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Kinetics of Facultative Heterochromatin and Polycomb Group Protein Association with the Herpes Simplex Viral Genome during Establishment of Latent Infection

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ABSTRACT The herpes simplex virus (HSV) genome is associated with heterochromatic histone modifications, including trimethylation of the lysine 27 residue of histone H3 (H3K27me₃), during latent infection of neurons. Here we have examined the kinetics of general chromatin and H3K27me₃ association with the viral genome during establishment of latent infection. Using both wild-type virus and a mutant virus that is unable to undergo replication in neurons, we found that histone H3 associates with viral gene promoters by 7 days postinfection (dpi). Levels of H3K27me₃ were low at 7 dpi but increased dramatically by 14 dpi. Hence, general chromatin association and/or other factors may play a key role(s) in the initial silencing of lytic genes, and H3K27me₃ may play a role in further suppression of the genome and/or the maintenance of latency. A component of Polycomb repressive complex 2 (PRC2), which mediates the addition of K27me₃ to histone H3 (Suz12), was also recruited by 14 dpi. We have shown previously that the levels of H3K27me₃ during latent infection are increased in the presence of the latency-associated transcript (LAT). However, the initial targeting of PRC2 was not found to be dependent on the LAT. We found that a component of the PRC1 complex (Bmi1), which binds to H3K27me₃, was not enriched at promoters found previously to be enriched for H3K27me₃. Our results are consistent with (i) chromatinization of viral DNA or other mechanisms causing the initial silencing of HSV lytic genes and (ii) facultative heterochromatin maintaining that silencing during latent infection of neurons.

IMPORTANCE The human pathogen herpes simplex virus (HSV) hides for the lifetime of the host in peripheral neurons. The mechanism by which HSV is able to shut off its gene expression and persist in neurons is not known. Here we show that the HSV DNA first associates with histone H3, with later recruitment of Polycomb repressor complex 2 (PRC2) and trimethylation of the lysine 27 residue of histone H3 (H3K27me₃), a modification associated with heterochromatin. This work indicates that the initial silencing of HSV gene expression is not correlated with enrichment of H3K27me₃ and that PRC2 may be recruited to already-silenced genes to further silence gene expression and/or maintain gene silencing. We demonstrate that recruitment of PRC2 is not dependent upon expression of the noncoding HSV latency-associated transcripts, indicating the presence of unknown triggers for PRC2 recruitment during the establishment of latent infection.

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Herpesviruses persist for the lifetime of an individual in the form of a latent infection. Latent herpes simplex virus 1 (HSV-1) DNA persists as an episome (1) assembled in nucleosomal chromatin (2) in sensory neurons within peripheral ganglia, where lytic cycle protein expression is restricted, and the only viral transcripts expressed to high levels are noncoding RNAs known as the latency-associated transcripts (LATs) (3) and microRNAs (miRNAs) (reviewed in reference 4). Periodic reactivation from latency allows transmission of HSV to a new host. Because all antivirals currently in use target only the lytic stage of infection, latent infection presents a major challenge to the treatment of HSV.

The mechanism(s) by which HSV-1 lytic gene expression is silenced within neurons is not fully understood, but a number of

mechanisms have been proposed. First, the inability of VP16, the viral immediate early (IE) gene transcriptional activator, to localize to the neuronal nucleus (5) and/or the cytoplasmic localization of HCF-1, the cellular activator for IE genes, in sensory neurons (6) may lead to the inability of the virus to form the transactivator complex that promotes IE gene expression (7). Second, cellular repressors, such as the restriction element 1 silencing transcription (REST) factor - cofactor of restriction element 1 silencing transcription (Co-REST) complex, have been shown to reduce viral replication in neurons (8). Furthermore, at least two mechanisms by which the RNAs expressed from the LAT locus actively limit lytic protein expression in neurons have been proposed. First, expression of the LATs has been associated with reduced expression of lytic gene transcripts during acute infection (9) or

latent infection (10). In latent infection, the viral lytic gene promoters are associated with heterochromatin, and expression of the LATs is associated with increased heterochromatin on the viral lytic gene promoters (11, 12). The viral genome is specifically enriched for chromatin containing the histone H3 lysine 9 trimethyl (H3K9me3), H3K9 dimethyl (H3K9me2), and H3 lysine 27 trimethyl (H3K27me3) modifications (11–13), and H3K27me3 was found to be a major form of heterochromatin on the viral genome (12). Interestingly, the association of H3K9me2 and H3K27me3 with lytic gene promoters was decreased in neurons latently infected with a *LAT* deletion mutant virus relative to their association in neurons infected with its rescued virus (11, 12). Therefore, a role for the LAT in promoting or maintaining heterochromatin levels is consistent with previous observations that LAT down-regulates lytic gene expression during the establishment and maintenance of latent infection. Second, miRNAs that are expressed from the *LAT* locus during lytic and latent infection can reduce the expression of certain HSV-1 lytic proteins in cotransfected cells (14). Expression of these miRNAs depends on the *LAT* promoter (15), so these miRNAs may contribute to the phenotype of a *LAT* promoter mutant virus.

H3K27me3 is a hallmark of cellular facultative heterochromatin (fHC). fHC is found on different regions of the cellular genome in different cell types and during different developmental stages (16). Because of its differential distribution on developmentally regulated genes, fHC is thought to be able to convert readily to euchromatin to allow gene expression to occur. The cellular proteins involved in the initiation and maintenance of fHC are known as Polycomb group proteins, and they form two complexes, Polycomb repressor complex 1 and 2 (PRC1 and -2) (reviewed in reference 17). PRC2 contains the proteins Ezh2, Suz12, Eed, and RdAp46/48 and is responsible for trimethylation of histone H3. PRC1 binds H3K27me3 and is responsible for maintaining and further compacting fHC. The Bmi1 protein, a component of PRC1, has previously been found on the *LAT* region of the HSV-1 genome during latent infection and at low levels on certain lytic genes (13).

Following corneal infection and spread of the virus to the trigeminal ganglia (TG) in the mouse model system, there is an initial period of acute replication in the ganglia (18). During the period of ongoing acute infection, a subpopulation of infected neurons does not appear to express lytic genes, and they are thought to enter quiescence and contribute to the pool of latently infected neurons (19, 20). However, recent evidence indicates that prior lytic gene expression in an individual neuron is also compatible with latent infection (20), and certain neurons express both lytic and latent transcripts during the establishment of latent infection (19, 21). Therefore, the pool of neurons latently infected with HSV-1 may originate by two pathways, one in which the genomes become silenced rapidly upon infection of the neuron and one in which lytic genes become silenced following an initial period of lytic gene expression.

Given the potential for two pathways leading to the establishment of latent infection, we were interested in determining the kinetics of total chromatin, H3K27me3, and PRC2 recruitment to the HSV-1 genome during the establishment and maintenance of latent infection. We found that histone H3 is associated with the viral genome at early times (7 days postinfection [dpi]) and that H3K27me3 becomes deposited on viral lytic gene promoters to high levels following the resolution of the acute infection (14 dpi).

We also found that recruitment of the Suz12 protein to lytic gene promoters also increased following the resolution of acute infection. Surprisingly, the association of Suz12 with lytic gene promoters was not affected by LAT expression, indicating that during the establishment of latency, the LAT is not required to target PRC2 to lytic gene promoters. Finally, we also investigated whether the PRC1 complex was recruited to viral promoters following the establishment of latent infection. However, we were unable to detect Bmi1, a component of PRC1, on lytic gene promoters during latency.

RESULTS

Kinetics of association of chromatin during the establishment of latent infection with wild-type (WT) HSV-1. We and others have found that HSV-1 lytic gene promoters are associated with H3K27me3 during latent infection (12, 13). We have also found that H3K27me3 association with the viral lytic gene promoters was decreased following infection with two independent *LAT* promoter deletion mutant viruses (12). We were interested in determining the mechanism by which the LAT either promotes or maintains the H3K27me3 modification on latent genomes. To this end, we first aimed to establish when H3K27me3 is targeted to the viral genome during the establishment of latent infection.

Previously, we found that H3 association with HSV DNA in TG was apparent by 7 dpi and that the H3K9me2 modification was apparent by 15 dpi (11). Therefore, we looked at this time period for the H3K27me3 modification. We infected mice with HSV-1, harvested the TG at 7, 10, and 14 dpi, and carried out chromatin immunoprecipitation (ChIP) using antibodies specific for histone H3 and H3K27me3. In previous experiments, we noticed that the efficiencies of histone H3 ChIP assays varied depending on the number of input viral genomes (results not shown). To control for this, we first quantified the numbers of copies of viral genomes in the chromatin extracts and used chromatin amounts representing equal numbers of viral genomes. The samples were made up to equivalent amounts of total chromatin by adding chromatin derived from TG of mice that were not infected with HSV.

To validate the ChIP for H3K27me3, we analyzed the *Rasgrf1*-imprinted locus, for which one copy of the gene is enriched for H3K27me3 (22). The association of H3K27me3 with the *GAPDH* promoter served as a cellular negative control. There was little immunoprecipitation of either *Rasgrf1* or *GAPDH* DNA with the IgG antibody control (see Fig. S1A in the supplemental material). In contrast, both cellular genes were associated with histone H3 (Fig. S1B). There was an approximate 6-fold increase in the fraction of *Rasgrf1* DNA associated with the H3K27me3 modification compared to the fraction of *GAPDH* DNA (Fig. S1C), demonstrating that the assay was specific for H3K27me3. We also did not detect any significant changes in the levels of H3K27me3 on the cellular controls at the three time points tested.

We then examined the kinetics of association of H3 and H3K27me3 with different regions of the viral genome, including the *LAT* promoter and 5' exon, the immediate early *ICP4* and *ICP0* promoters, the early *ICP8* and *TK* promoters, and the late *UL48* promoter. ChIP with the histone H3-specific antibody immunoprecipitated significant amounts of all 5 viral promoters by 7 dpi (Fig. 1B), compared to amounts immunoprecipitated with control antibody (Fig. 1A), indicating that histone H3 associated with all 5 viral promoters by 7 dpi. In contrast, the H3K27me3 modification was not observed until 14 dpi on the viral lytic gene

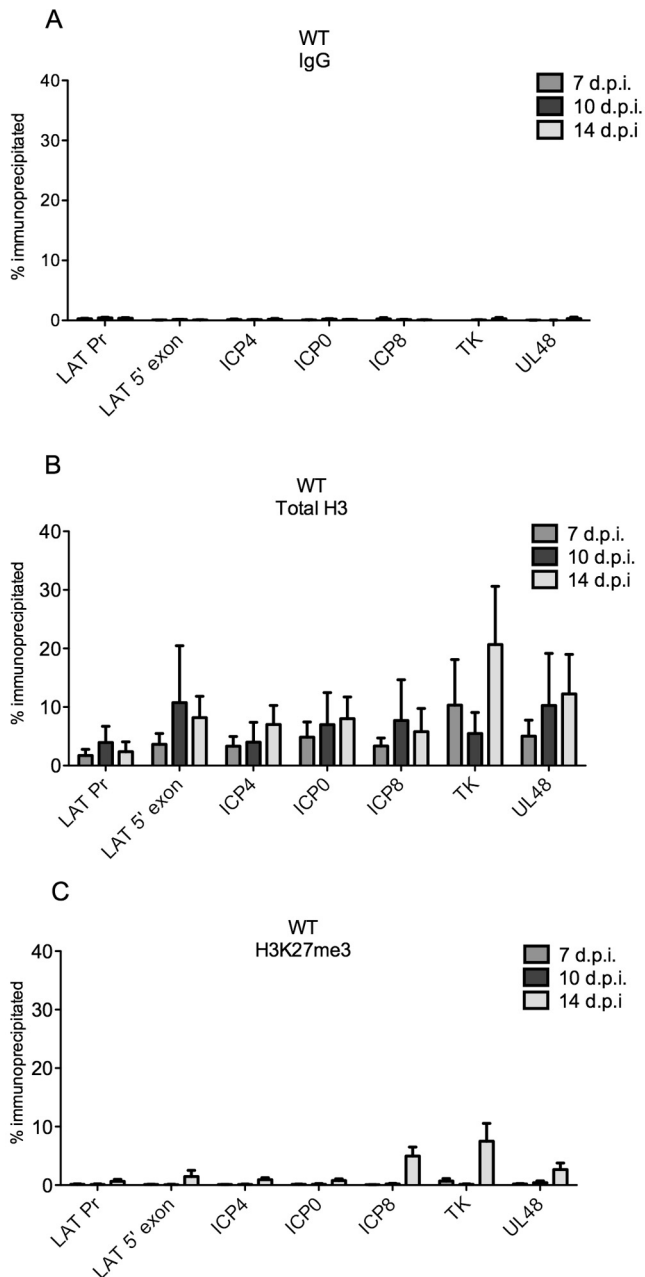


FIG 1 Association of histone H3K27me3 with the wild-type (WT) herpes simplex virus 1 (HSV-1) genome during establishment of latent infection. Mice were infected with WT HSV-1, and at 7, 10, or 14 dpi, the mice were sacrificed. Trigeminal ganglia were collected, and chromatin immunoprecipitation (ChIP) was carried out. Shown are percentages of viral DNA immunoprecipitated with IgG control antibody (A), with an antibody against total histone H3 (B), or with an antibody against H3K27me3 (C). Pr, promoter.

promoters. The *ICP8*, *TK*, and *UL48* promoters showed the highest levels of H3K27me3 (Fig. 1C). The differences in the association of H3K27me3 with the *ICP8*, *TK*, and *UL48* promoters from that of the *ICP0* promoter were 6.4-fold, 9.6-fold, and 3.4-fold, respectively.

Because it appeared that total H3 was present on the viral genomes at a time preceding the H3K27me3 modification, we analyzed the levels of H3 and H3K27me3 compared to their levels

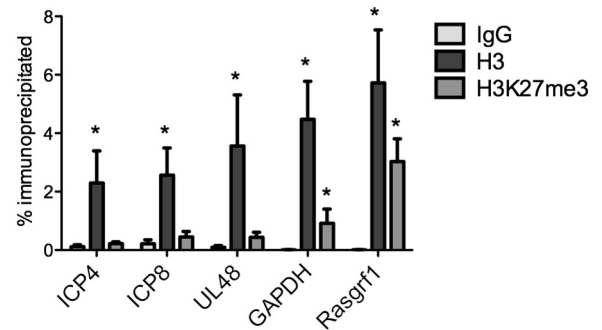


FIG 2 Association of HSV-1 lytic promoters with total chromatin and H3K27me3 at 7 dpi. Mice were infected with WT HSV-1, and at 7 dpi, the mice were sacrificed and trigeminal ganglia were collected. ChIP was carried out on trigeminal ganglia. The percentages of viral lytic gene promoters (*ICP4*, *ICP8*, and *UL48*) and cellular genes (*GAPDH* and *Rasgrf1*) immunoprecipitated with nonspecific IgG, total histone H3, and H3K27me3 were determined. Values that were statistically increased compared to the value for the nonspecific control ($P \leq 0.05$, one-tailed paired t test) are indicated (*).

with the IgG control at 7 dpi. Statistical analyses carried out on the ChIPs of 7-dpi TG revealed that the association of lytic genes with total histone H3 was increased significantly compared to their association with the IgG control on all the promoters tested (Fig. 2). In contrast, the association of viral promoters with the H3K27me3 modification was not increased significantly compared to that with the IgG control. Therefore, histone H3 association with viral lytic promoters was detectable earlier than H3K27me3 modification (18).

The association of H3K27me3 with viral lytic promoters increases between 10 and 14 dpi in the absence of viral DNA replication. HSV-1 undergoes a period of acute replication starting at 2 dpi following ocular infection of CD-1 mice, and the infection is then cleared (18). Therefore, it was possible that the increase in H3K27me3 levels seen after 10 dpi was due to clearance of viral genomes not associated with H3K27me3 following the resolution of the acute infection and not *de novo* formation of H3K27me3 on viral genomes. We therefore infected mice with the *dlsptk* thymidine kinase (TK)-null mutant virus, which does not undergo viral DNA replication in neurons (23, 24). Because viral DNA replication results in a decrease in the fraction of viral DNA associated with histone H3 (25), the use of a viral mutant that does not undergo DNA replication in neurons allowed us to enrich for viral genomes associated with H3K27me3 during an acute infection that might otherwise be masked by ongoing viral DNA replication.

The specificity of the ChIP carried out on *dlsptk* virus-infected TG was confirmed by analyzing the amount of cellular DNA immunoprecipitated with the H3- and H3K27me3-specific antibodies. Both *GAPDH* and *Rasgrf1* DNAs were enriched following immunoprecipitation with the histone H3 antibody compared to the nonspecific antibody control (see Fig. S2A and S2B in the supplemental material). There was an approximate 12-fold-increased association of *Rasgrf1* DNA with the H3K27me3 modification compared to *GAPDH* DNA (Fig. S2C). Although it appeared that the association of *Rasgrf1* with H3K27me3 increased between 10 and 14 dpi, the proportions of histone H3 with the K27me3 modification were similar for all three time points (Fig. S2D). Consistent with our observations for wild-type virus, histone H3 associ-

ated with the viral gene promoters by 7 dpi (Fig. 3B), whereas little viral DNA was immunoprecipitated with the IgG control (Fig. 3A). The levels of H3K27me3 on the *dlspstk* genome (Fig. 3C) appeared to be increased compared to those on the WT viral genome (compare Fig. 1C to 3C) by 7 dpi; however, the ChIP assays on WT- and *dlspstk*-infected TG (Fig. 1 and 3) were not carried out side by side. To determine if H3K27me3 levels were increased on the *dlspstk* genome, we infected mice with either WT or *dlspstk* virus, and the ChIP assays were carried out in parallel. We detected an approximately 2-fold increase in the proportion of H3 with the K27me3 modification following infection with *dlspstk* virus compared to that following infection with WT virus (results not shown). Therefore, the TK-null mutant virus showed an earlier although still limited association of H3K27me3 with its genome. Furthermore, the association of H3K27me3 with the *dlspstk* lytic promoters increased between 10 and 14 dpi (Fig. 3C). Because we saw some variation in the total H3 levels on the *dlspstk* promoters, we also normalized the proportion of histone H3 with the K27me3 modification (Fig. 3D). Following normalization to total H3, a dramatic increase in the K27me3 levels was evident between 10 and 14 dpi especially on the *ICP4*, *ICP0*, *ICP8*, and *TK* promoters. There was no increase in H3K27me3 levels on the *LAT* promoter or 5' exon during the same time period. Thus, even in the absence of viral replication during the acute stage of infection, H3K27me3 accumulated on the viral genome at a time later than the time of clearance of the wild-type virus acute infection, indicating that the increase in H3K27me3 association was not a result of the clearance of virus that had undergone DNA replication.

***ICP0* gene transcript levels are higher than those of the *ICP8* and *UL48* genes at 14 dpi.** Because we observed low levels of H3K27me3 on the *ICP4* and *ICP0* promoters at 14 days following infection with the wild-type virus, we examined whether there were differences in gene expression that correlated with H3K27me3 levels on lytic promoters. To this end, we carried out real-time-quantitative PCR (RT-qPCR) using primers specific for the *ICP0*, *ICP8*, and *UL48* gene transcripts. Following infection with WT virus, we observed an approximate 2-fold increase in *ICP0* RNA compared to *ICP8* RNA (see Fig. S3 in the supplemental material). We also observed higher *ICP8* RNA levels than *UL48* levels (approximately 3-fold more) (Fig. S3). Therefore, in TG isolated from infected mice, an increase in *ICP0* RNA levels compared to *ICP8* and *UL48* RNA levels correlated with reduced H3K27me3 association with the *ICP0* (italicized) promoter. However, the correlation was not complete, because we observed increased levels of H3K27me3 on the *ICP8* promoter compared to levels on the *UL48* promoter at 14 dpi but higher levels of *ICP8* RNA than *UL48* RNA.

Suz12 is recruited to the viral genome during the establishment of latency. Polycomb repressive complex 2 (PRC2) is responsible for the addition of H3K27me3 to cellular chromatin (26). We therefore tested whether we could detect PRC2 on viral promoters during the establishment of latent infection, at the time at which we first detected H3K27me3 on the viral genome. We first examined whether we could detect PRC2 on the WT viral genome at 14 dpi, because this was the earliest time at which the lytic gene promoters were found to be enriched for H3K27me3. To this end, we carried out ChIP using an antibody specific for Suz12, a component of PRC2. We first validated the antibody by analyzing the enrichment of Suz12 at cellular DNA. Following immunoprecipitations with Suz12, there was no enrichment of

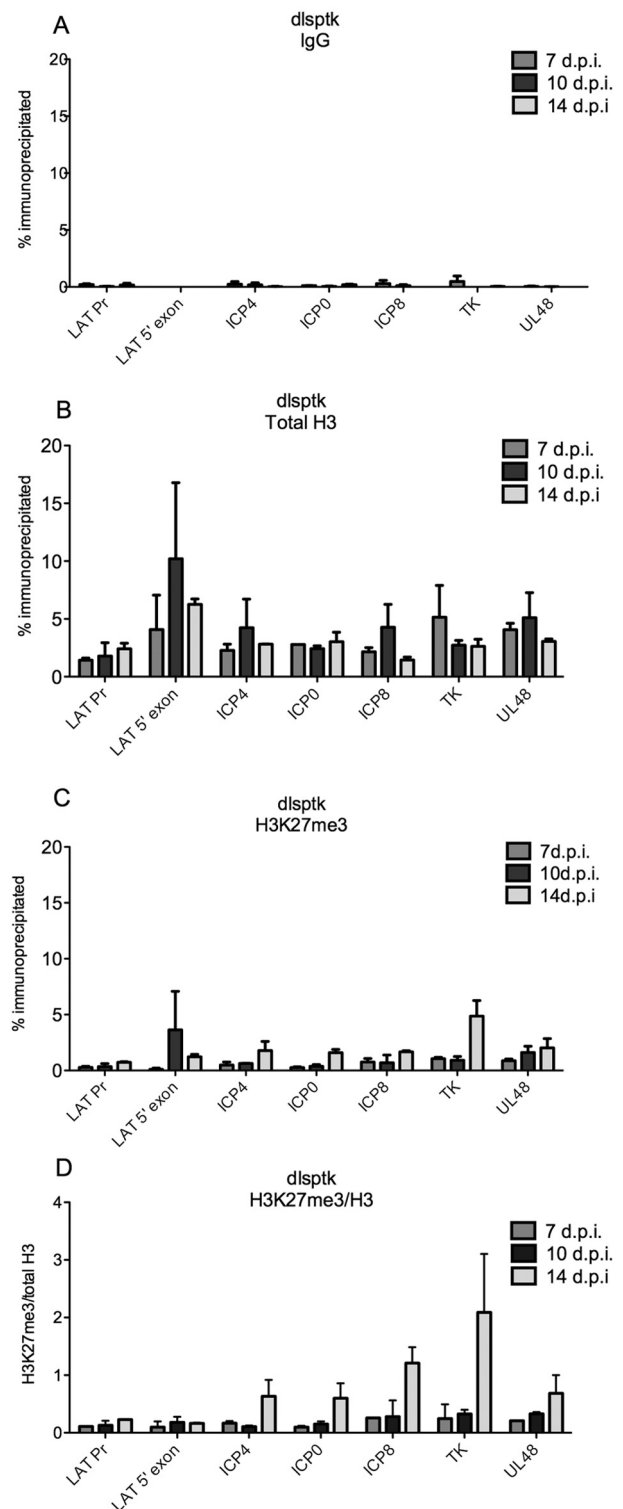


FIG 3 Association of H3K27me3 with the HSV-1 genome following infection with a TK-null mutant virus. Mice were infected with HSV-1 *dlspstk* virus, and at 7, 10, or 14 dpi the mice were sacrificed and trigeminal ganglia were collected. ChIP was carried out on trigeminal ganglia. Shown are the percentages of viral DNA sequences immunoprecipitated with IgG control antibody (A), with an antibody against total histone H3 (B), and with an antibody against H3K27me3 (C). (D) The percentages of viral DNA immunoprecipitated with an antibody against H3K27me3 were normalized to the percentages of DNA precipitated with an antibody against total histone H3.

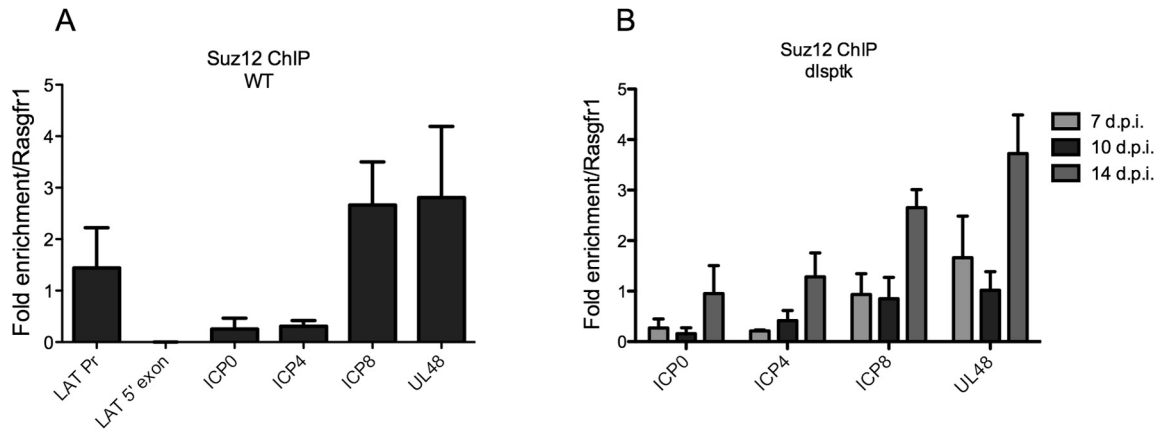


FIG 4 Association of the Polycomb group protein Suz12 with the HSV-1 genome. Mice were infected with either WT or *dlsptk* virus, and at 7, 10, or 14 dpi, the mice were sacrificed and trigeminal ganglia were collected. ChIP was carried out on trigeminal ganglia using an antibody specific for Suz12. (A) The percentages of viral DNA sequences immunoprecipitated from WT infected ganglia with an antibody against Suz12 were normalized to the percentages of *Rasgrf1* DNA immunoprecipitated in the same reaction. (B) The percentages of viral DNA sequences immunoprecipitated from *dlsptk*-infected ganglia with an antibody against Suz12 were normalized to the percentages of *Rasgrf1* DNA immunoprecipitated in the same reaction.

GAPDH DNA compared to levels with the nonspecific antibody control, whereas there was an approximate 5-fold increase in *Rasgrf1* DNA immunoprecipitated with the Suz12 antibody compared to that with the nonspecific antibody control (Fig. S4A). Because the Suz12 ChIP assay detected the association of cellular DNA correlating with H3K27me₃, we analyzed the enrichment of Suz12 at viral promoters in the same samples (Fig. 4A). We detected an enrichment of Suz12 on the *ICP8*, *U_L48*, and *LAT* gene promoters, compared to its level on the *Rasgrf1* sequences. Although detectable over levels with the nonspecific antibody control, the levels of Suz12 on the *ICP0* and *ICP4* promoters were less than in the cellular positive control. We could not detect an enrichment of Suz12 on the *LAT* 5' exon over levels with the nonspecific antibody control.

Having shown that Suz12 was present on the WT viral genome at 14 dpi, we examined whether the kinetics of Suz12 recruitment mirrored the increase in H3K27me₃ levels seen on the *dlsptk* genomes between 7 and 14 dpi. The levels of Suz12 on the *Rasgrf1* region of cellular DNA remained constant between 7 and 14 dpi and were enriched by approximately 10-fold over levels on *GAPDH* sequences (see Fig. S4B in the supplemental material). We observed an increase in Suz12 levels on all four lytic gene promoters tested between 10 and 14 dpi (*ICP0*, 6-fold; *ICP4*, 3-fold; *ICP8*, 3-fold; *U_L48*, 3.8-fold) (Fig. 4B). Consistently with the H3K27me₃ results, the levels of Suz12 were highest on the *ICP8* and *U_L48* gene promoters at all of the times tested.

Suz12 recruitment occurs independently of LAT expression. The increase in H3K27me₃ levels between 10 and 14 dpi argued for a potential trigger of Suz12 recruitment during this time period. Noncoding RNAs have been found to function as cofactors for the recruitment of PRC2 to regions of cellular chromatin (27–33). Using two independent LAT-null viruses, we previously identified a role for the LAT in promoting wild-type levels of H3K27me₃ on the lytic gene promoters during latent infection (12).

Proença et al. (20) found that the activity of the *LAT* promoter increased in ganglia between 5 and 15 dpi. We therefore examined whether levels of *LAT* intron and exon RNAs increased in our system during the same time frame, in which we observed in-

creases in H3K27me₃ levels. We carried out RT-qPCR analysis with RNA extracted from mice infected with WT virus at 7, 10, and 14 dpi (Fig. 5A). Primers specific for the *LAT* intron and 5' and 3' exons were used, and the RNA copy numbers were normalized to the viral DNA copy numbers in the samples extracted from the same mice. As expected, the *LAT* intron was more abundant than the 5' and 3' exons. We observed a slight increase (2.7-fold) in *LAT* intron expression following infection with WT virus between 10 and 14 dpi (Fig. 5A).

We further investigated whether Suz12 association with viral lytic gene promoters was affected by the presence or absence of the *LAT*. We infected mice with the KdLLAT *LAT* deletion mutant virus in parallel with the KFSLAT rescued virus. All experiments and ChIP assays for the two viruses were carried out side by side. The enrichment of Suz12 on the cellular *Rasgrf1* sequence was similar to that on the *GAPDH* sequence following infection with both viruses (see Fig. S5B in the supplemental material). The levels of Suz12 on the *ICP4*, *ICP8*, *TK*, and *U_L48* gene promoters were comparable following infection with either KdLLAT or KFSLAT virus (Fig. 5B). The slight increase in the levels of Suz12 on the *ICP0* gene promoter following infection with the *LAT* deletion virus was not statistically significant ($P = 0.137$, one-tailed paired *t* test). Importantly, we did not detect any change in Suz12 recruitment to the lytic gene promoters in the absence of the *LAT*.

Bmi1 is not detectable on lytic promoters previously found to be associated with H3K27me₃ during latent infection. Because it appeared that the *LAT* did not play a role in the initial recruitment of the PRC2 complex during the establishment of latent infection, we hypothesized that it might instead play a role in the recruitment of the PRC1 complex for maintenance of the H3K27me₃ modification. However, in the course of our experiments investigating Bmi1 recruitment to viral lytic gene promoters following infection with KdLLAT and KFSLAT, we noticed that the levels of occupancy by Bmi1 were much lower than the levels of occupancy on the cellular positive control (results not shown). Therefore, we decided to investigate whether Bmi1 was indeed recruited to WT genomes during the maintenance of latent infection. To this end, we infected mice with WT HSV and prepared chromatin from latently infected TG at least 28 dpi. A 3.2-fold

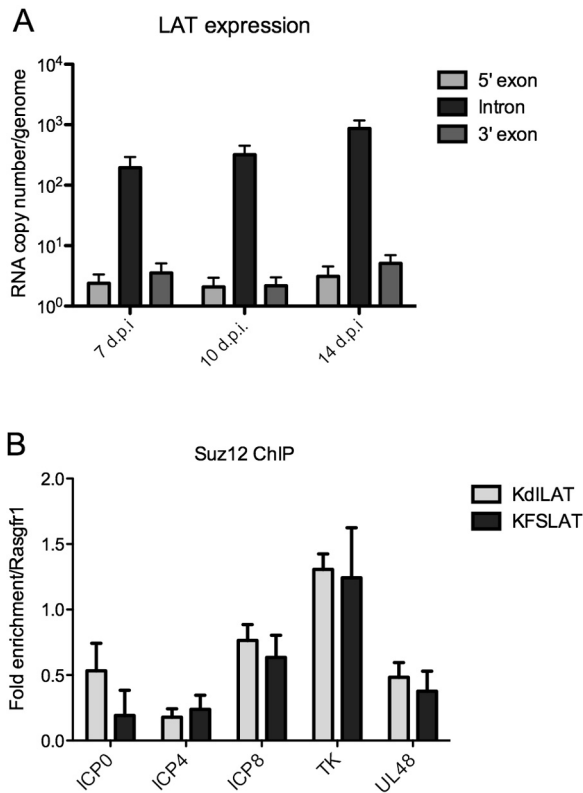


FIG 5 Role of latency-associated transcript (LAT) in the recruitment of Suz12 during the establishment of latent infection. (A) Kinetics of LAT expression by WT HSV-1. The absolute copy numbers of the LAT 5' exon, intron, and 3' exon transcripts were normalized to the copy numbers of viral genomes isolated from the same mouse following infection with wild-type HSV-1. (B) Mice were infected with either a KdlLAT *LAT* promoter deletion mutant virus or the KFSLAT rescued virus, and at 14 dpi, the mice were sacrificed and trigeminal ganglia were collected. ChIP was carried out on trigeminal ganglia using an antibody specific for Suz12. The percentages of viral DNA sequences immunoprecipitated from KdlLAT- or KFSLAT-infected ganglia with an antibody against Suz12 were normalized to the percentages of *Rasgrf1* DNA immunoprecipitated in the same reaction.

increase in the enrichment of *Rasgrf1* DNA compared to *GAPDH* DNA following immunoprecipitation with the Bmi1 antibody confirmed the specificity of the ChIP assay (see Fig. S6 in the supplemental material). The relative enrichment of viral genes following ChIP with Bmi1-specific antibody was normalized to the Bmi1 occupancy of the negative-control *GAPDH* gene. Of the regions of the viral genome tested, the only regions enriched at least 2-fold above their level in the cellular negative control were the *LAT* promoter, *LAT* intron, and *TK* promoter (Fig. 6). However, in a number of experiments, these regions were not enriched even compared to levels with the IgG control. Therefore, we carried out a direct comparison with the amount of viral DNA immunoprecipitated with the Bmi1 antibody and the nonspecific antibody control (Table 1). The only region of DNA analyzed that was significantly enriched for Bmi1, compared to IgG, was the cellular positive control, *Rasgrf1*. Thus, we did not detect significant association of the lytic promoters with Bmi1.

DISCUSSION

Lytic gene promoters on the HSV genome are associated with heterochromatin during latent infection of murine sensory gan-

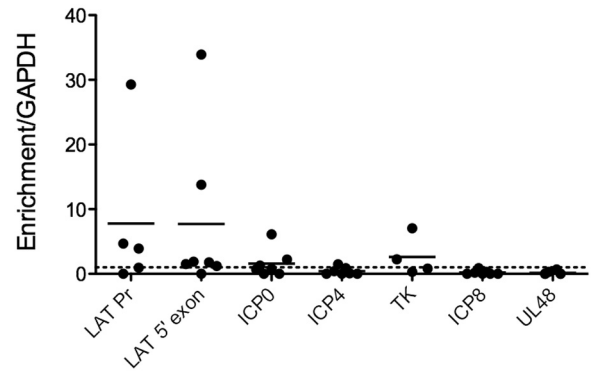


FIG 6 Association of the Polycomb group protein Bmi1 with the HSV-1 genome. Mice were infected with wild-type HSV-1, and at 28 dpi or later, TG were collected. Chromatin immunoprecipitation was carried out on the trigeminal ganglia using an antibody against Bmi1. The fractions of viral DNA sequences immunoprecipitated with the Bmi1-specific antibody are shown normalized to the fraction of cellular *GAPDH* DNA. Results from individual experiments are represented.

glia (11–13). We and others observed that one of the major heterochromatin marks associated with HSV latent chromatin is H3K27me3 (12, 13), a form of heterochromatin associated with developmentally regulated genes called facultative heterochromatin (16). The observation in this study that the PRC2 complex is recruited to the viral genome during the establishment of latent infection argues further that the virus utilizes this repressive mark, at least in part, to silence or maintain silencing of its genome during latent infection. The association of lytic promoters with fHC, which is thought to be dynamic and capable of being converted to euchromatin, would be advantageous for reactivation of HSV from latent infection. However, unlike with some forms of cellular facultative heterochromatin, we were unable to detect the PRC1 complex on viral lytic promoters during the maintenance of latent infection.

Little was known about the kinetics of association of various forms of chromatin with the HSV lytic genes beyond our original observations that the HSV-1 genome is associated with histone H3 by 7 dpi and that the heterochromatin marker H3K9me2 is associated with the viral genome by 15 dpi (11). In this study, we confirmed that during establishment of latent infection by HSV-1 in murine trigeminal ganglia, the viral lytic gene promoters are associated with histone H3 by 7 dpi. Histone H3K27me3 modification was detected on WT HSV lytic gene chromatin by 14 dpi, arguing that nucleosomes are deposited prior to K27 methylation. Hence, we hypothesize that there are multiple steps in the silencing and the maintenance of that silencing during latent infection.

Mechanisms of HSV gene silencing following initial infection of neurons. In this infection model, acute viral replication in the trigeminal ganglia largely declined by 7 dpi (18). Therefore, the bulk of H3K27me3 addition onto viral genomes appears to occur after resolution of the infection. This argues that other earlier events are responsible for the initial silencing of the viral genome. The initial mechanisms of silencing may involve the association of unmodified histones, other heterochromatin markers on HSV DNA, or other undefined mechanisms.

Other factors that have been hypothesized to explain the lack of lytic gene expression in sensory neurons include the following. (i) The transactivator complex is unable to localize in the nucleus of

TABLE 1 Immunoprecipitation of viral and cellular DNA with anti-Bmi1 and control antibodies

Primer	Mean fraction immunoprecipitated with:		Mean fold enrichment relative to IgG control	P value
	IgG	Bmi1		
LAT promoter	0.0748	0.416	5.6	0.3125
5' exon	0.0616	0.105	1.7	0.2188
ICP4	0.0808	0.0620	0.77	0.9375
ICP0	0.138	0.135	0.98	0.3750
ICP8	0.122	0.0529	0.43	0.6875
TK	0.0391	0.0806	2.1	0.1250
U _L 48	0.158	0.0551	0.35	0.2188
Rasgrfl	0.0113	0.128	11	0.0156
GAPDH	0.0244	0.0387	1.6	0.1094

an infected neuron or to stimulate IE gene expression (7). The transactivator complex includes VP16, the cellular HCF-1, Oct1, histone K9 demethylases, and histone K4 methyltransferases (7, 34). In addition to recruiting the transactivator complex, VP16 recruits ATP-dependent chromatin remodeling complexes to IE promoters (35). Hence, if VP16 is unable to recruit cellular factors to IE promoters in neurons, the result could be an increase in total chromatin, increased heterochromatin, and decreased euchromatin association with IE promoters. (ii) Initial silencing may be due to cellular repressive mechanisms. The cellular repressor REST-CoRest complex has been hypothesized to contribute to lytic gene silencing. Inactivation of this complex in infected cells resulted in increased viral replication *in vivo* by 7 dpi (36).

In this study, we did not detect a role for LATs in the initial recruitment of Suz12. However, silencing of lytic gene expression during the acute stage of infection may involve RNAs expressed from the *LAT* locus acting in a distinct manner, independently of Suz12 recruitment. Infection with a *LAT* deletion mutant virus resulted in increased numbers of neurons expressing lytic mRNAs by 3 to 5 dpi (9). Whether this was due to a direct effect of LAT RNAs down-regulating RNA expression, a result of deletion of *cis*-acting sequences in the *LAT* region, such as insulators, or an indirect effect due to viral miRNAs down-regulating lytic protein expression is not known. Clearly, more-detailed studies utilizing recombinant viruses lacking specific elements within the *LAT* region are required.

Thus, it is conceivable that other mechanisms restrict IE gene transcription as the initial acute infection is cleared and that facultative heterochromatin modifications are incorporated onto the histones associated with lytic promoters to maintain gene silencing. Consistently with this, a recent study found that dense chromatin increases H3K27 methylation (37), raising the possibilities that the initial silencing of the viral genome involves association of dense chromatin with the viral genome and, then, that H3K27me3 modification serves to maintain or further silence gene expression. More-detailed studies of the first days of infection could yield more information on these possibilities.

H3K27me3 association with lytic promoters during the establishment of latent infection. We hypothesize that H3K27 methylation plays a role in the maintenance of latent infection by silencing lytic gene expression. Although a limited number of genes have been surveyed thus far, it is noteworthy that the early *ICP8* and *TK* genes and the late *U_L48* gene had higher levels of H3K27me3 than the IE *ICP0* and *ICP4* genes. We also detected higher levels of *ICP0* RNA at 14 dpi than of *U_L48* and *ICP8* RNAs. Thus, this heterochromatic mark may have a greater silencing

effect on E and L genes than on IE genes, or H3K27me3 levels may be higher on E and L genes because they undergo lower levels of transcription. Noteworthy was the level of expression of *ICP0* gene transcripts, which was higher than those of *ICP8* and *U_L48* RNAs. One model of RNA interference (RNAi)-mediated transcriptional silencing involves complementary transcripts (38); therefore, it is conceivable that the *LAT* and *ICP0* gene transcripts form complementary RNAs that silence the rest of the genome. The presence of *ICP0* mRNA does not necessarily indicate the presence of *ICP0* protein, especially since an HSV-1 miRNA, miR-H2, which is expressed during both acute and latent infection of the TG (15), targets *ICP0* mRNA and prevents the accumulation of *ICP0* protein levels but not mRNA when tested in transfected cells (39).

The LAT and the mechanism of H3K27me3 modification of viral chromatin. Previous studies in our system have shown that viruses that lack *LAT* expression have reduced levels of the H3K27me3 modification on histones associated with HSV lytic gene promoters (12), although one other study comparing a mutant HSV-1 strain 17 with a wild-type strain 17 virus observed that this mutant showed increased H3K27me3 associated with viral promoters (13). In the current study, we observed that recruitment of Suz12, a component of PRC2 that serves as the methyltransferase complex, was not increased by *LAT* expression; instead, Suz12 was associated with the viral lytic gene promoters at equal levels in the presence and absence of *LAT* transcription. This argues that the *LAT* may promote the accumulation of H3K27me3 by a mechanism(s) other than recruitment of Suz12 to the viral lytic promoters. Most models for long noncoding RNA silencing show the PRC2 complex of Ezh2, Suz12, and Eed being recruited to chromatin as a complex involving the RNA (38). Based on our results, it is conceivable that a complex that does not involve Suz12 may be regulated by *LAT* or that components of the complex are recruited onto chromatin separately and that *LAT* promotes the association of a component other than Suz12. Alternatively, the *LAT* may regulate another step and shift the equilibrium by stimulating the activity of the PRC2 complex or inhibiting a demethylase enzyme.

Role of PRC1 in HSV lytic gene silencing. In this study, we were unable to detect significant enrichment of HSV lytic promoters with a component of the PRC1 complex, Bmi1. At first glance, this seems contradictory to the findings of a previous study (13). However, our results are similar to the results obtained in the study by Kwiatkowski et al. (13), which also found that the enrichment of lytic promoters following ChIP for Bmi1 was less than 2-fold more than that of the cellular negative control (*APRT*). The

two different conclusions reached in our study and the previous study highlight the challenges in using ChIP assays to determine the presence or absence of a protein on regions of DNA. It is possible that the PRC1 complex is present on the viral genome during latency at other sites not analyzed in this study. Global analysis of ChIP assays using antibodies to PRC1 proteins would be required to determine if PRC1 binds specific regions of the HSV genome during latent infection. It is also possible that Bmi1 is recruited at a time point later than 28 dpi.

Our results raise the possibility that the form of facultative heterochromatin on the viral lytic promoters is not bound by PRC1. A study by Ku et al. found that a proportion of bivalent genes enriched for H3K27me3 and H3K4me3 in embryonic stem cells were not enriched for PRC1 (40). Hence, H3K27me3 is not always bound by PRC1. The presence of the H3K27me3 modification in the absence of PRC1 binding is still predicted to maintain gene silencing because methylation of the lysine 27 residue of histone H3 has been found to prevent its acetylation (41). Lytic gene promoters that are associated with H3K27me3 in the absence of Bmi1 may also be more readily converted into euchromatin to allow reactivation to occur. Clearly, additional functional studies are required to investigate the contribution of both the PRC2 and PRC1 complexes to the initiation and maintenance of latent infection.

MATERIALS AND METHODS

Cells and viruses. Vero cells were maintained as described previously (25). The WT strain of HSV-1 (KOS) used in this study was grown and titrated as described previously (9). The HSV-1 KdlLAT LAT promoter deletion mutant virus, the KFLAT repaired virus, and their propagation and titration have been described elsewhere (9). The HSV-1 KOS *dlsptk* TK-null mutant virus has been described previously (23).

Mouse infections. Six-week-old male CD-1 mice (Charles River Laboratories) were anesthetized by intraperitoneal injection of ketamine hydrochloride (3.4 mg) and xylazine hydrochloride (0.5 mg). For the majority of experiments, the scarified corneas of mice were inoculated with 2×10^6 PFU/eye of virus (in a 5- μ l volume), as described previously (42). For *dlsptk* infections, mice were inoculated with 5×10^6 PFU of virus/eye. Mice were housed in accordance with institutional and National Institutes of Health guidelines on the care and use of animals in research, and all procedures were approved by the Institutional Animal Care and Use Committee of Harvard Medical School.

Chromatin immunoprecipitations. The following antibodies were used for chromatin immunoprecipitations (ChIP): anti-H3K27me3 (Millipore; catalog number 07-449), anti-histone H3 (Abcam; catalog number AB1791), anti-Bmi1 (Abcam; catalog number AB14389), anti-Suz12 (AB12093), and normal rabbit IgG (Millipore; catalog number 12-370).

See materials and methods in the supplemental material for detailed descriptions of chromatin immunoprecipitation and quantification of viral gene expression.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00590-12/-/DCSupplemental>.

Text S1, DOCX file, 0.1 MB.
Figure S1, DOCX file, 0.1 MB.
Figure S2, DOCX file, 0.1 MB.
Figure S3, DOCX file, 0.1 MB.
Figure S4, DOCX file, 0.1 MB.
Figure S5, DOCX file, 0.1 MB.
Figure S6, DOCX file, 0.1 MB.

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