JOURNAL OF VIROLOGY, Apr. 2011, p. 3424–3435 Vol. 85, No. 7
0022-538X/11/$12.00 doi:10.1128/JVI.02263-10

Reversal of Heterochromatic Silencing of Quiescent Herpes Simplex Virus Type 1 by ICP0▽

Michael W. Ferenczy and Neal A. DeLuca*

Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Received 28 October 2010/Accepted 22 December 2010

Persisting latent herpes simplex virus genomes are to some degree found in a heterochromatic state, and this contributes to reduced gene expression resulting in quiescence. We used a relatively long-term quiescent infection model in human fibroblasts, followed by provision of ICP0 in *trans*, to determine the effects of ICP0 on the viral chromatin state as gene expression is reactivated. Expression of ICP0, even at low levels, results in a reduction of higher-order chromatin structure and heterochromatin on quiescent viral genomes, and this effect precedes an increase in transcription. Concurrent with transcriptional activation, high levels of ICP0 expression result in the reduction of the heterochromatin mark trimethylated H3K9, removal of histones H3 and H4 from the quiescent genome, and hyperacetylation of the remaining histones. In contrast, low levels of ICP0 did not appreciably change the levels of histones on the viral genome. These results indicate that ICP0 activity ultimately affects chromatin structure of quiescent genomes at multiple levels, including higher-order chromatin structure, histone modifications, and histone association. Additionally, the level of ICP0 expression affected its ability to change chromatin structure but not to reactivate gene expression. While these observations suggest that some of the effects on chromatin structure are possibly not direct, they also suggest that ICP0 exerts its effects through multiple mechanisms.

Herpes simplex virus type 1 (HSV-1) latency is characterized by significantly reduced transcription of the viral genome relative to that seen in productive infection. One gene that appears to be selectively transcribed is that for the latency-associated transcript (LAT) (54, 56). This general repression of gene expression suggests that latent gene expression is controlled by epigenetic mechanisms. Since viral DNA is not extensively methylated (33), expression is probably repressed by chromatin structure on the viral genome. During latency, the viral genome is found in an endless, possibly circular, episomal structure, bound by nucleosomes (12, 30) and heterochromatin (12).

Periodically *in vivo*, HSV-1 reactivates, replicates, and can cause recurrent disease. Cellular stress (10, 11, 39, 49, 60) and decrease in immune function can both contribute to reactivation (19). Upon reactivation, the full repertoire of viral genes is eventually expressed. The exact order of gene expression upon reactivation is unclear, but latently infected explanted mouse trigeminal ganglia (TG) were shown to express genes in a temporal pattern different from that seen in productive infection (57), while there is also a decrease in LAT expression prior to, or concurrent with, lytic gene expression (55, 60). The relative contributions of viral activators of gene expression to different aspects of the reactivation process are unclear. However, one activator, the immediate-early (IE) protein ICP0, is required for efficient reactivation from latency *in vivo* (4–6, 26, 27, 31).

ICP0 is a promiscuous activator of gene expression at the level of RNA synthesis (15, 20, 31, 47, 50). Since it does not directly bind DNA, it has been postulated that ICP0 may exert its effects by interaction with proteins controlling transcription or influencing some general step prior to the assembly of transcription complexes on the viral genome. It has been shown that ICP0 contains an E3 ubiquitin ligase RING finger domain (3, 13). Expression of ICP0 leads to the degradation of a number of proteins, including the centromeric histone variants CENP-A (42), -B (41), and -C (16), as well as constituents of the PML nuclear bodies (17, 22), which are thought to be part of the innate antiviral defense (14, 58). ICP0 also interacts with, and redistributes, class II histone deacetylases (HDACs) (43), and the C terminus of ICP0 has also been shown to interact with and disrupt the repressive HDAC-containing complex of HDAC1/HDAC2/REST/CoREST/LSD1 (21, 23–25), which has been implicated in increased viral gene expression. Additionally, ICP0 may interact with the histone acetyltransferase (HAT) PCAF (37).

These observations suggest that ICP0 may mediate its transactivation function through manipulation of epigenetic control of gene expression by chromatin. Few studies have investigated the changes to viral chromatin upon reactivation of quiescent or latent virus. A study by Coleman et al. examined ICP0-mediated derepression in a fibroblast model of quiescence utilizing replication-deficient HSV mutants (9). After establishment of quiescence, followed by superinfection with an adenovirus providing ICP0, an increase in hyperacetylated histone H3 (AcH3) was found on the ICP0, ICP4, ICP27, VP16, gC, and LAT promoters, and a decrease in the repressive modification trimethylation of histone H3 lysine 9 (H3K9me3) was found on the ICP0, ICP27, VP16, and gC promoters. These results demonstrated that derepression of quiescent genomes by ICP0 induces a global change in chromatin structure, specifically, acetylation of histones associated with quiescent viral genomes.

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, 547 Bridgeside Point II, 400 Technology Dr., Pittsburgh, PA 15213. Phone: (412) 648-9947. Fax: (421) 624-1401. E-mail: ndeluca@pitt.edu.

▽ Published ahead of print on 29 December 2010.

In the current study, we explored the effects of ICP0 on gene expression and epigenetic structure of quiescent HSV genomes by using a cell culture model of HSV quiescence in HEL cells. The results suggest that there may be multiple mechanisms through which ICP0 exerts its effects and that these mechanisms may depend on the abundance of ICP0. In addition to its previously demonstrated effects on the acetylation and methylation of histones associated with quiescent genomes, the activity of ICP0 results in the removal of preformed nucleosomes from quiescent genomes. This may be a direct or indirect effect.

MATERIALS AND METHODS

Cells and viruses. Experiments were performed using MRC-5 (human embryonic lung) cells obtained from, and propagated as recommended by, the American Type Culture Collection (ATCC). The viruses used in this study were the HSV-1 IE mutants d105 (28, 52) and d109 (52), as well as the adenoviruses AdS.11D and AdS.11E4(ICP0) (28). d105 was propagated on E11 cells and d109 on F06 cells as previously described (52).

ChIP. Chromatin immunoprecipitation (ChIP) was carried out as previously described (18, 53), with a few modifications. MRC-5 cells (5×10^6) were plated in 100-mm dishes and were infected by d109 at a multiplicity of infection (MOI) of 10 at room temperature for 1 h. After adsorption, the inoculum was removed and 37°C 5% Dulbecco's modified Eagle's medium (DMEM) was added. Infected cells were maintained at 37°C for 24 h. At 24 h postinfection (hpi), the medium was replaced with fresh medium, and infected cells were maintained at 34°C. On day 4 postinfection, medium was again replaced with fresh medium. At day 7 postinfection, d109-infected cells were mock superinfected, superinfected with d105 at an MOI of 10, or superinfected with AdS.11D and AdS.11E4(ICP0) at 200 focus-forming units (FFU) per cell for 1 h at room temperature. After adsorption, the inoculum was aspirated and the conditioned medium (which was saved and maintained at 37°C) was replaced. This was considered time zero postsuperinfection. At various times postinfection, as indicated in the figures, cells were treated with 1% formaldehyde for 10 min at 37°C, washed 3 times with cold phosphate-buffered saline (PBS) containing protease inhibitors (67 ng/ml aprotinin, 1 ng pepstatin, 0.16 mM TLCK [*N*α-*p*-tosyl-L-lysine chloromethyl ketone], 1 mM phenylmethylsulfonyl fluoride [PMSF]), and scraped into PBS containing protease inhibitors. The cells were pelleted at 3,000 rpm for 10 min at 4°C, resuspended in cold SDS lysis buffer (100 μl per million cells) containing protease inhibitors (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1], 4 μg/ml aprotinin, 2 μg/ml pepstatin, 0.15 mM TLCK, and 0.6 mM PMSF), and incubated on ice for 30 min. All other procedures were as described (53). The antibodies used were anti-histone H3 (ab1791; Abcam), anti-histone H4 (05-858; Millipore), anti-acetyl histone H3 (06-599; Millipore), anti-acetyl histone H4 (06-866; Millipore), anti-trimethyl histone H3 lysine 9 (07-422; Millipore), and anti-heterochromatin protein 1γ (05-690; Millipore). A "no-antibody control" was included for each ChIP experiment. When ChIP results were calculated after quantitative PCR (qPCR), the value for the no-antibody control was subtracted from the immunoprecipitation results before the percent input of immunoprecipitation was calculated. Therefore, any values reported indicate an increase over the baseline. The baseline, or no-antibody controls, never resulted in amplifiable products with the number of cycles used.

Micrococcal nuclease digestion. MRC-5 cells were plated (7.5×10^6 cells per 100-mm dish) in 12 dishes. Four dishes were mock infected, and 8 were infected with d109 at an MOI of 20, for 1 h with rocking every 10 min at room temperature. After adsorption, the inoculum was removed and 37°C 5% DMEM was added. Infected cells were maintained at 37°C for 24 h. At 24 hpi, the medium was replaced with fresh medium, and infected cells were maintained at 34°C. On day 4 postinfection, medium was again replaced with fresh medium. At day 7 postinfection, 4 d109-infected dishes and 4 mock-infected dishes were superinfected with d105 at an MOI of 10, while the remaining 4 d109-infected dishes were mock superinfected, at room temperature for 1 h. After adsorption, the inoculum was removed and the conditioned medium, which had been saved at 37°C, was replaced. This was considered time zero postinfection. Eight hours postsuperinfection, nucleoprotein complexes were digested by *in situ* micrococcal nuclease (MN) digestion (61) (protocol 1). Briefly, cells were permeabilized with lysolecithin (0.5 mg/ml) in permeabilization solution 1 (150 mM sucrose, 80 mM KCl, 35 mM HEPES, pH 7.4, 5 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$) for 1 min at 37°C. Chromatin from each of the three groups of 4 infections was then digested with 2.5 ml permeabilization solution 2 (150 mM sucrose, 50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 2 mM $CaCl_2$) with 0, 300, 1,000, or 3,000 gel units micrococcal nuclease (M0247S; New England BioLabs) for 5 min at room temperature. Gel units are as defined by New England BioLabs. Cells were scraped into 500 μl NDPK buffer (20 mM Tris-Cl, pH 7.4, 0.2 M NaCl, 3 mM EDTA, 1% SDS, 0.2 mg/ml proteinase K) and digested at 37°C overnight. DNA was isolated by phenol chloroform extraction and ethanol precipitation, RNase A treated, separated on a 2% agarose gel, transferred to a Nytran membrane, probed with 2 μg ^{32}P-labeled nick-translated green fluorescent protein (GFP) fragment of pEGFP-C1 digested with AseI and BglII (New England BioLabs), and exposed to Hybond film.

RNA isolation and reverse transcription (RT). RNA was isolated with an Ambion RNaqueous 4 PCR kit, following the included protocol. Briefly, 5×10^6 MRC-5 cells in 100-mm plates were infected with d109 at an MOI of 10 for 1 week and superinfected as described for the ChIP experiments. RNA was harvested at the time points indicated in the figures by addition of 500 μl lysis/binding buffer. Cells were scraped and vortexed. Equal volumes of 67% ethanol were added, and the solution was added to a filter, which was centrifuged at 12,500 rpm at 4°C for 1 min. The bound RNA was washed with wash buffers 1 and 2/3, which were provided with the kit. RNA was eluted with 60 μl 65°C elution solution. The RNA was treated with DNase I at 37°C for 30 min to degrade any residual DNA.

Reverse transcription was performed using an Ambion reverse transcription kit by following the included instructions. RNA (2 μg total) was reverse transcribed in a reaction volume of 20 μl containing RNase inhibitor, oligo(dT) primers, 1 μl Moloney murine leukemia virus (MMLV) reverse transcriptase, and 2 μl 10× reaction buffer. The reaction tube was incubated at 85°C for 3 min to remove RNA secondary structure, and the reverse transcription reaction was carried out for 1 h at 44°C. After the reverse transcription reaction was complete, the reaction tube was incubated at 95°C for 10 min in order to inactivate the reverse transcriptase. cDNA (8 μl) was diluted 1:8 by addition of 40 μl DNase/RNase-free H_2O for use in qPCRs. Additionally, 1 μg RNA was diluted into a total of 60 μl DNase/RNase-free H_2O for use as a negative control in qPCRs.

qPCR. Reactions for ChIP or cDNA quantification were performed in triplicate using 2.5 μl of DNA for each reaction mixture as described previously (53), with a few modifications. Before the 96-well reaction plate was set up, a master mix containing 0.3125 μl of each primer (stock concentration, 1 mM), 6.25 μl Applied Biosystems SYBR green super mix with 1.0 μM 6-carboxy-X-rhodamine (Bio-Rad), and 3.125 μl of water for a total of 10 μl for each reaction was made. The final reaction volume was 12.5 μl, including the DNA. The primers used for ChIP and cDNA quantification and their locations relative to the transcription start site of the gene to be analyzed are as previously published (18). d106 DNA was also included in each plate, in a standard curve of 1:10 dilutions from 250,000 to 25 copies per well, which covers the threshold cycle values for the ChIP DNA samples tested. d106 is a mutant virus genome that contains the same ICP27/human cytomegalovirus (HCMV) enhanced GFP (EGFP) locus as d109 (52). qPCR was run on a StepOne Plus real-time PCR machine. The conditions for the run were as follows: stage 1, 95°C for 10 min, and stage 2, 40 cycle repeats of 95°C for 15 s and 60°C for 1 min. At the end of the run, a dissociation curve was completed to determine the purity of the amplified products. Results were analyzed using StepOne v2.1 software from Applied Biosystems.

RESULTS

RNA expression from quiescent genomes upon reactivation by ICP0. Immediate-early genes are not expressed during infection with d109. This is because both copies of the ICP4 and ICP0 genes are deleted, as is the ICP27 gene. In addition, the TAATGARAT elements that make the IE promoter responsive to VP16 have been removed from the ICP22 and ICP47 promoters. As a consequence, gene expression is repressed and the viral genome persists in cells (52). The virus contains a model transgene consisting of the EGFP gene driven by the HCMV IE promoter in the deleted ICP27 locus. GFP expression is reduced over time in d109-infected cells, with the greatest level of repression in fibroblasts (18, 28, 52, 59). This repression of GFP expression can be reversed in fibroblasts at 7 days postinfection (dpi) by provision of ICP0 (28, 59). In order to characterize the effects of epigenetic changes on the quies-

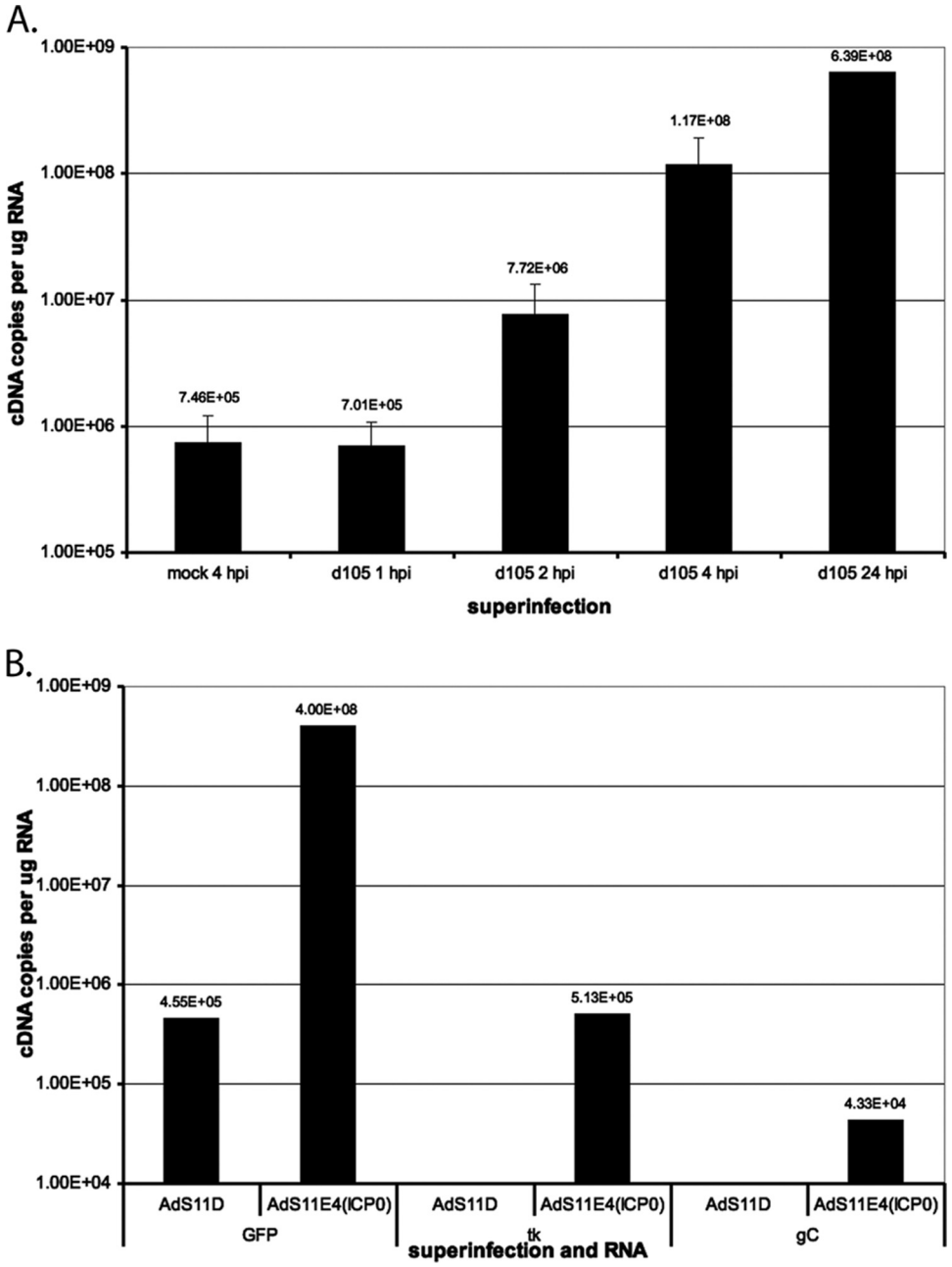

FIG. 1. Abundance of GFP, tk, or gC mRNA in d109-infected MRC-5 cells after superinfection. Infections, RNA isolation, cDNA preparation, and RT-PCR were performed as described in Materials and Methods. Quiescent infection by d109 was established for 1 week. The graphs indicate the numbers of RNA molecules of GFP per μg RNA at the indicated time points after superinfection by d105 (A) or at 24 h after the indicated adenovirus infection (B).

cent HSV genome induced by ICP0, it was important to measure the kinetics in GFP RNA expression upon reactivation. It was also of interest to examine the induction of other viral promoters to determine whether the effects of ICP0 were specific for the HCMV promoter or were more generally targeted to the HSV-1 genome.

After quiescent infection was established with d109 for 7 days, MRC-5 cells were superinfected with d105 or an adenovirus vector expressing ICP0, and RNA levels were determined at various time points by RT-PCR (Fig. 1A). d105 has the same mutations as d109 except that (i) both copies of the ICP0 gene are intact and (ii) there is not an EGFP transgene in the deleted ICP27 locus. The steady-state level of expression of GFP driven by the HCMV promoter of d109 was approximately 7×10^5 copies per μg of total RNA, or approximately an average of 10 molecules per cell. This level of expression was reached by approximately 24 hpi (18) and can be found after 7 dpi. Induction of GFP expression by superinfection with d105 was seen by 2 hpi, at which time GFP mRNA was increased 10-fold. There was a continuous increase in expression

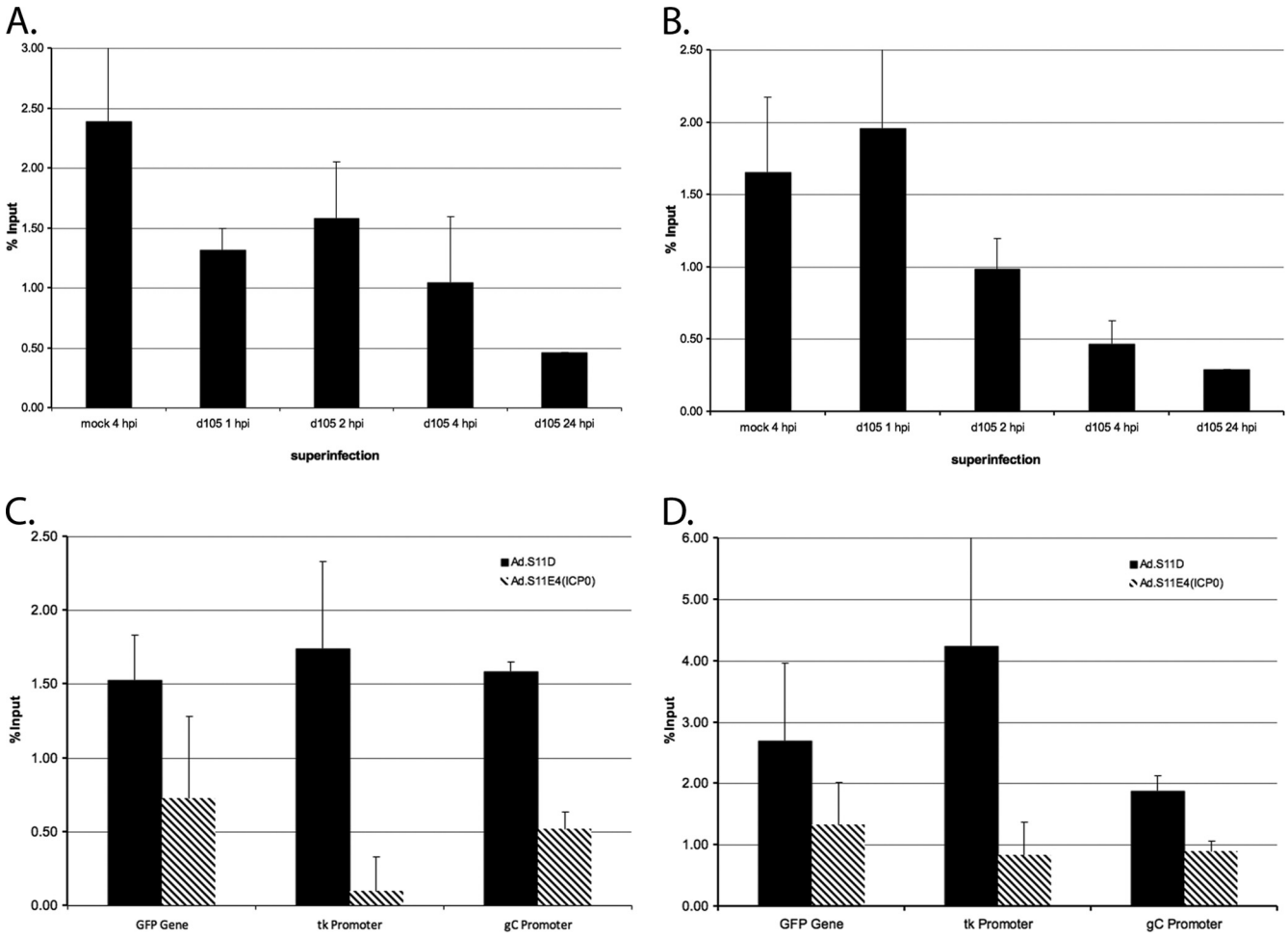

FIG. 2. Repressive chromatin modifications associated with the tk, gC, and HCMV promoters and the GFP 5′ region of d109 in MRC-5 cells after superinfection. Chromatin immunoprecipitation (ChIP) with antibodies to HP1γ (A and C) and H3K9me3 (B and D), followed by RT-PCR with primer pairs corresponding to the indicated promoter regions, was performed as described in Materials and Methods. Graphs show the percentages of total genomes bound after superinfection with d105 at the indicated time points (A and B) or 24 h after superinfection with the indicated adenovirus (C and D). Error bars represent standard errors of the means from 4 experiments.

over time after 2 hpi, and by 24 h after infection with d105, GFP mRNA levels were induced 1,000-fold.

We also explored the effects of an adenovirus vector expressing ICP0 on reactivation of quiescent gene expression in this system. One of the important differences between the provision of ICP0 by d105 and that by the adenovirus vector used is that the amount of ICP0 expression from the adenovirus is about 1/1,000 that from d105 and yet it is still sufficient to reactivate quiescent genomes (28). The same pattern of induction was seen when ICP0 was provided by the adenovirus vector (Fig. 1B) as when ICP0 was provided by d105. By 24 hpi, GFP mRNA increased 1,000-fold. Levels of tk and gC mRNA, which were undetectable in AdS.11D-superinfected cells, increased by greater than 4 orders of magnitude (compared to the lower limit of detection) upon infection with AdS.11E4(ICP0). The different baseline levels of expression of the three genes are probably reflective of the different architectures, and hence strengths, of the tk, gC, and HCMV IE promoters. They are also distributed across the HSV genome. These data indicate that the effects of ICP0 may be general across the entire viral genome and independent of promoter composition and structure.

Removal of higher-order chromatin structure and repressive epigenetic marks upon expression of ICP0. It has previously been shown that expression of ICP0 prevents the accumulation of heterochromatin on the viral genome at representative promoters (8). Specifically, expression of ICP0 prevented accumulation of the heterochromatin mark H3K9me3, as well as deposition of HP1γ (heterochromatin protein 1γ), at the tk, gC, and HCMV promoters, and these effects were correlated with transcriptional activation (18). We wished to test whether ICP0 could also remove heterochromatin marks from repressed, quiescent genomes, once a highly repressed state was established. ChIP assays were performed following d105 and AdS.11E4(ICP0) superinfection reactivation.

ChIP assays for HP1γ and H3K9me3 on the HCMV promoter were performed on d109-infected cells that were superinfected for different lengths of time with d105 (Fig. 2A and B). Expression of ICP0 from d105 caused the removal of HP1γ

beginning at 1 h postsuperinfection (Fig. 2A), which preceded a detectable increase in GFP RNA (Fig. 1). HP1γ levels continued to decrease over time and by 24 h postinfection were approximately one-fifth the level of that found in mock-superinfected cells. H3K9me3 levels did not decrease until 2 h after superinfection with d105 (Fig. 2B). This is presumably because access to histone modifications is limited when they are found in higher-order heterochromatin structures, and binding proteins such as HP1γ must first be removed. H3K9me3 was reduced by 2 h postsuperinfection and also continued to decrease over time. The ICP0-mediated reduction in heterochromatin on the d109 HCMV promoter was concurrent with reactivation of gene expression.

In order to investigate whether the removal of heterochromatin was a general effect or limited to the HCMV promoter, we tested the effects of superinfection with adenovirus-provided ICP0 on the quiescent d109 genome. After d109 quiescence was established for 7 days, cells were superinfected with either AdS.11D or AdS.11E4(ICP0). Twenty-four hours postinfection, ChIP was performed, and the levels of heterochromatin marks on the tk and gC promoters were determined. Additionally, the EGFP coding sequence was assayed. This was necessary because AdS.11D and AdS.11E4(ICP0) contain the secretory alkaline phosphatase reporter gene under the control of the HCMV promoter (28).

Expression of ICP0 from the adenovirus vector caused a reduction in HP1γ on all three classes of viral promoter, with the greatest decrease on the tk promoter (Fig. 2C). Additionally, H3K9me3 was reduced on all three promoters (Fig. 2D). These results demonstrate that ICP0 expression results in a decrease in heterochromatin on quiescent genomes, that this reduction is not dependent on the context in which ICP0 is provided, and that the reduction of heterochromatin may be general across the quiescent viral genome.

Removal of histones upon expression of ICP0. The basic unit of chromatin structure is the nucleosome, which is an octamer of 4 histone proteins, H2A, H2B, H3, and H4, around which approximately 150 bp of DNA is wrapped (44). Nucleosomes have been found to be associated with quiescent HSV DNA (30), while transcriptionally active HSV DNA is most likely not bound by stable classical nucleosomes (32, 34, 36). Previously, it has been demonstrated that expression of ICP0 during productive infection prevents the accumulation of histone H3 on the viral genome (8) and that this effect is also seen during the establishment of quiescence (18). In order to determine whether histones are removed during ICP0-mediated reactivation, ChIP was performed for histones H3 and H4 upon ICP0-mediated reactivation of quiescent d109.

As described for previous experiments, quiescence of d109 was established for 1 week in MRC-5 cells, and cells were superinfected with d105 for 1, 2, 4, or 24 h (Fig. 3). Histone H3 and H4 occupancy of the d109 HCMV promoter was reduced upon expression of ICP0 (Fig. 3). This reduction in histone occupancy was coincident with RNA expression (Fig. 1).

In order to extend these results and further probe the nucleosomal structure of quiescent genomes and the changes induced upon ICP0 expression, *in situ* micrococcal nuclease digestion was performed. Quiescent infection with d109 was established for 1 week, and cells were either superinfected with d105 or mock superinfected. Eight hours postsuperinfection, the cells were permeabilized, and increasing concentrations of micrococcal nuclease were added. Total cell-associated DNA was isolated, size fractionated on an agarose gel, and stained with ethidium bromide to visualize bulk cellular DNA. The DNA was transferred onto a nylon membrane and probed with ^{32}P-labeled GFP DNA. Therefore, the observed MN patterns represent the chromatin structure of the gene unique to the quiescent genomes. The ethidium bromide-stained gel showed the standard nucleosomal pattern of the bulk cellular DNA (Fig. 4). The hybridization signals showed that the quiescent d109 genomes were more resistant to MN digestion than bulk cellular DNA (Fig. 4), implying that these genomes are packaged in a higher-order structure. If quiescent genomes are packaged in regular nucleosomes, additional structure may mask this by preventing access by micrococcal nuclease. Provision of ICP0 by d105 resulted in a chromatin configuration that was degraded by low concentrations of micrococcal nuclease. The GFP gene of ICP0-reactivated d109 is at least as sensitive to MN digestion as bulk cellular DNA. These results are consistent with the ChIP data demonstrating removal of both heterochromatin and histones from quiescent d109 upon ICP0 expression.

In a study by Coleman et al. (9), ICP0 provided by a superinfecting adenovirus failed to cause the removal of histones from quiescent HSV genomes during reactivation, which contrasts with the effects seen when ICP0 was expressed by d105. We therefore tested whether ICP0 expressed from AdS.11E4(ICP0) caused the reduction of histone occupancy on the quiescent d109 genome. By ChIP analysis, it was seen that ICP0 expression from AdS.11E4(ICP0) did not cause appreciable reduction of histone H3 on either the tk or the gC promoter or the EGFP gene, compared to the level for the empty adenoviral vector (Fig. 5). Histone H4 occupancy was reduced a relatively small amount (0 to 40%) on all three regions tested (Fig. 5). Despite the lack of histone removal from quiescent genomes during AdS.11E4(ICP0) superinfection (Fig. 5), GFP expression was activated to the same extent as in d105 superinfection (Fig. 1). This implies that the removal of histones from quiescent genomes is not required for significant ICP0-mediated reactivation.

Hyperacetylation of histones is a result of ICP0 expression. It has been shown in a number of studies that infection with HSV results in global hyperacetylation of histones (32) and that ICP0 expression causes hyperacetylation of histones bound to the HSV genome (8, 9, 18). ICP0 has also been shown to cause the disruption of the repressive complex HDAC1/HDAC2/REST/CoREST/LSD1 (21, 23–25). In addition, the effects of ICP0 expression seem to share some similarities to those of HDAC inhibitors, such as trichostatin A (29).

In order to determine the effects of ICP0 on histone acetylation during activation of quiescent HSV, we performed ChIP at different times following d105 superinfection and compared the results with expression data at the same time points. In order to account for the reduction of histones on the quiescent genome upon reactivation, AcH3 and AcH4 levels were normalized to the bulk amount of histones H3 and H4 as determined by the ChIP experiments with anti-H3 and -H4. As has been seen previously, ICP0 expression by d105 caused hyperacetylation of the histones

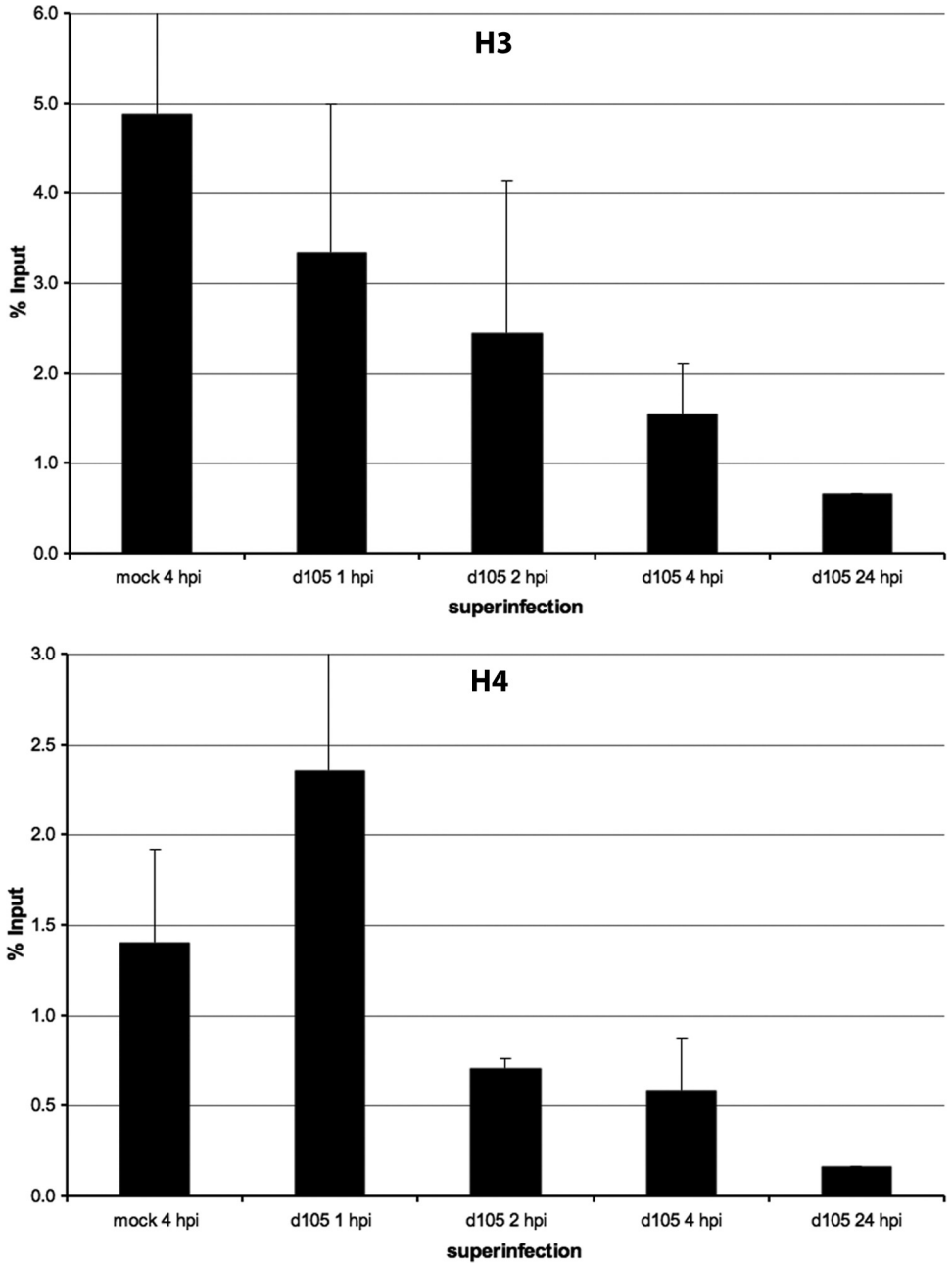

FIG. 3. Binding of histones H3 and H4 to the HCMV promoter of d109 in MRC-5 cells after superinfection with d105. ChIP with antibodies to histones H3 and H4 and RT-PCR were performed as described in the legend for Fig. 2 to determine the percentages of genomes bound by histones H3 and H4. Graphs show the percentages of total genomes bound at the indicated times after infection with d105. Error bars represent standard errors of the means from 4 experiments.

that were bound to the d109 HCMV promoter (Fig. 6). However, the activation of transcription under these conditions (Fig. 1A) preceded the appearance of acetylated histones. This may indicate that hyperacetylation of the histones bound to the reactivating quiescent genome is not an event required for the initiation of transcriptional reactivation. It could be significant in that it may act to prevent reestablishment of repression and allow for continuous access to the genome by the transcriptional apparatus.

ChIP experiments were also performed 24 h after adenovirus superinfection (Fig. 7). Expression of ICP0 from AdS.11E4(ICP0) caused an increase in acetylation of histone H3 and H4 on all three genes tested. However, this increase was modest and variable, with a significant increase seen only on the gC promoter. These results indicate that expression of ICP0 may result in the hyperacetylation of histones at different loci on the viral genome, which has also been seen previously (9). However, this may not be the primary activity responsible for activation. Therefore, the effects of ICP0 on the acetylation and higher-order chroma-

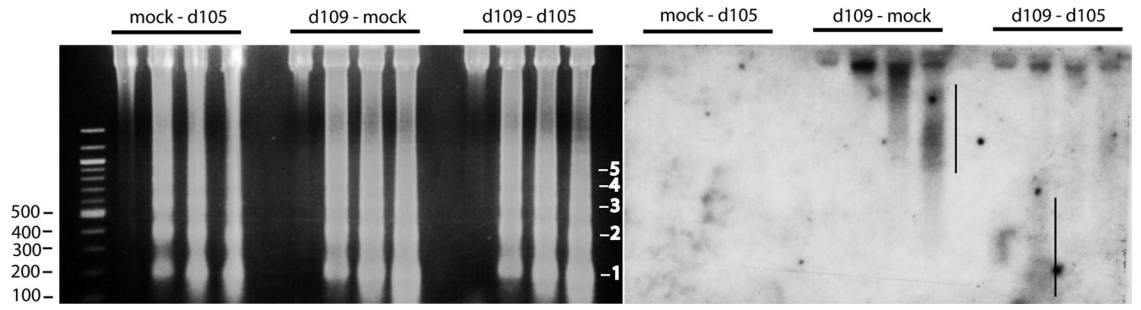

FIG. 4. Micrococcal nuclease digestion of d109-infected MRC-5 cells after superinfection. MRC-5 cells were mock or d109 infected for 1 week, followed by superinfection with the indicated virus. Cells were permeabilized and digested for 5 min with 0, 200, 1,000, or 3,000 units micrococcal nuclease. The DNA was isolated from the nuclei at 8 h postinfection, purified, and fractionated on 2.0% agarose gels. The agarose gels were stained with ethidium bromide (left) and transferred to Nytran membranes for Southern blot hybridization as described in Materials and Methods. The Southern blots were probed with ^{32}P-labeled GFP DNA, washed, and exposed to X-ray film. Shown are the autoradiographic images of the probed blots (right). Sizes for marker fragments are given in base pairs.

tin structure of quiescent HSV genomes may be mediated through different mechanisms.

DISCUSSION

ICP0 is a promiscuous transactivator of gene expression and is one of several viral proteins that can reactivate gene expression from quiescent genomes. Some of the effects of ICP0 are similar to those of the HDAC inhibitor trichostatin A (29, 59), but as quiescence is established, inhibition of histone deacetylases is not sufficient to activate gene expression (59). However, ICP0 maintains the ability to reactivate the quiescent virus (59). Additionally, replicating HSV lacking ICP0 cannot participate in recombination with quiescent HSV-1 (46, 59), implying that the state of quiescent genomes is relatively inaccessible to viral proteins and the recombination machinery in the absence of ICP0. Therefore, it appears that ICP0 is involved in the removal of a repressive mechanism and that this mechanism involves perturbation of higher-order chromatin structure. In this study, quiescence was established with the HSV mutant d109, which expresses no IE proteins and cannot replicate in noncomplementing cells. After 1 week, gene expression from the d109 genome was reactivated by providing ICP0 in *trans*, either by the HSV-1 mutant d105 or by an adenovirus vector expressing ICP0. RNA expression was quantified, and ChIP was used to determine the relationship of chromatin structure to gene expression patterns upon reactivation of viral gene expression. We determined whether heterochromatin was present on the quiescent genomes, whether histones were removed, and whether remaining histones were acetylated and correlated the chromatin structure temporally with gene expression, both from the HCMV IE promoter and from representative early (E) and late (L) viral genes.

Gene expression upon ICP0-induced reactivation. The steady-state level of GFP expression from quiescent d109 in MRC-5 cells was approximately 10 mRNA copies per cell. The majority of this expression likely occurs in the few (less than 0.1%) cells where the genomes do not become repressed (59). Upon provision of ICP0 by d105 superinfection, expression of GFP mRNA increased 10-fold by 2 h, 150-fold by 4 h, and approximately 1,000-fold by 24 h postsuperinfection (Fig. 1).

Due to the absence of ICP4, ICP0 is overexpressed in the d105 background. In contrast, because its transcription is directed from the adenovirus E4 promoter, the amount of ICP0 expressed in AdS.11E4(ICP0)-infected cells at 24 h postinfection is less than 1,000 times that expressed in d105-infected cells and approximately equal to that seen in the first hour of infection by wild-type (wt) virus (28). As seen in Fig. 1, the levels of mRNA GFP accumulating at 24 hpi were equivalent in d105 and AdS.11E4(ICP0) superinfection. As we have shown previously (28), this result reinforces that even small amounts of ICP0 can alleviate repression to an extent that allows for full transcriptional activation. Additionally, superinfection with AdS.11E4(ICP0) allowed the measurement of detectable levels of the E and L representative genes tk and gC. The levels of activation of these genes varied by the relative promoter strength, an effect which has been seen previously (18).

Removal of heterochromatin as a function of ICP0. It has been shown that ICP0 prevents the association of HP1γ with incoming viral genomes as quiescence is established (18). In this study, it was determined that ICP0 expression in *trans* can cause the removal of HP1γ from highly heterochromatic quiescent genomes. This effect was seen in both d105 and AdS.11E4(ICP0) superinfection, indicating that even small amounts of ICP0 can facilitate the removal of HP1γ. Additionally, during AdS.11E4(ICP0) superinfection, all three promoter classes tested showed a reduction in HP1γ, indicating that this may be a general effect across the genome. The removal of HP1γ occurred by 1 hpi, which precedes a measurable increase of RNA levels. This is presumably because higher-order chromatin structure must be removed before the transcriptional machinery can access the DNA.

A previous report (9) determined that HP1α was enriched at viral promoters after superinfection with HSV-2, while no large change in HP1α levels was observed after ICP0 expression by an adenovirus. The differences between these observations and our results can be explained by several possibilities. First and most obvious is that although the three isoforms of HP1 share homology and similar roles in gene silencing, they have different localization characteristics and may have different interaction partners (40, 45). Thus, HP1α localizes exclusively to heterochromatin, while HP1γ has been shown to localize to both heterochromatin and silenced euchromatic

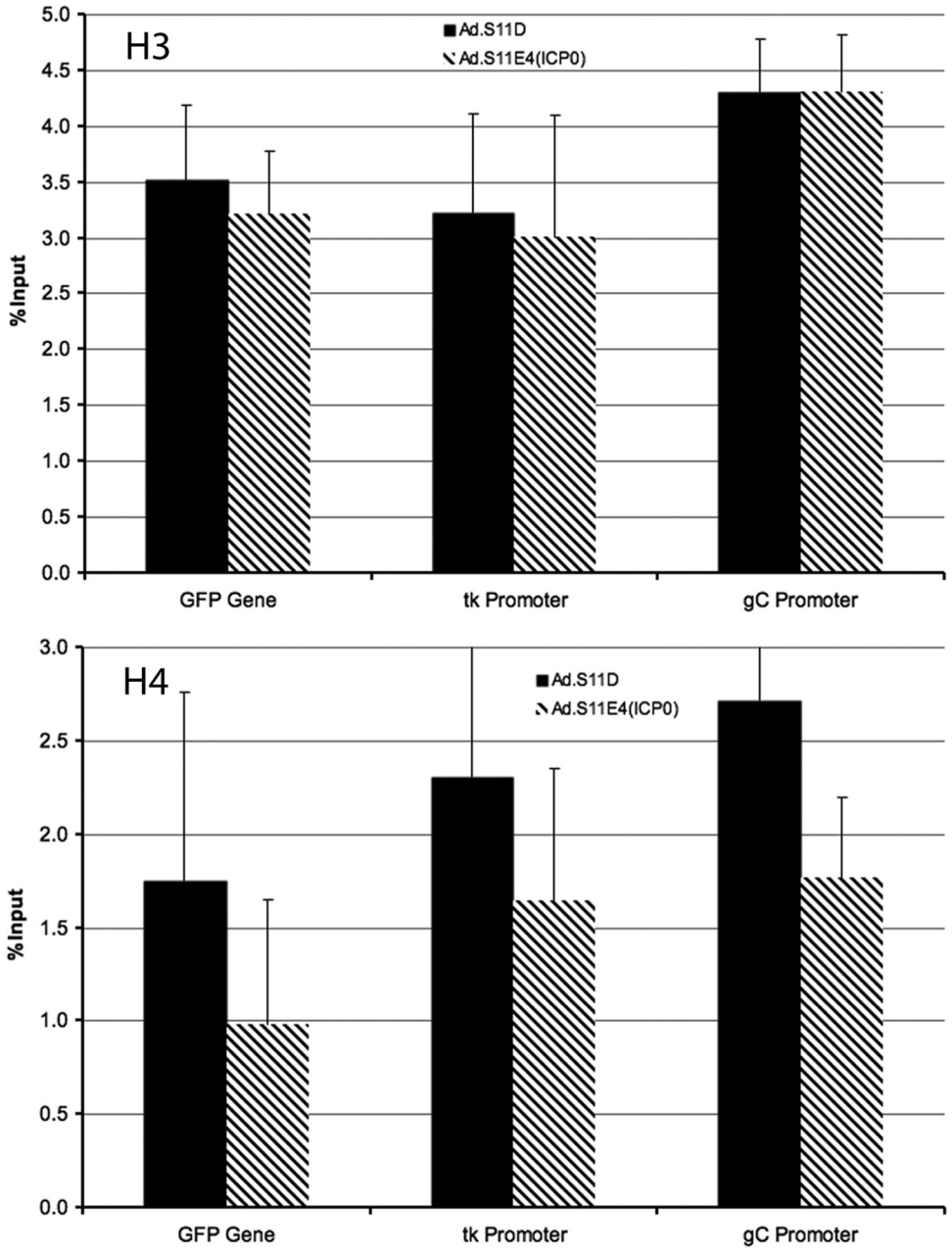

FIG. 5. Binding of histones H3 and H4 to the tk and gC promoters and the GFP 5′ region of d109 in MRC-5 cells after superinfection with adenovirus. ChIP with antibodies to histones H3 and H4 and RT-PCR were performed as described in the legend for Fig. 2 to determine the percentages of d109 genomes bound by histones H3 and H4. Graphs show the percentages of total genomes bound at 24 h after infection with the indicated adenovirus. Error bars represent standard errors of the means from 4 experiments.

regions. This may indicate that the HP1 isoforms play different roles in HSV-1 silencing during quiescence. Additionally, the previous study employed phosphonoacetic acid to prevent replication in HSV-2 superinfection and doxycycline to drive expression during adenovirus superinfection. These chemicals may have altered the chromatin dynamics on quiescent virus, as we have seen that even replacing conditioned medium with fresh medium can change histone occupancy levels on quiescent viral genomes (data not shown).

Trimethylation of histone H3 lysine 9 is a mark of repressed transcription (2) and recruits HP1 (1). After superinfection with d105, a reduction of H3K9me3 on the HCMV promoter was seen by 2 hpi, concurrent with a detectable rise in GFP RNA levels. As expected, the change in H3K9me3 levels followed a reduction in HP1γ association with the quiescent genome. The reduction of H3K9me3 was also found on gC and tk promoters after AdS.11E4(ICP0) superinfection, showing that the reduction of heterochromatin is a general effect across the quiescent genome. The temporal order of heterochromatin removal suggests that HP1γ removal is required before

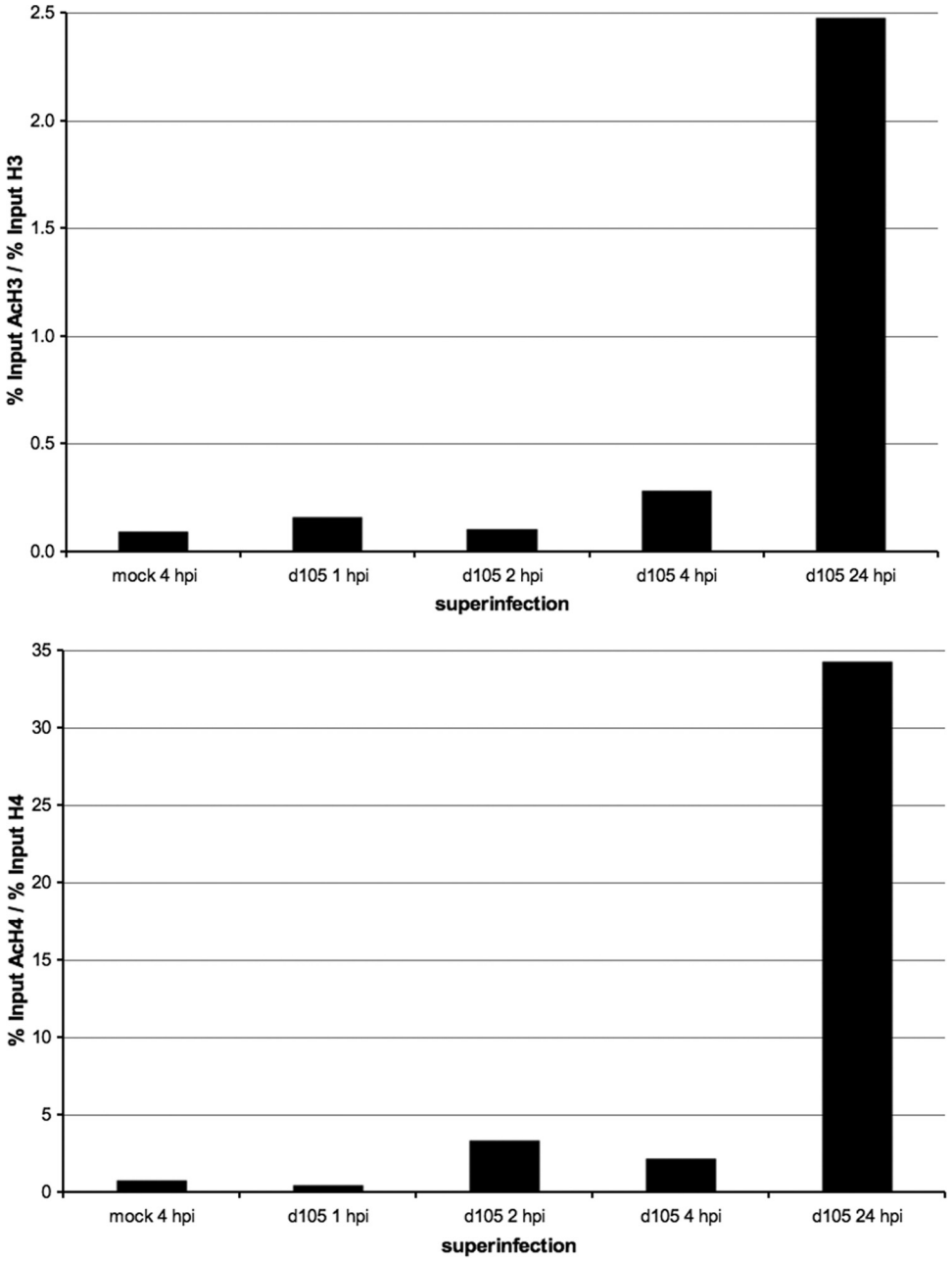

FIG. 6. Binding of hyperacetylated histone H3 (AcH3) and hyperacetylated H4 (AcH4) to the HCMV promoter of d109 in MRC-5 cells after superinfection with d105. ChIP with an antibody to AcH3 and AcH4 and RT-PCR were performed as described in the legend for Fig. 2 to determine the percentages of d109 genomes bound by AcH3 or AcH4 at the indicated times after infection with d105. AcH3 was normalized to the amount of histone H3 and AcH4 was normalized to the amount of histone H4 on the genome by dividing the percentage of acetylated histone by the percentage of total histone (Fig. 3). Error bars represent standard deviations from 4 experiments.

changes to histone modifications can occur and before transcriptional activation can be induced.

The abundance of ICP0 may affect the removal and acetylation of histones from quiescent genomes. Previously, it has been seen that superinfection with an adenovirus expressing ICP0 does not result in the removal of histone H3 from quiescent HSV-1 (9), while the presence of ICP0 prevents the accumulation of histone H3 and quiescence (18). These results taken together suggest that ICP0 expression can prevent histone deposition on incoming viral genomes but that it cannot cause the removal of histones from previously chromatinized quiescent genomes. Further analysis, however, reveals a more complex picture of ICP0 function.

When ICP0 was overexpressed in *trans* from d105, histones H3 and H4 were removed from the HCMV promoter of the quiescent d109 genome, concurrent with gene expression, as well as with the decrease of H3K9me3. Micrococcal nuclease analysis of the GFP gene revealed that d105 infection resulted

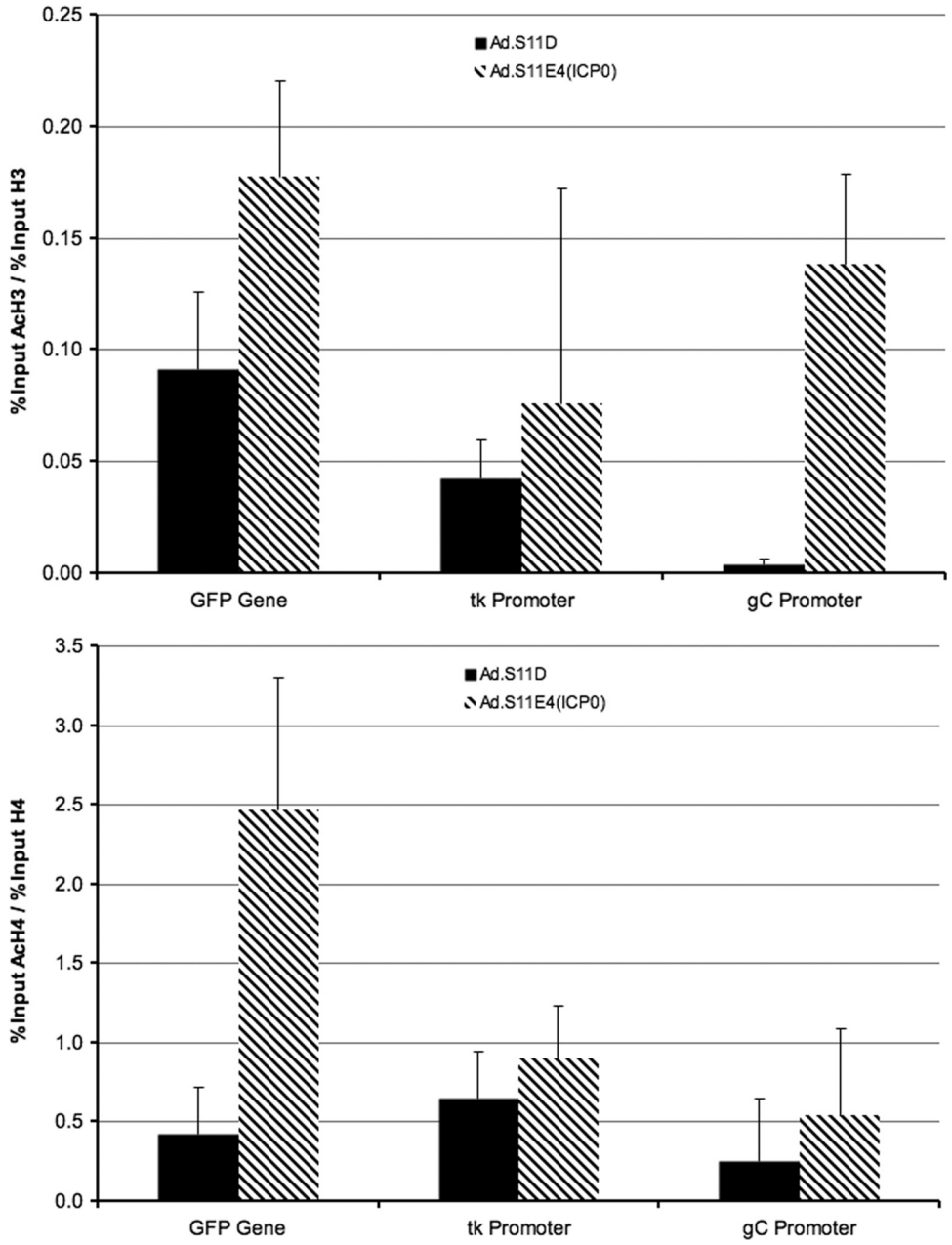

FIG. 7. Binding of hyperacetylated histone H3 (AcH3) and hyperacetylated H4 (AcH4) to the tk and gC promoters and the GFP 5′ region of d109 in MRC-5 cells after superinfection with adenovirus. ChIP with an antibody to AcH3 and AcH4 and RT-PCR were performed as described in the legend for Fig. 2 to determine the percentages of d109 genomes bound by AcH3 or AcH4. AcH3 was normalized to the amount of histone H3 and AcH4 was normalized to the amount of histone H4 on the indicated regions of the genome by dividing the percentage of acetylated histone by the percentage of total histone (Fig. 3). Error bars represent standard deviations from 4 experiments.

in greater susceptibility to micrococcal nuclease than was seen with cellular DNA and showed no detectable banding pattern, indicating that both higher-order chromatin structure and nucleosomes were removed from the GFP gene on the d109 genome upon ICP0 expression. However, when ICP0 was expressed at low levels from AdS.11E4(ICP0), which more closely mimic those seen early in HSV infection, histone H3 was not removed from the HCMV, tk, or gC promoter of d109, while there was a slight reduction in histone H4 occupancy at these promoters. While the levels of removal of histones may vary with the amount of ICP0 expressed, levels of transcriptional activity remain approximately equal after ICP0-induced reactivation. This indicates that full histone removal is not essential for activation of quiescent genomes.

A possible explanation for this phenomenon lies in the multifunctional nature of ICP0. ICP0 has at least two distinct regions that are required for its full transcriptional activation phenotype. The RING finger region is an E3 ubiquitin ligase and is responsible for the degradation of multiple proteins, many of which are involved in the cellular antiviral response, as

well as heterochromatin maintenance (7, 16, 41, 42) and the DNA damage response (35, 38, 48). When the RING finger is disrupted, this activity is lost. Thus, the enzymatic activity of the RING finger is essential for ICP0 function. However, a number of studies (4–6, 62) have shown that regions of the C terminus of ICP0 are required for full activation of gene expression. When the C terminus is truncated, the RING finger portion of ICP0 has a reduced ability to activate gene expression.

This phenotype may be explained, in part, by recent work demonstrating that a region of the C terminus is responsible for disrupting the repressive complex formed by HDAC1/HDAC2/REST/CoREST/LSD1 (21, 23–25, 51). A region of ICP0 has homology to CoREST, and this region has the ability to physically disrupt and cause the translocation of the CoREST repressive complex. The ability of ICP0 to disrupt this complex may require relatively large amounts of ICP0 compared to those required for enzymatic function and may explain the differences seen in removal of histones from quiescent genomes.

Abundant expression of ICP0 was also required for full hyperacetylation of histones H3 and H4. Upon d105 superinfection, the histones H3 and H4 remaining on the quiescent genome were hyperacetylated. When ICP0 was provided by superinfection with AdS.11E4(ICP0), however, only the gC promoter showed a large increase in the hyperacetylation of remaining histone H3. Hyperacetylation of histone H4 was prominent only on the GFP gene after AdS.11E4(ICP0) superinfection. Taken together with the lack of significant histone removal when ICP0 is expressed at low levels, this lack of full hyperacetylation of histones supports the hypothesis that these activities, which possibly result from the physical disruption of the CoREST complex, may require more abundant levels of ICP0, while the enzymatic functions of ICP0 occur even at low expression levels.

The results of these studies indicate that ICP0 expression results in the disruption of heterochromatin on quiescent HSV-1 genomes. The loss of HP1γ and a higher-order chromatin structure precedes reactivation of gene expression, while the decrease in the heterochromatin mark H3K9me3 occurs concurrently with detectable transcriptional activation. Additionally, large amounts of ICP0 were able to facilitate the removal of histones from quiescent virus, while relatively small amounts were not, suggesting that ICP0 may exert its function through multiple mechanisms. This also demonstrates that full transcriptional activation does not require full histone removal. Lastly, hyperacetylation of histones temporally followed derepression of gene expression, suggesting either that this is a bystander effect of the other functions of ICP0 or that acetylation of histones is a means by which the virus maintains a euchromatic state during reactivation.

ACKNOWLEDGMENTS

This work was supported by NIH grant R01 AI044821 to N.A.D. M.W.F. was funded by NIH training grant T32 AI 049820.

REFERENCES

1. **Bannister, A. J., et al.** 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature **410:**120–124.
2. **Barski, A., et al.** 2007. High-resolution profiling of histone methylations in the human genome. Cell **129:**823–837.
3. **Boutell, C., S. Sadis, and R. D. Everett.** 2002. Herpes simplex virus type 1 immediate-early protein ICP0 and its isolated RING finger domain act as ubiquitin E3 ligases in vitro. J. Virol. **76:**841–850.
4. **Cai, W., et al.** 1993. The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. J. Virol. **67:**7501–7512.
5. **Cai, W., and P. A. Schaffer.** 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J. Virol. **66:**2904–2915.
6. **Cai, W. Z., and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. J. Virol. **63:**4579–4589.
7. **Chelbi-Alix, M. K., and H. de The.** 1999. Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. Oncogene **18:**935–941.
8. **Cliffe, A. R., and D. M. Knipe.** 2008. Herpes simplex virus ICP0 promotes both histone removal and acetylation on viral DNA during lytic infection. J. Virol. **82:**12030–12038.
9. **Coleman, H. M., et al.** 2008. Histone modifications associated with herpes simplex virus type 1 genomes during quiescence and following ICP0-mediated de-repression. J. Gen. Virol. **89:**68–77.
10. **Danaher, R. J., R. J. Jacob, M. D. Chorak, C. S. Freeman, and C. S. Miller.** 1999. Heat stress activates production of herpes simplex virus type 1 from quiescently infected neurally differentiated PC12 cells. J. Neurovirol. **5:**374–383.
11. **Danaher, R. J., R. J. Jacob, and C. S. Miller.** 2006. Reactivation from quiescence does not coincide with a global induction of herpes simplex virus type 1 transactivators. Virus Genes **33:**163–167.
12. **Deshmane, S. L., and N. W. Fraser.** 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J. Virol. **63:**943–947.
13. **Everett, R. D.** 2000. ICP0 induces the accumulation of colocalizing conjugated ubiquitin. J. Virol. **74:**9994–10005.
14. **Everett, R. D.** 2006. Interactions between DNA viruses, ND10 and the DNA damage response. Cell. Microbiol. **8:**365–374.
15. **Everett, R. D.** 1984. Trans activation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. EMBO J. **3:**3135–3141.
16. **Everett, R. D., W. C. Earnshaw, J. Findlay, and P. Lomonte.** 1999. Specific destruction of kinetochore protein CENP-C and disruption of cell division by herpes simplex virus immediate-early protein Vmw110. EMBO J. **18:**1526–1538.
17. **Everett, R. D., et al.** 1998. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. J. Virol. **72:**6581–6591.
18. **Ferenczy, M. W., and N. A. DeLuca.** 2009. Epigenetic modulation of gene expression from quiescent herpes simplex virus genomes. J. Virol. **83:**8514–8524.
19. **Freeman, M. L., B. S. Sheridan, R. H. Bonneau, and R. L. Hendricks.** 2007. Psychological stress compromises CD8+ T cell control of latent herpes simplex virus type 1 infections. J. Immunol. **179:**322–328.
20. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. U. S. A. **82:**5265–5269.
21. **Gu, H., Y. Liang, G. Mandel, and B. Roizman.** 2005. Components of the REST/CoREST/histone deacetylase repressor complex are disrupted, modified, and translocated in HSV-1-infected cells. Proc. Natl. Acad. Sci. U. S. A. **102:**7571–7576.
22. **Gu, H., and B. Roizman.** 2003. The degradation of promyelocytic leukemia and Sp100 proteins by herpes simplex virus 1 is mediated by the ubiquitin-conjugating enzyme UbcH5a. Proc. Natl. Acad. Sci. U. S. A. **100:**8963–8968.
23. **Gu, H., and B. Roizman.** 2009. Engagement of the lysine-specific demethylase/HDAC1/CoREST/REST complex by herpes simplex virus 1. J. Virol. **83:**4376–4385.
24. **Gu, H., and B. Roizman.** 2007. Herpes simplex virus-infected cell protein 0 blocks the silencing of viral DNA by dissociating histone deacetylases from the CoREST-REST complex. Proc. Natl. Acad. Sci. U. S. A. **104:**17134–17139.
25. **Gu, H., and B. Roizman.** 2009. The two functions of herpes simplex virus 1 ICP0, inhibition of silencing by the CoREST/REST/HDAC complex and degradation of PML, are executed in tandem. J. Virol. **83:**181–187.
26. **Halford, W. P., C. D. Kemp, J. A. Isler, D. J. Davido, and P. A. Schaffer.** 2001. ICP0, ICP4, or VP16 expressed from adenovirus vectors induces reactivation of latent herpes simplex virus type 1 in primary cultures of latently infected trigeminal ganglion cells. J. Virol. **75:**6143–6153.
27. **Halford, W. P., and P. A. Schaffer.** 2001. ICP0 is required for efficient reactivation of herpes simplex virus type 1 from neuronal latency. J. Virol. **75:**3240–3249.
28. **Hobbs, W. E., D. E. Brough, I. Kovesdi, and N. A. DeLuca.** 2001. Efficient activation of viral genomes by levels of herpes simplex virus ICP0 insufficient to affect cellular gene expression or cell survival. J. Virol. **75:**3391–3403.
29. **Hobbs, W. E., and N. A. DeLuca.** 1999. Perturbation of cell cycle progression

and cellular gene expression as a function of herpes simplex virus ICP0. J. Virol. **73:**8245–8255.
30. **Jamieson, D. R., L. H. Robinson, J. I. Daksis, M. J. Nicholl, and C. M. Preston.** 1995. Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants. J. Gen. Virol. **76:**1417–1431.
31. **Jordan, R., and P. A. Schaffer.** 1997. Activation of gene expression by herpes simplex virus type 1 ICP0 occurs at the level of mRNA synthesis. J. Virol. **71:**6850–6862.
32. **Kent, J. R., et al.** 2004. During lytic infection herpes simplex virus type 1 is associated with histones bearing modifications that correlate with active transcription. J. Virol. **78:**10178–10186.
33. **Kubat, N. J., R. K. Tran, P. McAnany, and D. C. Bloom.** 2004. Specific histone tail modification and not DNA methylation is a determinant of herpes simplex virus type 1 latent gene expression. J. Virol. **78:**1139–1149.
34. **Lacasse, J. J., and L. M. Schang.** 2010. During lytic infections, herpes simplex virus type 1 DNA is in complexes with the properties of unstable nucleosomes. J. Virol. **84:**1920–1933.
35. **Lees-Miller, S. P., et al.** 1996. Attenuation of DNA-dependent protein kinase activity and its catalytic subunit by the herpes simplex virus type 1 transactivator ICP0. J. Virol. **70:**7471–7477.
36. **Leinbach, S. S., and W. C. Summers.** 1980. The structure of herpes simplex virus type 1 DNA as probed by micrococcal nuclease digestion. J. Gen. Virol. **51:**45–59.
37. **Li, W., et al.** 2009. The transactivating effect of HSV-1 ICP0 is enhanced by its interaction with the PCAF component of histone acetyltransferase. Arch. Virol. **154:**1755–1764.
38. **Lilley, C. E., et al.** 2010. A viral E3 ligase targets RNF8 and RNF168 to control histone ubiquitination and DNA damage responses. EMBO J. **29:**943–955.
39. **Loiacono, C. M., N. S. Taus, and W. J. Mitchell.** 2003. The herpes simplex virus type 1 ICP0 promoter is activated by viral reactivation stimuli in trigeminal ganglia neurons of transgenic mice. J. Neurovirol. **9:**336–345.
40. **Lomberk, G., L. Wallrath, and R. Urrutia.** 2006. The heterochromatin protein 1 family. Genome Biol. **7:**228.
41. **Lomonte, P., and E. Morency.** 2007. Centromeric protein CENP-B proteasomal degradation induced by the viral protein ICP0. FEBS Lett. **581:**658–662.
42. **Lomonte, P., K. F. Sullivan, and R. D. Everett.** 2001. Degradation of nucleosome-associated centromeric histone H3-like protein CENP-A induced by herpes simplex virus type 1 protein ICP0. J. Biol. Chem. **276:**5829–5835.
43. **Lomonte, P., et al.** 2004. Functional interaction between class II histone deacetylases and ICP0 of herpes simplex virus type 1. J. Virol. **78:**6744–6757.
44. **Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond.** 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature **389:**251–260.
45. **Maison, C., and G. Almouzni.** 2004. HP1 and the dynamics of heterochromatin maintenance. Nat. Rev. Mol. Cell Biol. **5:**296–304.
46. **Minaker, R. L., K. L. Mossman, and J. R. Smiley.** 2005. Functional inaccessibility of quiescent herpes simplex virus genomes. Virol. J. **2:**85.
47. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53:**751–760.
48. **Parkinson, J., S. P. Lees-Miller, and R. D. Everett.** 1999. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. J. Virol. **73:**650–657.
49. **Preston, C. M., and M. J. Nicholl.** 2008. Induction of cellular stress overcomes the requirement of herpes simplex virus type 1 for immediate-early protein ICP0 and reactivates expression from quiescent viral genomes. J. Virol. **82:**11775–11783.
50. **Quinlan, M. P., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. **5:**957–963.
51. **Roizman, B., H. Gu, and G. Mandel.** 2005. The first 30 minutes in the life of a virus: unREST in the nucleus. Cell Cycle **4:**1019–1021.
52. **Samaniego, L. A., L. Neiderhiser, and N. A. DeLuca.** 1998. Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. J. Virol. **72:**3307–3320.
53. **Sampath, P., and N. A. Deluca.** 2008. Binding of ICP4, TATA-binding protein, and RNA polymerase II to herpes simplex virus type 1 immediate-early, early, and late promoters in virus-infected cells. J. Virol. **82:**2339–2349.
54. **Spivack, J. G., and N. W. Fraser.** 1987. Detection of herpes simplex virus type 1 transcripts during latent infection in mice. J. Virol. **61:**3841–3847.
55. **Spivack, J. G., and N. W. Fraser.** 1988. Expression of herpes simplex virus type 1 latency-associated transcripts in the trigeminal ganglia of mice during acute infection and reactivation of latent infection. J. Virol. **62:**1479–1485.
56. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science **235:**1056–1059.
57. **Tal-Singer, R., et al.** 1997. Gene expression during reactivation of herpes simplex virus type 1 from latency in the peripheral nervous system is different from that during lytic infection of tissue cultures. J. Virol. **71:**5268–5276.
58. **Tavalai, N., P. Papior, S. Rechter, M. Leis, and T. Stamminger.** 2006. Evidence for a role of the cellular ND10 protein PML in mediating intrinsic immunity against human cytomegalovirus infections. J. Virol. **80:**8006–8018.
59. **Terry-Allison, T., C. A. Smith, and N. A. DeLuca.** 2007. Relaxed repression of herpes simplex virus type 1 genomes in murine trigeminal neurons. J. Virol. **81:**12394–12405.
60. **Wilcox, C. L., R. L. Smith, C. R. Freed, and E. M. Johnson, Jr.** 1990. Nerve growth factor-dependence of herpes simplex virus latency in peripheral sympathetic and sensory neurons in vitro. J. Neurosci. **10:**1268–1275.
61. **Zaret, K.** 2005. Micrococcal nuclease analysis of chromatin structure. Curr. Protoc. Mol. Biol. **21:**21.1.
62. **Zhu, X. X., J. X. Chen, C. S. Young, and S. Silverstein.** 1990. Reactivation of latent herpes simplex virus by adenovirus recombinants encoding mutant IE-0 gene products. J. Virol. **64:**4489–4498.