenetics, herpes simplex virus, latent infection, regulation of gene expression

Herpes simplex virus 1 (HSV-1) undergoes a lytic infection cycle at the primary mucosal site of infection, expresses approximately 80 lytic genes, and then spreads to sensory neurons, where it establishes a latent infection and expresses a minimal number of viral genes (1). Viral gene products recruit host epigenetic complexes to regulate the viral genome during lytic and latent infection (2, 3). HSV-1 persists as a latent infection in sensory ganglia, during which lytic genes are epigenetically silenced, and the only viral gene products expressed abundantly are a family of noncoding RNAs known as the latency-associated transcripts (LATs) and microRNAs (miRNAs) (4–8). The LAT gene is transcribed to yield a primary 8.3-kb transcript from which stable 1.5- and 2.0-kb introns and a number of miRNAs are processed (6–9). The LATs promote gene silencing and increased heterochromatin at lytic genes and have been associated with...
a reduction in lytic gene transcripts in both acute and latent infections of neurons (10–14). In addition, LAT-dependent gene repression during latent infection in a mouse model has been implicated in promoting neuronal survival and suppressing reactivation (15). No strong evidence for proteins encoded by the LAT genes has been found (16); however, long noncoding RNAs in other systems are known to mediate assembly of heterochromatin and maintenance through both direct and indirect mechanisms (16). In transfection assays, miRNAs originating from the LAT region can also repress expression of viral lytic proteins (6, 17–19).

During establishment of latency, lytic gene expression and viral replication are repressed and the viral genome progressively accumulates histones and heterochromatin on lytic genes (20, 21). Viral lytic genes are associated with histones that are hypoacetylated and enriched for markers of heterochromatin, such as histone H3 lysine 9 trimethyl (H3K9me3), which is a hallmark of constitutive heterochromatin, and H3 lysine 27 trimethyl (H3K27me3), which is a hallmark of facultative heterochromatin (9, 20–23). Elements of the LAT transcriptional unit, which include upstream regulatory sequences, a neuron-specific promoter, and a downstream enhancer, appear to be the exception to this chromatin phenotype (22–28). The LAT gene is the only viral region known to be enriched for acetylated histones and other markers of active euchromatin, while also maintaining association with markers of heterochromatin (22, 23). Upon reactivation in vitro, this pattern is reversed, with lytic genes associating increasingly with acetylated histones and markers of euchromatin and accumulating transcripts, while the LAT gene exhibits a corresponding decrease in euchromatin and transcript levels (29–32). Chromatin control of viral lytic gene expression is therefore thought to act as a regulator of the transitions between lytic infection, latent infection, and reactivation.

Transcribed from the strand antisense to the LAT gene in each of the long component repeats is the ICP0 gene (Fig. 1), which encodes an immediate-early (IE) protein that serves many functions to promote lytic infection, including the transactivation of viral genes and repression of the innate immune system and intrinsic resistance (33–39). ICP0 counters host-mediated chromatin silencing, intrinsic resistance, and innate immune responses through several mechanisms, including the degradation of promyelocytic leukemia (PML) protein in nuclear domain 10 (ND10) bodies (40), degradation of interferon-inducible protein 16 (IFI16) (36), and inhibition of the histone deacetylase (HDAC) RE1-silencing transcription factor (REST) corepressor to REST (CoREST)-HDAC repressor complex (41, 42). In latently infected neuronal populations, ICP0 is largely repressed despite its proximity to the LAT enhancer sequences and

![FIG 1](https://example.com/fig1.png)

**FIG 1** Map of the LAT transcriptional unit of an HSV-1 viral mutant with a deletion of the CTCF-binding sites from the LAT intron sequences. (A) Schematic map of the HSV-1 genome with an expanded view showing the LAT coding region, with the LAT promoter (LAP), LAT enhancer (LTE), CTCF-binding sites (CTRL2), and ICP0 promoter (ICP0 P). Restriction endonuclease cleavage sites (H, HpaI; B, BamHI) used to generate the ΔCTRL2 virus are indicated. (B) The locations of qPCR primers are indicated as open arrows connected by dashed lines: I, LAP; II, LAT intron; III, ICP0 P. (C) Locations of the primary LAT, stable 2.0-kbp LAT intron, ICP0 transcript, and miRNAs.
abundant expression from the adjacent LAT region (43); however, we recently found that ICP0 promotes LAT expression and latent infection (44).

The host factors regulating latent viral chromatin have not been well defined. One candidate, CCCTC-binding factor (CTCF), is an 11 zinc-finger DNA-binding protein that is essential, ubiquitously expressed, and highly conserved among metazoan species (45, 46). CTCF was initially classified as a transcriptional repressor with the ability to bind diverse DNA target sequences (45–48) and was later recognized as a transcriptional activator as well as an enhancer blocker and insulator (49–51). Consequently, CTCF emerged as an important chromatin regulator responsible for a diverse range of activities, including affecting pausing of RNA polymerase II (RNAPII) and RNA splicing, directing the specific positioning and phasing of nucleosomes (52, 53), and generating chromatin loops and mediating long-range chromatin interactions through manipulation of the chromatin three-dimensional (3D) architecture (54–56).

Importantly, CTCF is the only identified vertebrate insulator protein. As such, it acts as a boundary element to isolate adjacent domains of active and inactive chromatin by directing enhancer activity to prevent interaction with nearby but inappropriate promoters and by blocking the linear spread of heterochromatin (51, 57–59). Insulators are crucial in the spatial organization of complex genomes of both metazoan species and herpesviruses (60–63), throughout which essential active regions of transcription are frequently interspersed with silenced domains.

CTCF-binding sites have been identified in a number of herpesviruses and are associated with functions such as regulating latent gene expression in two gamma-herpesviruses, Epstein-Barr virus (EBV) (60) and Kaposi’s sarcoma-associated herpesvirus (KSHV) (61, 62), as well as regulating expression of the major immediate-early gene in the betaherpesvirus human cytomegalovirus (HCMV) (63). A number of CTCF-binding sites have been identified in the HSV-1 genome, including the CTRL2 sites, which are located between the LAT and ICP0 promoter regions in the terminal repeats of the long component (Fig. 1) (64, 65). CTCF binds at the CTRL2 sites during latent infection but is lost during reactivation (66), and this site has been hypothesized to regulate distinct expression from the adjacent genetic elements (64–66). Additionally, in transfection assays, CTRL2 is capable of enhancer blocking, silencing, and prevention of heterochromatin spreading (64, 65). Indirect evidence also suggests that removal of CTCF-binding sites can alter gene expression from lytic promoters in cell culture latency models (67).

Interestingly, in lytic infection, CTCF is not detected at CTRL2 or other latency-associated binding sites; however, CTCF does bind extensively to other regions of the viral genome and may promote viral transcription and prevent epigenetic silencing (68).

We were therefore interested in determining whether the presence of CTCF binding at the LAT intron is critical for establishing and/or maintaining the latent gene expression pattern and chromatin structure in the LAT region during latency. In this study, we eliminated the CTRL2 sites from HSV-1 to assess CTCF function in a mouse model system of latency. We found that the CTRL2 sites are essential for maintaining the chromatin at the LAT and ICP0 regions that promotes efficient reactivation.

RESULTS

Construction of an HSV-1 CTRL2 binding site deletion mutant virus. Previous studies identified the CTRL2 DNA elements within the HSV-1 genome and demonstrated that CTRL2 was able to bind CTCF both in vitro and in vivo (64–66) and function as an insulator capable of blocking enhancer-promoter interactions (64) and the spread of heterochromatin in transfection assays (65). To test whether the CTRL2 region has specific effects on in vivo HSV-1 infection, we constructed a CTRL2 deletion mutant virus by removing a 370-bp fragment from within the 2.0-kbp LAT intron to generate the HSV-1 ΔCTRL2 mutant virus (Fig. 1). A control virus, called CTRL2R, was constructed in parallel by restoring an intact LAT-CTRL2 region in KOSΔLAT1.8eGFP virus, the parent of ΔCTRL2 mutant virus.
Growth kinetics in human foreskin fibroblast (HFF) and HeLa cells following infection at a low multiplicity of infection (0.1) confirmed that there were no differences in lytic replication between the ΔCTRL2 mutant and control CTRL2R viruses (Fig. 2).

Analysis of CTCF binding to the ΔCTRL2 mutant and rescued viral genomes. To confirm the loss of CTCF binding during in vivo infection due to deletion of the CTRL2 site and to validate the mutant virus, we infected mice via corneal scarification with 2 × 10^5 PFU/eye of ΔCTRL2 or CTRL2R virus and performed chromatin immunoprecipitation (ChIP) for CTCF. At 28 days postinfection (dpi), trigeminal ganglia (TGs) were removed and processed for ChIP analysis with a CTCF-specific antibody, and we quantified the immunoprecipitated DNA sequences by using quantitative PCR (qPCR) with primers specific to the LAT promoter, LAT intron, and ICP0 promoter (Fig. 3). ChIP analysis performed in parallel with a nonspecific rabbit IgG confirmed specificity. Statistical analyses were performed on results from 7 independent ChIP experiments from 4 independent infections, using Friedman’s test with Dunn’s post hoc test to account for multiple comparisons. Consistent with the absence of putative CTCF-binding sites, CTCF was not enriched at the LAT promoter (LAP) or at the cellular GAPDH pseudogene relative to the nonspecific antibody control upon infection with either ΔCTRL2 or CTRL2R virus (Fig. 3A and D). Comparison of the ΔCTRL2 mutant viral chromatin to control CTRL2R viral genome showed a significant decrease in CTCF binding at the LAT intron based on ChIP using primers specific to a region less than 500 bp downstream of the CTRL2 site (P = 0.003) (Fig. 3B). Interestingly, ΔCTRL2 viral genomes also showed a significant reduction of CTCF binding within the ICP0 promoter region (P ≤ 0.05) (Fig. 3C). CTCF enrichment at the downstream ICP0 promoter site was consistent with a potential CTCF-binding site within the ICP0 promoter region or detection of CTCF binding to another CTCF-binding site, such as the CTa\”m site, which is ~1.5 kbp away from CTRL2 (64). Collectively, these results demonstrated that deletion of the CTRL2

![Viral Replication](image_url)

**FIG 2** Analysis of ΔCTRL2 and CTRL2R lytic viral replication. HeLa or HFF cells were infected at 0.1 PFU/cell with ΔCTRL2 or CTRL2R virus. Progeny virus was collected at 12, 24, and 36 hours postinfection and titrated on Vero cells.

![Comparison of CTCF association at the LAT region during latent infection with ΔCTRL2 or CTRL2R virus in mice](image_url)

**FIG 3** Comparison of CTCF association at the LAT region during latent infection with ΔCTRL2 or CTRL2R virus in mice. Mice were infected with ΔCTRL2 or CTRL2R virus. At 28 dpi, the mice were sacrificed, and ChIP experiments were carried out on harvested TGs by using antibodies specific for CTCF. Three viral regions were queried: LAP (A), the LAT intron (B), and the ICP0 promoter (C). The cellular GAPDH sequences (D) were also analyzed. Percentages of immunoprecipitated DNA are shown as means and standard deviations from 7 ChIP experiments from 4 independent infections. Statistical significance was evaluated using Friedman’s test along with Dunn’s posttests for ΔCTRL2 versus CTRL2R (controlling for multiple comparisons); significant P values are shown on top of the brackets.
sequences in the ΔCTRL2 virus reduced CTCF association at the LAT intron sequences and at the ICP0 promoter region relative to that with the control CTRL2R virus.

**Acute infection of mice with ΔCTRL2 and CTRL2R viruses.** We first examined acute infection in mice infected with the ΔCTRL2 and CTRL2R viruses by collecting shed virus with eye swabs for the initial 5 dpi and titrating the virus on Vero cells. We observed similar levels of viral shedding from mice infected with ΔCTRL2 and CTRL2R viruses at days 1 to 4; however, at day 5, virus levels collected from mice infected with ΔCTRL2 virus were slightly but significantly lower than in mice infected with CTRL2R (P < 0.005) (Fig. 4A). To allow establishment of latent infection, we maintained infected mice for 28 days. For CTRL2R-infected mice, 90% of the mice survived to day 28 and generally did not succumb to infection before day 10. In contrast, for ΔCTRL2-infected mice, we observed slightly increased mortality beginning at day 7, with 15% fatality by day 10 and 19% fatality by day 28 (P = 0.05 by log-rank test) (Fig. 4B). In general, acute viral replication and survival were largely similar for the mice infected with mutant or restored mutant viruses.

**Trigeminal ganglion infection with ΔCTRL2 and CTRL2R viruses.** To test whether the ΔCTRL2 mutation affected the viral genome load in the ganglia during acute or latent infection, we isolated total ganglion DNA and quantified viral genomes at 7 and 30 dpi from TGs of mice infected with ΔCTRL2 or CTRL2R virus. Viral DNA levels, when normalized to cellular DNA levels, showed no significant difference in genomes per TG for ganglia acutely infected with ΔCTRL2 or CTRL2R viruses at day 7 or for ganglia latently infected with ΔCTRL2 or CTRL2R viruses at day 30 (Fig. 5A), showing that acute infection of the trigeminal ganglia was similar with the two viruses and there were similar levels of latent infection with the ΔCTRL2 and CTRL2R viruses.

To measure latent viral gene expression with the two viruses, total RNA was also extracted from each sample and measured with primers specific for the viral LAT intron, the viral miRNAs, including miR-H2, miR-H4, and miR-H6, the lytic viral transcripts, including ICP0, ICP27 (IE), and the genes for thymidine kinase (tk; early kinetic class [E]), and glycoprotein C (gC; late kinetic class [L]), and also host GAPDH transcripts and let-7a miRNA. Viral transcripts were first normalized to cellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Total RNA levels were then normalized to total viral genome copy numbers for each mouse to calculate transcript levels per genome. Similarly, viral miRNAs were normalized to let-7a levels and then to the viral genome copy number for each mouse. In ganglia infected with the ΔCTRL2 virus, compared to those infected with the CTRL2R virus, we observed a small (∼3-fold) but significant increase in levels of LATs at days 7 and 30 (Fig. 5B) (P < 0.0001 by the Mann-Whitney test); however, the levels of LAT became indistinguishable during reactivation of ΔCTRL2 and CTRL2R viruses (Fig. 5B).
miR-H2 and miR-H4 are both derived from the primary LAT transcript ([6, 17, 69]); while miR-H6 is encoded upstream of the LAT transcription unit, its expression is dependent on a 200-bp sequence that includes the LAT promoter ([6, 69]). At day 7, miRNAs showed no significant difference; however, at day 30, we observed a small (~1.8-fold) increase in miR-H2 (Fig. 5C) in ΔCTRL2-infected ganglia, based on the Mann-Whitney test. Levels of miR-H4 (Fig. 5D) and miR-H6 (Fig. 5E) were not significantly different (~1.2-fold and ~1.4-fold higher, respectively). In addition, lytic transcript levels were not significantly different at day 7 (Fig. 5F to I). Because lytic transcript levels were too low for quantification at day 30, ganglia were scored as positive or negative for detectable transcripts. We did not find significant differences in the fraction of ganglia with detectable lytic viral transcripts between ΔCTRL2 and CTRL2R infections (Table 1). These results indicated that loss of CTCF binding at the CTRL2 site increased the accumulation of LATs slightly but did not affect the low levels of lytic gene

**TABLE 1** Expression of viral lytic transcripts in mouse TG at 30 days

| Virus   | ICP0  | ICP27 | tk    | gC    |
|---------|-------|-------|-------|-------|
| ΔCTRL2  | 8/34  | 8/34  | 7/34  | 7/34  |
| CTRL2R  | 8/24  | 5/24  | 4/24  | 4/24  |
Increased H3K27me3 histone modification on the LAT region of ΔCTRL2 mutant virus. To examine the potential role of CTRL2 in regulating viral chromatin during latency and its potential function as an insulator, we collected TGs from mice latently infected with ΔCTRL2 or CTRL2R viruses and performed ChIP analysis with antibodies specific for total histone H3 (A), H3K9me3 (B), or H3K27me3 (C). Three viral regions, the LAT promoter (LAP), LAT intron, and ICP0 promoter (ICP0 P), were examined, and the results are expressed as means and standard deviations of the percent viral chromatin immunoprecipitated relative to immunoprecipitation of the cellular GAPDH region. Asterisks indicate significance (P < 0.05), which was evaluated using the Wilcoxon matched-pairs signed-rank test.

When we compared the chromatin of the ΔCTRL2 virus directly to that of the CTRL2R virus, we observed a significant increase of H3K27me3 heterochromatin marker accumulation at LAP and the LAT intron (P ≤ 0.05), but not at the ICP0 promoter (Fig. 6C). We also observed slight but not significant increases in total histone H3 or H3K9me3 accumulation. These results showed that CTCF binding to CTRL2 may prevent the spread of specific heterochromatin markers, such as H3K27me3, to the LAT region encompassing LAP and the LAT intron.

Explant reactivation from TGs infected with ΔCTRL2 virus is reduced. To determine whether loss of CTCF binding at the CTRL2 sites affected the ability of the ΔCTRL2 mutant virus to reactivate, we harvested TGs latently infected with ΔCTRL2 or CTRL2R virus and explanted them onto a monolayer of Vero cells. We tested individual ganglia from two independent infections of 5 mice and 10 mice per virus, respectively. We assessed the emergence of infectious virus for 7 days postexplant by collecting overlay medium and, on day 7, also the underlying Vero cell monolayer and replating each on a fresh Vero monolayer. Infectious virus was first detected at 3 days from
CTRL2R virus-infected ganglia but appeared first at 4 days from ΔCTRL2 virus-infected ganglia (Fig. 7). By 7 days postexplant, 77% of CTRL2R virus-infected ganglia produced infectious virus, while only 53% of ΔCTRL2 virus-infected ganglia produced detectable virus ($P = 0.03$, by log-rank test). At 7 days, the underlying cells were also sampled, and ΔCTRL2 never reached the reactivation frequency of the CTRL2R virus, as evaluated by the log-rank Mantel-Cox test. These results indicated that despite similar numbers of latent viral genomes present at day 28, genomes from ΔCTRL2 virus infection showed significantly reduced explant reactivation.

**DISCUSSION**

The LAT and ICP0 transcriptional units are encoded on opposite strands of the HSV-1 genome (Fig. 1) but are regulated independently during latent infection, so that they maintain suppression of ICP0 gene transcription while allowing persistence of LAT transcription (4). The divergent expression patterns correlate with different chromatin modifications found at the LAT and ICP0 promoters that are maintained despite the relative proximity of these genetic elements (21–23, 70, 71). While ICP0 and other lytic gene promoters are associated with high levels of heterochromatin during latent infection, the LAT region also shows enrichment of euchromatin (23, 70). CTCF is a key mediator of the ability to maintain independently regulated but adjacent chromatin domains within mammalian and viral genomes (51, 72). Of the various sites on the HSV-1 genome that bind CTCF during latent infection, CTRL2 has been of particular interest due to its position between the transcriptionally active LAT promoter and the silenced ICP0 promoter (64–66).

In this study, we constructed a mutant HSV-1 virus, ΔCTRL2, with deletions of the CTRL2 CTCF-binding sites to examine the role of CTCF binding to CTRL2 during *in vivo* latent infection in a mouse model system. The ΔCTRL2 mutant virus produced a relatively normal acute infection in the cornea and trigeminal ganglia and established latent infection to the same level as the CTRL2R virus. The ΔCTRL2 mutant virus showed slightly higher levels of LAT expression during latent infection, but this was indistinguishable during reactivation. Despite equal latent DNA genome loads per TG, the mutant virus showed reduced reactivation relative to that by the CTRL2R virus. Because the mutant virus showed higher levels of H3K27me3 heterochromatin on the LAT promoter and intron sequences, we hypothesize that CTCF binding at the CTRL2 site serves as a chromatin barrier to keep at least certain forms of heterochromatin off the LAT promoter/regulatory sequences.

CTCF is associated with the LAT intron sequences in a CTRL2-dependent manner. We confirmed that deletion of the CTRL2 region was sufficient to reduce CTCF binding to the LAT intron to background levels. CTCF is known to mediate long-range,
three-dimensional chromatin interactions through simultaneous association with distant binding sites; therefore, the loss of one binding site has the potential to alter CTCF binding to distant sites (54, 73–75). Our study also identified a reduction of CTCF binding at the ICP0 promoter region after deletion of the CTRL2 sites, suggesting that CTRL2 and a region near the ICP0 promoter may bind CTCF cooperatively. An interaction between these sites could form a closed chromatin loop surrounding the 5’ end of the ICP0 gene and separating the ICP0 promoter from the LAT promoter. However, there are CTCF-binding sites at many regions of the HSV-1 genome, and further work is required to characterize these sites, their role in the HSV-1 3D chromatin architecture, and their interaction with the LAT region.

Removal of CTCF binding to CTRL2 decreases viral reactivation in a mouse model. Deletion of the CTCF binding site at the CTRL2 region did not affect viral replication in lytic infection of cultured HeLa or HFF cells or acute infection at the ocular epithelium for the first 4 days of infection. These results argue that lytic infection of nonneuronal cells is not affected by CTCF binding to CTRL2. This is consistent with the findings of a recent study that did not detect CTCF binding at the CTRL2 site during lytic infection (68). Although infection with the ΔCTRL2 virus showed a reduction in shed virus in the eyes of mice on one day, at 5 dpi, total viral genomes per TG were not significantly different between ΔCTRL2 and CTRL2R viruses at 7 or 30 dpi. However, the mutant virus showed reduced reactivation upon ganglionic explant, arguing that CTCF binding at CTRL2 promotes reactivation. Slightly higher levels of LAT were observed during latent infection with the mutant virus compared to the repaired virus, but during reactivation, the levels of LAT were equivalent. Therefore, the major effect of the CTRL2 sites on viral infection seems to be promotion of reactivation.

Results from related herpesviruses, EBV and KSHV, have also indicated that CTCF has a complex role in promoting latent infection. Removal of CTCF-binding sites from the intron of the LMP2A gene of EBV resulted in a higher viral genome copy number in latent infection, possibly as a result of partial lytic replication or aberrant latent replication (76). However, results with KSHV indicated that removal of CTCF-binding sites from the intron of its major latency-associated transcript reduced the latent genome copy number due to reduced viral episome maintenance (61). Given that CTCF binding has such diverse effects among related herpesviruses, it is possible that CTCF binding exhibits both positive and negative regulation during HSV-1 latent infection. To address these questions, future studies that address the progression of latency establishment before 28 days and the maintenance of long-term latent viral genomes are needed to differentiate between the initial viral dose entering the ganglia, viral replication within the neural tissue, and long-term viral genome maintenance.

Histone modifications at the LAT promoter sequences are affected by CTCF binding at the CTRL2 site. The increased accumulation of H3K27me3-modified heterochromatin at LAP and the LAT enhancer regions of the ΔCTRL2 mutant viral genome is consistent with the hypothesis that CTCF binding at CTRL2 functions as an insulator to block the linear spread of heterochromatin from the lytic ICP0 region to the LAT transcripational regulatory regions. Previous results demonstrated that the H3K27me3 modification accumulates gradually at viral lytic promoters from days 10 to 14 during the establishment of latent infection and is enhanced by LATs (20, 23). Therefore, increased LAT accumulation at 7 dpi with the ΔCTRL2 virus may also contribute to higher levels of H3K27me3 accumulation at the LAT sequences by day 28.

Furthermore, H3K27me3 can be found in bivalent chromatin domains (77) and poised chromatin domains (78) on developmentally regulated genes and genes in pluripotent stem cells. Our previous study found that increased levels of H3K27me3 at the ICP8 promoter relative to the viral UL48 promoter correlated with higher levels of ICP8 RNA than UL48 RNA (20). Therefore, H3K27me3 alone may be insufficient to silence transcription, or additional histone modifications, such as H3K4me3, may exert a dominant effect to maintain active transcription. Alternatively, a phospho switch proposed for reactivation (79) may work effectively on chromatin that has H3K27me3
Model for mechanism of action for CTCF binding at the CTRL2 site. We have shown that binding of CTCF to the CTRL2 sites causes a statistically significant reduction in H3K27me3 and a trend toward reduction of H3K9me3 modification of chromatin on the LAT promoter and intron sequences. Furthermore, the CTRL2 sites promote the reactivation of latent virus upon explant in culture. We propose a model in which CTCF bound at CTRL2 dimerizes with a CTCF molecule bound at another site to form a 3D structure that promotes reactivation. The most likely targets, VP16 and ICP0, are viral genes whose products are involved in early stages of reactivation (1, 12, 80). We envision at least two ways in which CTCF promotes their expression during reactivation. First, we have shown that CTCF binding at the CTRL2 sites promotes CTCF binding in the ICP0 promoter, which could place the 5’ end of the ICP0 gene in a chromatin loop. Because CTCF-mediated chromatin loops are believed to often enclose inducible gene regulatory domains (81), enclosing the ICP0 gene promoter in a chromatin loop may promote its expression when reactivation is triggered. Second, CTCF may dimerize with a CTCF molecule bound near the VP16/UL48 gene promoter to keep the VP16 promoter in a chromatin state that can be readily induced when reactivation is triggered. Further studies are needed to define the 3D chromatin structure of viral chromatin that is dependent on CTCF binding at the CTRL2 sites and to test these hypotheses.

Establishing and maintaining a poised latent infection. To establish a successful latent infection, HSV-1 must balance suppression of lytic gene expression with maintenance of a latent state that is poised for reactivation. The results in this study indicate that HSV-1 exploits the cellular protein CTCF to maintain its genome in a state that can be efficiently reactivated. Large domains of H3K27me3 with short domains of H3K4me3 histone modifications are known to define a bivalent chromatin structure that is silenced but poised to be reactivated at the appropriate time (77). We propose a model to explain how the CTRL2 sites can promote reactivation. In addition to the CTRL2 sites, HSV-1 gene products contribute to promoting H3K27me3 on HSV-1 lytic gene chromatin. HSV-1 LAT promotes H3K27me3 on lytic gene chromatin (23) as well as reactivation (12; P. Raja, J. S. Lee, and D. M. Knipe, unpublished results). Furthermore, ICP0 promotes LAT expression and H3K27me3 modifications on lytic gene chromatin (44) as well as reactivation (82, 83). Therefore, CTCF, LAT, and ICP0 may all be acting to promote the correct form of viral chromatin so that a poised latent infection is established and maintained and reactivation can take place at the appropriate time.

Therefore, viral gene products play an active role in not only promoting epigenetic silencing of DNA viral genomes for latent infection (84) but also keeping them in a poised form that is capable of efficient reactivation, a state that we call poised latency.

MATERIALS AND METHODS

Cells and viruses. Vero, HeLa, and HFF cells were obtained from ATCC. The ΔCTRL2 virus has a deletion of CTRL2 sites from within the LAT intron of both TR, repeats (HSV-1 KOS KT899744 bp 120136 to 120508 [85]).

The control CTRL2R virus was constructed in parallel by cotransfection of the WT DNA fragment. All viruses were propagated and titered in parallel on Vero cells.

Mouse infections. Mice were housed in accordance with institutional and National Institutes of Health guidelines on the care and use of animals in research. Ocular infection was carried out with 2 × 105 PFU/eye of virus (86). Eyes were swabbed on days 1 to 5 postinfection, and virus collected from tear films was titered on Vero cells (87). Mice were monitored for survival for at least 28 dpi.

ChIP assays. Immunoprecipitations were carried out on chromatin prepared from TGs (20) with 5 to 10 μg of anti-CTCF (catalog number 07-729; Millipore), 2.5 μg of anti-histone H3 (ab1791; Abcam, Inc.), 2.5 μg of anti-H3K27me3 (39156; Active Motif, Inc.), 2.5 μg of anti-H3K9me3 (ab8580; Abcam, Inc.), or normal rabbit IgG (12-370; Millipore). Immunoprecipitated DNA was quantitated using the qPCR primers listed in Table 2.

Quantification of viral genomes and transcripts. Nucleic acids were isolated using the Allprep RNA/DNA minikit (Qiagen), and RNA was reverse-transcribed with specific primers using the QuantiTect RT kit (Qiagen). Viral DNA and transcripts were quantified relative to host adipsin DNA and GAPDH mRNA (44, 88). For miRNA quantification, RNAs from TGs were purified using an RNeasyPlus minikit (Qiagen) and quantified using TaqMan miRNA assays (Applied Biosystems). Viral miRNA levels were normalized to cellular let-7a.
TABLE 2 Primer sequences

| Target       | Purpose      | Sequence (5’-3’/) | Forward         | Reverse          |
|--------------|--------------|-------------------|-----------------|------------------|
| LAT promoter | qPCR         | 5’-CCGCGCGCCCGCAT-3’ | 5’-GAACCGCCGCGGCTT-3’ |
| LAT intron   | qPCR         | 5’-GGGTTAGAGGCTGTGC-3’ | 5’-GTGGAGCGACGGGAACACAT-3’ |
| ICP8 promoter | qPCR        | 5’-CGCTTCCCGGAAGAAATCT-3’ | 5’-CCGTCATGAAACCCGGCTT-3’ |
| ICP34 promoter | qPCR     | 5’-GAGACCCGGGTTGGGAAATG-3’ | 5’-CCCCCGGGGTGTTGTCGAAAG-3’ |

Reactivation. TGs isolated from mice latently infected with HSV-1 ΔCTRL2 or CTRL2R viruses were biopsied and explanted onto a confluent monolayer of Vero cells in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) fetal bovine serum and 0.25 μg/ml amphotericin B. Culture medium was sampled daily for 7 days, and after 7 days (7 +) the entire Vero monolayer and ganglia were collected, frozen, and replated onto a fresh Vero monolayer to score the number of ganglia that showed detectable infectious virus (see Text S1 for additional details on our methods).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02372-17.

TEXT S1, DOCX file, 0.1 MB.

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