Herpes Simplex Virus 1 DNA Is in Unstable Nucleosomes throughout the Lytic Infection Cycle, and the Instability of the Nucleosomes Is Independent of DNA Replication

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Herpes simplex virus 1 (HSV-1) DNA is chromatinized during latency and consequently regularly digested by micrococcal nuclease (MCN) to nucleosome-size fragments. In contrast, MCN digests HSV-1 DNA in lytically infected cells to mostly heterogeneous sizes. Yet HSV-1 DNA communoprecipitates with histones during lytic infections. We have shown that at 5 h postinfection, most nuclear HSV-1 DNA is in particularly unstable nucleoprotein complexes and consequently is more accessible to MCN than DNA in cellular chromatin. HSV-1 DNA was quantitatively recovered at this time in complexes with the biophysical properties of mono- to polynucleosomes following a modified MCN digestion developed to detect potential unstable intermediates. We proposed that most HSV-1 DNA is in unstable nucleosome-like complexes during lytic infections. Physiologically, nucleosome assembly typically associates with DNA replication, although DNA replication transiently disrupts nucleosomes. It therefore remained unclear whether the instability of the HSV-1 nucleoprotein complexes was related to the ongoing viral DNA replication. Here we tested whether HSV-1 DNA is in unstable nucleosome-like complexes before, during, or after the peak of viral DNA replication or when HSV-1 DNA replication is inhibited. HSV-1 DNA was quantitatively recovered in complexes fractionating as mono- to polynucleosomes from nuclei harvested at 2, 5, 7, or 9 h after infection, even if viral DNA replication was inhibited. Therefore, most HSV-1 DNA is in unstable nucleosome-like complexes throughout the lytic replication cycle, and the instability of these complexes is surprisingly independent of HSV-1 DNA replication. The specific accessibility of nuclear HSV-1 DNA, however, varied at different times after infection.

Eukaryotic DNA is packaged into nucleoprotein complexes known as chromatin. The basic unit of chromatin is the nucleosome, 146 bp of DNA wrapped 1.75 turns around a histone octamer composed of two copies of each histone 2A (H2A), H2B, H3, and H4 (33). Linker histone H1 binds at the core nucleosome entry and exit points, stabilizing the core nucleosome, and to linker DNA in between nucleosomes. H1 also promotes further compaction of chromatin into higher-order structures (23, 50, 51). The chromatin structure is classically probed with nucleases (61). The endonuclease micrococcal nuclease (MCN), for example, preferentially cleaves linker DNA between nucleosomes (2, 19). Mild MCN digestions first cleave linker DNA sparsely, releasing polynucleosomes, which are eventually cleaved to mononucleosomes. Therefore, MCN digestion of regularly chromatinized DNA results in protection of poly- and mononucleosome-size DNA fragments. Under more stringent digestion conditions, MCN eventually degrades even the DNA in mononucleosomes.

MCN has also been used to characterize specialized chromatin structures and intrinsically unstable nucleosomes. Centromeres, for example, are specialized chromatin structures responsible for the equal segregation of chromosomes at mitosis. They contain specialized nucleosomes with the centromere-specific histone H3 variant, CENP-A (called CenH3 in Drosophila melanogaster). Dalal et al. have shown that CenH3-containing centromeric nucleosomes in Drosophila melanogaster protect only 120 bp of DNA from MCN digestion (9). Such atypical protection was shown to result from unique nucleosome structures called “hemisomes” (8, 9). Hemisomes contain only one copy of CenH3, H2A, H2B, and H4, assembled in a tetrameric structure. Hemisomes, however, still show a typical “beads-on-a-string” appearance in electron microscopy. However, they resist condensation under physiological conditions. Hemisomes have only half the height of canonical octameric nucleosomes, as determined by atomic-force microscopy. These particular structures result in protection of only 120 bp (9).

In addition to the characterization of specialized chromatin structures, MCN has also been used in the characterization of particularly unstable cellular (27, 28) or viral (42) chromatin. Jin and Felsenfeld reported highly unstable hybrid nucleosomes containing the histone variants H2A.Z and H3.3 (27). H3.3 is the H3 variant synthesized in G1, S, G2, and G0 (79). H3.3 is consequently assembled into nucleosomes mostly via replication-independent pathways (73). Nucleosomes containing either H2A.Z or H3.3 and the remaining canonical core histones (H2B, H3, and H4 or H2A, H2B, and H4, respectively) were readily detected (by chromatin immunoprecipitation [ChIP] assays) in nucleosomes prepared from MCN-digested nuclei. However, hybrid nucleosomes containing both H2A.Z and H3.3 were not detected under standard conditions (27). Hybrid nucleosomes containing H2A.Z and H3.3 could be detected only when cells were cross-linked with formaldehyde prior to MCN digestion (28). Jin et al. have further shown that nucleosomes containing both H2A.Z and H3.3 actu-
ally mark the so-called “nucleosome-free regions” of active promoters and other regulatory regions (28).

Classically, the study of the chromatinization state of herpes simplex virus 1 (HSV-1) genomes during lytic infection used standard MCN time course digestion assays (43, 55, 56). The HSV-1 DNA released from these digestions displays unique patterns of DNA fragments compared to the DNA fragments released from bulk cellular chromatin (43, 55, 56). HSV-1 DNA was digested primarily to heterogeneously sized fragments, detected as a broad “smears” on Southern blot analyses, whereas chromatinized cellular DNA was digested to nucleosome-size pieces, detected as the typical “nucleosome ladders.” HSV-1 DNA was therefore classically considered to be mostly not assembled in nucleosomes during lytic infections (43, 55, 56, 69). More recently, however, ChIP assays have demonstrated that histones, and histone-modifying proteins, associate with HSV-1 DNA during lytic infections (5, 13–15, 20, 21, 24, 25, 29, 38, 39, 45, 49, 57, 63, 65, 76, 80). Such different results are consistent with the differences between nuclease protection and ChIP assays. Whereas MCN protection requires the protein-DNA interactions to be maintained during the entire digestion time, for example, ChIP requires them to interact for just long enough to be cross-linked. It is therefore not possible to quantitatively compare the results obtained with each of these techniques. Qualitatively, however, the assays are consistent in that MCN assays show that HSV-1 DNA is protected, in a small percentage, to nucleosome sizes (29, 42), and ChIP assays show that different histone modifications associate with HSV-1 genes that are differentially transcribed (1, 4, 29, 35, 58, 64). Together with the chromatin-modifying activities of the HSV-1 transcriptional regulators VP16 and ICP0, exerted by their associated cellular proteins (15, 21, 22, 24, 34, 48, 49, 63, 74, 76), and the effects on HSV-1 transcription and replication of modulators of chromatin-modifying proteins (20, 25, 45, 80), these results suggest that chromatin and histone modifications participate in the regulation of HSV-1 gene expression during lytic infections (5, 13–15, 20, 21, 24, 25, 29, 38, 39, 45, 49, 57, 63–65, 76, 80).

The results from the ChIP assays are vastly consistent with much smaller percentages of HSV-1 than cellular DNA associating with histones (5, 13–15, 20, 21, 24, 25, 29, 38, 39, 45, 49, 57, 63, 65, 76, 80), and only a small percentage of HSV-1 DNA is consistently protected to nucleosome sizes in standard MCN digestions (13, 29, 42, 43, 55, 56). Most models, therefore, proposed that only a small percentage of HSV-1 DNA was in nucleosomes, whereas most was proposed to be non-nucleosome-associated (30, 40, 52, 64). The implicit hypotheses in the analyses of the results of ChIP and MCN digestion assays are that the histone-HSV-1 DNA interactions are equivalent to those in cellular nucleosomes and that the HSV-1 DNA coimmunoprecipitated with the histones, or protected from MCN digestion, is representative of all the HSV-1 DNA in the cells. These assumptions are as yet untested. Moreover, neither ChIP nor standard MCN protection assays have been optimized to evaluate DNA-histone interactions more transient than those between cellular DNA and histones. We therefore used classic chromatin characterization techniques to evaluate the actual nature of the HSV-1 nucleoprotein complexes during lytic infections. Using this approach, we showed that most of the nuclear HSV-1 DNA is in unstable nucleoprotein complexes at 5 h after infection and consequently more accessible to MCN than the DNA in most cellular chromatin. Such unstable nucleosomes are consistent with both the low percentage of HSV-1 DNA immunoprecipitated with histones in ChIP assays and the unique pattern of DNA fragments consistently seen in classic MCN digestions. Nuclear HSV-1 DNA was quantitatively recovered at 5 h postinfection (hpi) in the unstable nucleosome-like complexes using a modified “serial” MCN digestion protocol designed to detect unstable digestion intermediates (42). However, the nature of HSV-1 nucleoproteins complexes at other times of infection remained unknown. Furthermore, it remained unclear whether HSV-1 nucleosome stability affects, or is affected by, HSV-1 transcription or DNA replication.

We here evaluated the HSV-1 nucleoprotein complexes released as soluble chromatin throughout the lytic infection cycle. Physiologically, nucleosome assembly occurs via DNA replication-independent and -dependent processes. The former is related to transcription, DNA repair, and other processes that require access to the DNA and uses mostly the histone H3 variant H3.3. The latter chromatinizes the newly replicated DNA and uses mostly the histone H3 variant H3.1. However, DNA replication itself transiently disrupts nucleosomes. It therefore remained unclear whether the instability of the HSV-1 nucleosome-like complexes observed at 5 hpi was related to the viral DNA replication ongoing at 5 h. We therefore tested here whether HSV-1 DNA was in unstable nucleosome-like complexes before, during, or after the peak of viral DNA replication or in the presence of the viral DNA polymerase inhibitor phosphonoacetic acid (PAA). Using our modified MCN digestion protocol, HSV-1 DNA was quantitatively recovered in complexes fractionating as mono- to polynucleosomes from nuclei harvested at 2, 5, 7, or 9 h after infection or from nuclei of cells infected for 7 h in the presence of PAA. Therefore, most HSV-1 DNA is in unstable nucleosome-like complexes throughout the lytic replication cycle. The general instability of the HSV-1 nucleosome-like nucleoprotein complexes was independent of HSV-1 DNA replication. However, the specific accessibility of nuclear HSV-1 DNA did vary at different times after infection.

MATERIALS AND METHODS

**Cells and viruses.** Vero CCL-81 African green monkey kidney fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 50 mM/mL penicillin, and 50 ng/mL streptomycin (42). A low-passage-number (p10) HSV-1 strain, KOS, was used throughout this study. Viral stocks were propagated and titrated on monolayers of Vero cells following standard procedures (12). Vero cells were infected at a multiplicity of infection (MOI) of 5 or 10 PFU per cell in serum-free DMEM, as described previously (12).

**Drugs.** PAA stocks (100 mM) were prepared in serum-free DMEM, neutralized with NaOH, and filter sterilized. Aliquots were stored at −20°C and thawed prior to use. The stock was diluted to a concentration of 400 μM in complete medium and added to cells after adsorption.

**Isolation of nuclei.** Cells were rinsed with phosphate-buffered saline (PBS) at 4°C, trypsinized, and resuspended in 20 mL of DMEM–5% FBS. Cells were then pelleted by centrifugation (3,200 × g for 10 min at 4°C), resuspended in hypotonic RSB buffer (10 mM Tris [pH 7.5], 10 mM NaCl, and 5 mM MgCl₂), and lysed with 0.5% (vol/vol) Igepal CA-630 detergent (Sigma-Aldrich). Nuclei were then isolated by differential centrifugation (1,800 × g for 25 min at 4°C).

**Standard MCN digestion.** Nuclei were resuspended to 1 × 10⁹ nuclei/μL in MCN digestion buffer (10 mM Tris [pH 8] and 1 mM CaCl₂) containing 0.0005 U MCM per 1 × 10⁹ nuclei. Digestions were carried out at 39°C and stopped at the indicated times by the addition of EGTA to a...
final concentration of 5 mM. Proteins were then digested with proteinase K, and the DNA was isolated by phenol-chloroform extractions.

**Chromatin fractionation.** MCN-digested nuclei were lysed by adding 1 volume of chromatin extraction buffer (CEB) (2 mM Tris [pH 8.0], 3 mM MgCl₂, 1 mM EDTA, and 2% Triton X-100) and incubated with rotation for 10 min at 4°C. So-called “soluble” and “insoluble” chromatin fractions were then separated by differential centrifugation (8,000 × g for 20 min at 4°C).

**Identification of unstable nucleosomes by serial MCN digestion.** Undigested nuclei were lysed for 10 min at 4°C with rotation in one volume of CEB. The undigested chromatin was then pelleted by centrifugation (8,000 × g for 20 min at 4°C). Resuspension of the pelleted chromatin for subsequent washes and digestion was facilitated by mechanical disruption. Pelleted chromatin was washed once in 80 μl of MCN buffer without MCN (10 mM Tris [pH 8.0] and 1 mM CaCl₂). The washed chromatin pellet was then subjected to serial MCN digestion (42). Briefly, the chromatin pellet was resuspended in 80 μl of MCN buffer (containing 0.625 U MCN/ml per 1 × 10⁷ nuclei) and digested for 5 min during differential centrifugation. The supernatant (soluble chromatin) was then removed and quenched with 5 μl of 0.5 M EDTA to prevent further digestion of the DNA in the unstable nucleosomes released in the soluble chromatin fraction. Meanwhile, the pellet (insoluble chromatin) was resuspended with fresh MCN digestion buffer (containing 0.625 U MCN/ml per 1 × 10⁷ nuclei), and the entire procedure was repeated six times. Soluble chromatin fractions from the serial MCN digestions were pooled and resolved together in sucrose gradients.

**Sucrose gradient ultracentrifugation.** Continuous 0 to 10% sucrose gradients were prepared using a Gradient Master instrument (Biocomp, Frederickton, New Brunswick, Canada) with sucrose gradient buffer (SGB) (10 mM Tris [pH 8.0], 1.5 mM MgCl₂, and 0.5 M EDTA) containing 450 mM NaCl. Soluble chromatin was loaded on top of preformed gradients and centrifuged at 284,000 × g for 180 min at 4°C. One-milliliter fractions were then collected from the bottom of the tube. The pellet was recovered after the removal of the final fraction in 1 ml of STE (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA). Fractions were digested with proteinase K, and the DNA was isolated by phenol-chloroform extractions.

**Southern blot hybridization.** DNA resolved on 2% agarose gels was transferred by Southern blotting. The positively charged nylon was prehybridized for a minimum of 1 h in rapid hybrid buffer (Amersham Biosciences, Piscatawy, NJ) at 75°C or 60°C for HSV-1 or cellular DNA, respectively. HSV-1 DNA was detected using the JK fragment from the HSV-1 EcoRI library (42), a generous gift from the late P. A. Schaffer, University of Pennsylvania, and cellular DNA with bulk Vero cell DNA. Denatured probes, labeled by random priming (Amersham Biosciences), and 2 μg of unlabeled “cold” blocking DNA (HSV-1 EcoRI library for cellular hybridization or Vero DNA for HSV-1 hybridizations) were added to 5 ml of hybridization buffer at 75°C for HSV-1 DNA or 60°C for cellular DNA and hybridized at the same temperatures for a minimum of 12 h. Membranes were washed twice for 15 min in 300 mM NaCl, 30 mM sodium citrate (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), and 0.1% SDS at room temperature, exposed to Kodak PhosphorImager screens, and quantitated using the Bio-Rad molecular imager FX (Bio-Rad).

**RESULTS**

**Serial MCN digestions trap unstable HSV-1 nucleoprotein complexes.** Serial MCN digestions “trap” the unstable nucleosomes released in the soluble chromatin fraction by the immediate inactivation of MCN after each round of limited digestion (42). The DNA in the unstable nucleosomes, which are promptly released into the soluble chromatin fraction, is therefore prevented from further MCN digestion. Using serial digestions, we have shown that most HSV-1 DNA at 5 h postinfection (hpi) is in unstable nucleosome-like complexes, which are recovered mostly as poly-nucleosomes (42). However, it was unclear from the previous experiments what percentage of nuclear HSV-1 DNA is in such unstable chromatin-like structures.

We therefore evaluated the efficiency of the recovery of nuclear HSV-1 DNA after serial MCN digestions. Briefly, Vero cells were infected with 5 PFU of HSV-1 per cell, and their nuclei were harvested at 5 hpi and lysed. The chromatin was then subjected to serial MCN digestions at 23°C, inactivating the MCN after each digestion to prevent further digestion of the DNA in the complexes released as soluble chromatin. The digestions were repeated six times, and the soluble chromatin fractions from each digestion were pooled together for analyses. The DNA was then purified from the soluble and insoluble chromatin fractions and quantitated. The recovery of HSV-1 DNA was calculated as the fraction of total HSV-1 DNA in the nucleus, normalized to the recovery of cellular DNA. We had previously repeated the serial digestions nine times (42). However, we have since established that the vast majority of cellular and HSV-1 DNA is released during the first three digestion cycles (data not shown). We therefore reduced the number of cycles from nine to six for all experiments presented herein.

Approximately 80% of nuclear HSV-1 DNA was effectively recovered using the serial MCN digestions. The soluble and insoluble chromatin fractions accounted for 62 and 20% of the total HSV-1 DNA, respectively (Fig. 1, Serial). Therefore, most nuclear HSV-1 DNA is in chromatin-like complexes at 5 hpi. Furthermore, the majority (75%) of the recovered HSV-1 DNA is in accessible chromatin-like complexes, which are recovered in the soluble chromatin fraction, as in our previous experiments (42). Under these conditions, nonchromatinized DNA is immediately digested by MCN, whereas HSV-1 DNA inside capsids is fully protected (43).

To test the stability of the complexes protecting the nuclear HSV-1 DNA, we performed parallel serial digestions without inactivating the MCN in the collected supernatants until all six digestions had been completed, for a total of 30 min of digestion at 20°C (Fig. 1, Multiple). In contrast to the previous conditions, and as expected, only 57% of nuclear HSV-1 DNA was recovered under these (still limited) MCN digestion conditions (Fig. 1, Multiple). Moreover, the soluble chromatin fractions accounted for only 31% of the total HSV-1 DNA, whereas the insoluble fraction still contained 26% of the total DNA, similar to the results after the serial digestions. Therefore, the complexes released as soluble chromatin were subject to further degradation under these conditions, in which MCN was not quenched, whereas the insoluble chromatin fraction remained relatively constant. These results are consistent with most of the HSV-1 DNA released into the soluble chromatin fraction being in the most unstable complexes, which are thus subject to further digestion when MCN is not rapidly inactivated.

In contrast to HSV-1 DNA, bulk cellular nuclear DNA was recovered to basically the same extent regardless of whether MCN was immediately inactivated or not (Fig. 1B, Serial or Multiple). These results are consistent with the known stability of most of the nucleosomes in cellular chromatin.

We next identified a set of conditions for continuous MCN digestions (only 2.5 min at 39°C) that result in quantitative recovery of the HSV-1 DNA in chromatin-like complexes similar to that for the 30-min serial digestions. Nuclei from HSV-1-infected cells harvested at 5 hpi were then subjected to continuous MCN diges-
At this time, all kinetic classes of serial MCN digestion at 5 hpi fractionate as cellular mono-, di-, and trinucleosomes (42). Therefore, similar levels of HSV-1 DNA were protected from serial or continuous MCN digestion if the digestion time for the continuous digestion was shortened from 30 to 2.5 min. The soluble chromatin fractions also appeared to differ. The insoluble HSV-1 chromatin-containing complexes do change somewhat over the course of the infection. The relative amounts of HSV-1 DNA in the insoluble complexes containing cellular mono-, di-, and trinucleosomes (Fig. 2A, 2 hpi, ii). These different patterns are most likely the result of the unavoidable minor experimental variations (such as residual volumes after washes, temperature, timing since seeding the cells, etc.), which affect the precise rate at which each infection starts. At these earliest times, small differences in the exact timing and rate of the early stages of infection result in different amounts of the viral DNA still in capsids, just entered into the nucleus but not yet being transcribed, or already undergoing transcription, as well as in the detection or not of the limiting amounts of viral DNA in each of the fractions.

A single predominant pattern of fractionation was observed in the nuclei isolated at 5 hpi. The HSV-1 DNA-containing complexes released as soluble chromatin were detected in all fractions throughout the gradient (fractions 1 to 12), although the vast majority was in complexes that resolved to fractions 8 to 11, very much like cellular DNA (Fig. 2A, 2 hpi, i). The fractionation pattern therefore very closely resembled that of DNA in cellular chromatin (Fig. 2A, compare Cellular, 2 hpi, HSV, and HSV, 2 hpi, i). In the other half of the experiments (2 of 4), most of the HSV-1 DNA in the soluble chromatin fraction was in complexes that resolved primarily to fractions 9 to 11, the fractions containing cellular mono-, di-, and trinucleosomes (Fig. 2A, 2 hpi, ii). In half of the experiments (2 of 4), HSV-1 DNA was also detected in the nucleosome-containing complexes released as soluble chromatin at 2, 5, or 9 hpi and evaluated the nucleoprotein complexes released as soluble chromatin following serial MCN digestions.

Briefly, nuclei from HSV-1-infected cells were harvested at 2, 5, or 9 hpi and lysed. Their chromatin was then subjected to serial digestions, in which the MCN is inactivated after each digestion. The soluble chromatin fractions from each digestion were pooled and resolved in sucrose gradients. The DNA was purified from each fraction and analyzed by Southern blot hybridization.

The fractionation patterns of the cellular DNA-containing complexes released as soluble chromatin at 2, 5, or 9 hpi were basically the same (Fig. 2A, Cellular). Under these conditions, the cellular DNA released as soluble chromatin fractionated as mono-, di- (fractions 10 to 11), and polynucleosomes (fractions 1 to 9), as expected for regularly chromatinized DNA (Fig. 2A, Cellular).

HSV-1 DNA was also detected in the nucleosome-containing fractions at 2, 5, and 9 hpi. However, the HSV-1 complexes released as soluble chromatin changed somewhat over the course of infection (Fig. 2A, HSV). At 2 hpi, we observed two distinct patterns of fractionation of the HSV-1 DNA-containing complexes released as soluble chromatin (Fig. 2A, 2 hpi, i and ii). In the fractionation patterns of the cellular DNA-containing complexes releasing soluble chromatin following serial MCN digestions (quenched after each incubation). Averages ± SD are shown; n = 4. ***, P < 0.001; ns, P > 0.05 (Student’s t test).
DNA. Briefly, nuclei from cells infected with HSV-1 and harvested at 2, 5, or 9 hpi were subjected to serial MCN digestions. The DNA from the soluble and insoluble chromatin fractions was then purified and quantitated. The recovery of HSV-1 DNA was calculated as a percentage of total HSV-1 DNA in the nucleus, normalized to the recovery of cellular DNA.

Effectively all nuclear HSV-1 DNA (within error) was recovered from nuclei harvested at 2 or 9 hpi and subjected to this restricted serial MCN digestion. As previously shown, however, only $\sim 80\%$ of HSV-1 DNA was recovered from nuclei isolated at 5 hpi (Fig. 2B and Table 1). Less HSV-1 DNA was therefore recovered at 5 than at 2 or 9 hpi, suggesting an increase in accessibility of HSV-1 DNA at middle times postinfection.

The soluble and insoluble chromatin fractions contained 56 and 44% of the HSV-1 DNA recovered from nuclei isolated at 2 hpi, respectively, whereas they contained 75 or 25% of the recovered HSV-1 DNA recovered from nuclei isolated at 5 and 9 hpi, respectively (Fig. 2B and Table 1). These results are globally consistent with the qualitative levels of HSV-1 DNA in the insoluble fractions at 2, 5, or 9 hpi in Fig. 2A. Furthermore, they suggest that the accessibility of HSV-1 DNA increases after 2 hpi, resulting in less HSV-1 DNA fractionating to the fractions containing the most protected DNA.

**Accessibility of HSV-1 DNA changes throughout the lytic infection cycle.** The relative levels of HSV-1 DNA in the chromatin-like complexes released by serial MCN digestion into the insoluble chromatin fraction changed over the course of infection, from more than half at 2 hpi to less than a quarter at 5 hpi. Such changes are consistent with changes in accessibility of HSV-1 DNA to MCN. We therefore further evaluated the accessibility of HSV-1 DNA over the course of infection using standard time course MCN digestions (42). Since the first change in accessibility had already occurred before 5 hpi, we added an additional time point at 4 hpi.

**TABLE 1** Recovery of nuclear HSV-1 DNA through the course of lytic infections following serial MCN digestion

<table>
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<tr>
<th>Time postinfection (h)</th>
<th>% recovery of nuclear HSV-1 DNA</th>
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<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>2</td>
<td>124 ± 20</td>
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<td>5</td>
<td>82 ± 17</td>
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<td>9</td>
<td>117 ± 5</td>
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$^a$ Percentage of total, soluble, or insoluble HSV-1 DNA recovered following serial MCN digestion of nuclei harvested at 2, 5, or 9 hpi, normalized to the recovery of cellular DNA. Values are averages ± SD; $n = 3$ (2 and 9 hpi) or 4 (5 hpi).
Nuclei of infected cells were isolated at 2, 4, 5, or 9 hpi and digested with MCN (0.05 U/1 × 10^4 cells) for 0.5, 2.5, 5, 15, 30, or 60 min at 39°C. DNA was purified, resolved by agarose gel electrophoresis, and analyzed by ethidium bromide staining and Southern blot hybridization. Hybridizations with cellular probes are shown in standard exposures. Hybridizations with HSV-1 probes are also shown overexposed, to highlight the absence of detectable lower-molecular-weight HSV-1 DNA at 2 hpi in half of the experiments (Fig. 3A).

As expected for regularly chromatinized cellular DNA, standard MCN digestion resulted in the digestion of cellular DNA to sizes of the typical nucleosome ladder (corresponding to multiples of ~160 bp; Fig. 3A, Cellular). The accessibility of cellular chromatin remained relatively constant throughout the course of infection (Fig. 3B and Table 2).

In contrast to that of cellular DNA, the accessibility of HSV-1 DNA changed significantly over the course of infection. First, we again observed two distinct patterns of digestion of HSV-1 DNA at 2 hpi (Fig. 3A, 2 hpi, i and ii), as expected at these early times when any small variability in the infection conditions leads to the possibility or not of detecting the limited amounts of HSV-1 DNA in each fraction. In half of the experiments (2 of 4), HSV-1 DNA was detected only as a homogeneous large-molecular-weight band at the top of the gel (Fig. 3A, HSV, 2 hpi, i), whereas some still large but heterogeneously sized fragments were also detected in the other half of the experiments (Fig. 3A, HSV, 2 hpi, ii). The change in the digestion pattern was accompanied by a 33-fold change in the time required to digest 50% of the DNA ($T_{50}$), from an estimated 104 min for the first pattern to a measured 3.2 min for the second (Fig. 3C and Table 2). Consistently, the percentage of HSV-1 DNA resolving as the large-molecular-weight band migrating at the top of the gel after 60 min of digestion decreased from 97% (HSV, 2 hpi, i) to 16% (HSV, 2 hpi, ii) of the total HSV-1 DNA (Fig. 3A and C). However, HSV-1 DNA fragments consistent with mono-, di-, and shorter polynucleosomes (as observed following serial MCN digestion) were never observed following standard continuous MCN digestions, consistent with HSV-1 DNA being in unstable chromatin-like complexes that are digested to completion following such digestions. Therefore, some HSV-1 DNA is in unstable and very accessible chromatin-like structures as early as 2 hpi.

The accessibility of HSV-1 DNA changed between 2 and 4 hpi. At 4 hpi, the majority of the HSV-1 DNA was rapidly digested and released as heterogeneously sized fragments (Fig. 3A), very similar to those characteristically observed after standard MCN digestion of nuclei from HSV-1-infected cells (42, 43, 56). Also consistent with previous reports (29, 42), a minor population of HSV-1 DNA was protected to nucleosome-size fragments (Fig. 3A). Similar digestion patterns were also observed at 5 hpi (Fig. 3A, 5 hpi). The $T_{50}$ at 4 and 5 hpi increased somewhat from the accessible state at 2 hpi, from 3.2 min to 7.2 and 10.5 min, respectively. Consistently, more HSV-1 DNA digestion intermediates were detected at 4 and 5 hpi than at 2 hpi, including DNA fragments protected to mononucleosome size.

Standard time course MCN digestion of nuclei harvested at 9 hpi resulted in HSV-1 DNA fragments that were also protected to dinucleosome sizes, in addition to the mononucleosome-size fragments, the large fragments migrating to the top of the gel, and the heterogeneous-size fragments detected at 4 and 5 hpi (Fig. 3A, compare lane 4 from 9 hpi to 4 and 5 hpi). Consistent with the trend at 4 and 5 hpi, the $T_{50}$ at 9 hpi increased to 12.7 min (Table 2).

Stability of HSV-1 DNA chromatin-like complexes changes throughout the lytic infection cycle. As expected, serial and standard time course MCN digestion gave rise to distinct patterns of HSV-1 DNA fragments. Consistent with the ability to “trap” unstable digestion intermediates, serial MCN digestions recovered the majority of nuclear HSV-1 DNA in chromatin-like complexes, whereas standard time course MCN digestions recovered only a minor percentage of HSV-1 DNA in nucleosome sizes. We next evaluated the HSV-1 DNA-containing chromatin-like complexes released as soluble chromatin at 2, 5, or 9 hpi following the 2.5-min continuous MCN digestions.

As expected, fractionation of the cellular DNA did not change significantly throughout the lytic infection cycle (Fig. 4A). The cellular DNA released as soluble chromatin fractionated as mono-, di-, (fractions 10 and 11), and polynucleosomes (fractions 1 to 9) (Fig. 4A). In contrast, the HSV-1 DNA-containing complexes released as soluble chromatin did change throughout the course of infection. At 2 hpi, no HSV-1 DNA was ever detected in the soluble chromatin fraction following continuous MCN digestion in any experiment (whereas cellular DNA was detected in all sucrose gradient fractions [Fig. 4A and B, 2 hpi]). These results are consistent with the absence of detectable nucleosome-size HSV-1 digestion intermediates following continuous MCN digestion during the time course analyses (Fig. 3A, 2 hpi, lane 2) and the instability of the HSV-1 DNA nucleoprotein complexes. Moreover, the HSV-1 DNA-containing complexes released as soluble chromatin at 5 or 9 hpi resolved primarily to the fractions containing mono- or dinucleosomes (fractions 9 to 11; Fig. 4A).

Inhibition of HSV-1 DNA replication changes the accessibility of HSV-1 DNA to MCN digestion. The accessibility of HSV-1 DNA therefore changes throughout the course of lytic infection. DNA-dependent processes, such as transcription and DNA replication, regulate chromatin accessibility. HSV-1 DNA replication has been implicated in playing a role in regulating the accessibility of HSV-1 DNA (43), and different histone H3 variants, which tend to form nucleosomes of different stability, associate with HSV-1 DNA before or after DNA replication (65). We therefore investigated the roles of HSV-1 DNA replication in the changes in accessibility of HSV-1 DNA. We used phosphonoacetic acid (PAA) to inhibit the HSV-1 DNA polymerase and therefore HSV-1 DNA replication (67).

Briefly, nuclear DNA from HSV-1-infected cells was isolated at 2, 3, 4, 5, 7, or 9 hpi or at 7 hpi from cells infected in the presence of PAA. The HSV-1 DNA in untreated cells was normalized to the levels of HSV-1 DNA in cells infected for 2 h (Fig. 5). The levels of nuclear HSV-1 DNA at 2 and 3 hpi were 1.5- and 1.9-fold higher than those in infected cells treated with PAA for 7 h (Fig. 5). The levels of HSV-1 DNA then increased faster, to 3.7-, 5.3-, 7.5-, and 9.4-fold at 4, 5, 7, and 9 hpi, respectively. The rate of HSV-1 DNA replication therefore accelerated between 3 and 4 hpi, before the changes in HSV-1 DNA accessibility we had observed at 4 or 5 h. We therefore evaluated the effect of HSV-1 DNA replication on the accessibility of HSV-1 DNA. Briefly, cells were infected with 10 PFU of HSV-1 per cell and treated with no drug or PAA. Infected cell nuclei were isolated at 7 hpi and digested with 0.05 U of MCN per 1 × 10^7 cells for 0.5, 2.5, 5, 15, 30, or 60 min. Nuclear DNA was purified, resolved by agarose gel electrophoresis, and analyzed by ethidium bromide staining and Southern blot hybridization. Hy-
FIG 3 The accessibility of HSV-1 DNA changes throughout the lytic infection cycle. Nuclei of infected cells isolated at 2, 4, 5, or 9 hpi were digested for 0.5, 2.5, 5, 15, 30, or 60 min with 0.05 U MCN per 1 × 10^7 nuclei. The DNA was analyzed by Southern blot hybridization with HSV-1 or cellular probes. (A) Images of the ethidium bromide-stained gels (Total) or membranes hybridized with cellular (Cellular) or HSV-1 (HSV)-specific probes. M, molecular weight marker. Two different hybridizations representing the different patterns observed at 2 hpi are shown [2 hpi (i) and (ii)]. For HSV-1 DNA, standard and overexposures (bottom panels), in which the absence of nucleosome-sized HSV-1 DNA is more obvious, are shown. To achieve comparable signal intensities, only 67% of each sample was loaded for 0.5-min digestions. (B and C) Line graphs of the quantitated Southern blot hybridizations presenting the levels of cellular (B) or HSV-1 (C) DNA against digestion time, as percentages of DNA at time zero. Results from one experiment (4 and 9 hpi) or averages ± ranges for two [2 (i), 2 (ii), and 5 hpi] are shown.
bridizations with cellular probes are shown in standard exposures. Hybridizations with HSV-1 probes are shown in overexposures, to highlight that only a minimal percentage of the HSV-1 DNA in nuclei of cells treated with PAA was digested to heterogeneously sized small fragments (Fig. 6A).

As expected, MCN digested the cellular DNA to the typical “nucleosome ladder,” corresponding to multiples of ~160 bp (Fig. 6A). Also as expected, this pattern was largely unaffected by PAA (Fig. 6B and Table 3). In contrast to that of cellular DNA, the accessibility of HSV-1 DNA was altered in cells treated with PAA. The pattern of digestion of HSV-1 DNA in the nuclei of untreated cells at 7 hpi was similar to that observed at 9 hpi in the previous experiments (compare Fig. 6A, No Drug, to Fig. 3A, 9 hpi). In contrast, HSV-1 DNA in the nuclei of infected cells treated with PAA was detected primarily as a large-molecular-weight band at the top of the gels, with only a very minor population of heterogeneously sized fragments (Fig. 6A). The pattern of HSV-1 DNA fragments was somewhat in between the two different patterns of HSV-1 DNA at 2 hpi (compare Fig. 6A, PAA, and Fig. 3A, 2 hpi). The decrease in accessibility of HSV-1 DNA in cells treated with PAA is reflected in the 3.1-fold change in $T_{50}$, from 14.3 min in untreated cells to 44.9 min in cells treated with PAA (Table 4). Despite the decrease in accessibility, however, the HSV-1 DNA in cells infected and treated with PAA for 7 hpi was still approximately 2.3-fold more accessible than that in the most inaccessible state in cells infected for 2 h in the absence of any drug ($T_{50}$, 44.9 and an estimated 104 min, respectively).

HSV-1 DNA is in nucleosome-like complexes when HSV-1 DNA replication is inhibited. During the time course of MCN digestion of PAA-treated nuclei, no HSV-1 DNA of sizes consistent with chromatin-like complexes was detected. However, HSV-1 DNA was digested and therefore accessible to MCN. It was therefore possible that unstable nucleosomes in the PAA-treated cells had failed to protect the HSV-1 DNA under continuous exposure to MCN. We therefore evaluated whether the HSV-1 DNA released by MCN was in nucleosome-like complexes in the ab-

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**TABLE 2** Accessibility of nuclear HSV-1 DNA changes throughout the lytic infection cycle

<table>
<thead>
<tr>
<th>Time postinfection (h)</th>
<th>Cellular DNA</th>
<th>HSV DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (i)</td>
<td>39.0</td>
<td>104†</td>
</tr>
<tr>
<td>2 (ii)</td>
<td>29.9</td>
<td>3.2</td>
</tr>
<tr>
<td>4</td>
<td>60.1†</td>
<td>7.2</td>
</tr>
<tr>
<td>5</td>
<td>42.2</td>
<td>10.5</td>
</tr>
<tr>
<td>9</td>
<td>44.6</td>
<td>12.7</td>
</tr>
</tbody>
</table>

*Digestion times required to degrade 50% ($T_{50}$) of cellular or HSV-1 DNA, graphically calculated from the average digestion curves. †, $T_{50}$ estimated by extrapolation. Results are from one experiment (4 and 9 hpi) or are averages for two (2 and 5 hpi). "i" and "ii" represent two distinct patterns of digestion of HSV-1 DNA at 2 hpi.

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**FIG 4** The stability of the HSV-1 DNA chromatin-like complexes changes throughout the lytic infection cycle. Nuclei of infected cells harvested at 2, 5, or 9 hpi were subjected to continuous MCN digestions. (A) DNA from each fraction was analyzed by Southern blotting with HSV-1 or cellular DNA-specific probes. At top, images of the membranes hybridized with cellular (Cellular) or HSV-1 (HSV) DNA-specific probes are shown. To achieve comparable signal intensities, only 50% of the insoluble fraction was loaded. Two different hybridizations representing the patterns observed at 2 hpi are shown (i and ii). At bottom, line graphs presenting cellular and HSV-1 DNA in each fraction, expressed as percentages of total DNA in the gradient, are shown. No HSV-1 DNA was detected in any fraction of the gradient at 2 hpi; therefore, there is no HSV-1 DNA line in this graph.

**FIG 5** HSV-1 DNA levels throughout the lytic infection cycle. Line graph showing the relative levels of nuclear HSV-1 DNA isolated from nuclei of infected cells harvested at 2, 3, 4, 5, 7, or 9 hpi. The purified DNA was quantitated by Southern blotting, and the HSV-1 DNA was normalized to the levels at 2 hpi.
sence of DNA replication. To this end, serial MCN digestions were performed on nuclei harvested at 7 hpi from untreated cells or from cells treated with PAA. Basically the same types of cellular DNA-containing complexes were released as soluble chromatin from the nuclei of cells treated or not with PAA. Under these conditions, the cellular DNA released as soluble chromatin fractionated as mono-, di-, tri- (fractions 10 to 12), and polynucleosomes (fractions 6 to 9) (Fig. 7A). The HSV-1 DNA in the nuclei of untreated cells was also detected

**TABLE 3** Accessibility of nuclear HSV-1 DNA in the presence or absence of PAA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cellular DNA</th>
<th>HSV DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>67.5†</td>
<td>14.3</td>
</tr>
<tr>
<td>PAA</td>
<td>54.2</td>
<td>44.9</td>
</tr>
</tbody>
</table>

*a Digestion times required to degrade 50% (T_{50}) of cellular or HSV-1 DNA in the absence (no drug) or presence of PAA, graphically calculated from the average digestion curves. †, T_{50} estimated by extrapolation. Average, n = 2.*

FIG 6 The accessibility of HSV-1 DNA decreases in the absence of HSV-1 DNA replication. Nuclei of infected cells untreated (No Drug) or treated with PAA were isolated at 7 hpi and digested for 0.5, 2.5, 5, 15, 30, or 60 min ( ) with 0.05 U MCN per 1 x 10^7 nuclei. DNA was analyzed by Southern blot hybridization with HSV-1 or cellular probes. (A) Images of the ethidium bromide-stained gels (Total) or membranes hybridized with cellular (Cellular) or HSV-1 (HSV) DNA-specific probes. M, molecular weight marker. Exposures in which the MCN-resistant and nucleosome-size HSV-1 DNA are most clearly visible are shown. To achieve comparable signal intensities, only 67% of each sample was loaded for 0.5 min. (B and C) Line graphs presenting the quantitation of the Southern blot hybridizations, presenting levels of cellular (B) or HSV-1 (C) DNA against digestion time, as percentages of DNA at time zero. Averages ± ranges are shown; n = 2.
TABLE 4 Percent recovery of nuclear HSV-1 DNA following serial MCN digestiona

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% recovery of nuclear HSV-1 DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble</td>
</tr>
<tr>
<td>No drug</td>
<td>73 ± 13</td>
</tr>
<tr>
<td>PAA</td>
<td>45.8 ± 8</td>
</tr>
</tbody>
</table>

a Soluble or insoluble HSV-1 DNA, expressed as percentage of total HSV-1 DNA, recovered following serial MCN digestion of nuclei harvested at 7 hpi from untreated cells (no drug) or cells treated with PAA. Averages ± ranges are given; n = 2 (no drug) or 5 (PAA).

in the nucleosome-containing fractions at 7 hpi (Fig. 7A). Most HSV-1 DNA-containing complexes released as soluble chromatin resolved to the same fractions as the DNA in cellular nucleosomes (9 to 12; Fig. 7A). The HSV-1 DNA was also recovered in chromatin-like complexes in the absence of HSV-1 DNA replication. Serial MCN digestion of nuclei of infected and treated with PAA released HSV-1 DNA-containing complexes that resolved to fractions 6 to 11, very much like the cellular DNA (Fig. 7A), or the fractionation pattern of the HSV-1 DNA in half of the experiments with cells infected for only 2 h. HSV-1 DNA is therefore in nucleosome-like complexes independently of HSV-1 DNA replication.

We next evaluated the percentage of nuclear HSV-1 DNA released as soluble or insoluble chromatin in the presence or absence of DNA replication following serial MCN digestions. Effectively all nuclear HSV-1 DNA (within standard error) was recovered from nuclei harvested at 7 hpi in the presence or absence of PAA and subjected to serial MCN digestion. We then analyzed the percentage of nuclear HSV-1 DNA recovered as soluble or insoluble chromatin. The soluble and insoluble chromatin fractions accounted for 73 and 27%, respectively, of the HSV-1 DNA recovered from cells treated with no drug. In contrast, the soluble and insoluble chromatin fractions accounted for 48 and 52%, respectively, of the HSV-1 DNA recovered from cells treated with PAA (Fig. 7B and Table 4). The distribution of soluble and insoluble HSV-1 DNA in nuclei infected for 7 h in the presence of PAA was somewhat similar to that in the nuclei of untreated cells infected for 2 h (Fig. 2B).

HSV-1 chromatin-like complexes are unstable when HSV-1 DNA replication is inhibited. We next tested the stability of the HSV-1 DNA-containing chromatin-like complexes in the nuclei of cells infected for 7 h in the presence of PAA. To this end, we evaluated the HSV-1 chromatin-like complexes released as soluble chromatin by the 2.5-min continuous MCN digestions.

The fractionation of the cellular DNA did not change significantly in the absence HSV-1 DNA replication (Fig. 7C). As expected, the cellular DNA released as soluble chromatin fractionated as mono-, di- (fraction 11), and polynucleosomes (fractions 1 to 10) (Fig. 7C).

The vast majority of the HSV-1 DNA-containing complexes released as soluble chromatin in untreated cells at 7 hpi resolved to fractions 9 to 11 (Fig. 7C), differently from the fractionation of the cellular DNA in the same cells (fractions 3 to 11; Fig. 7C, Cellular) and consistent with the fractionation of HSV-1 DNA from cells infected for 5 or 9 h. Likewise, the vast majority of HSV-1 DNA released as soluble chromatin in cells treated with PAA resolved to fractions 9 to 11, most similarly to the fractionation in untreated cells and differently from the fractionation of the cellular DNA in the same cells (fractions 2 to 11; Fig. 7C, Cellular) or the fractionation of the HSV-1 DNA after serial MCN digestion in cells infected for 7 h and treated or not with PAA (Fig. 7A, No Drug and PAA). Therefore, the HSV-1 DNA-containing nucleoprotein complexes released as soluble chromatin are particularly unstable regardless of whether HSV-1 DNA replication is ongoing or inhibited.

DISCUSSION

In contrast to earlier models proposing that most HSV-1 DNA was not associated in nucleosomes during lytic infections (43, 55, 56, 69), most current models propose that chromatin and chromatin modifications play important roles in the regulation of HSV-1 gene expression during lytic infections (30, 34, 38, 40, 46, 47, 52, 59, 62, 64). However, the physical evidence for the chromatinization of HSV-1 DNA during lytic infection has only recently started to be understood (42). Using classical chromatin characterization techniques, we had shown that at 5 hpi, most HSV-1 DNA is in particularly unstable nucleoprotein complexes and consequently more accessible to MCN than most DNA in cellular chromatin (42). Using serial MCN digestion, a protocol that allows the detection of unstable nucleosome-like complexes (Fig. 1), we showed here that HSV-1 DNA is in unstable chromatin-like complexes throughout the lytic infection cycle (Fig. 2), although the specific accessibility of HSV-1 DNA in chromatin-like complexes changes over the course of lytic infection (Fig. 3 and 4). The instability of these complexes surprisingly requires no HSV-1 DNA replication, although DNA replication does influence their accessibility (Fig. 6 and 7). As an intrinsic limitation, techniques that analyze nuclear HSV-1 DNA globally, such as our modified serial MCN protection assays, cannot differentially analyze the chromatinization state of very small genome subpopulations, such as the very small subpopulation of genomes transcribed at early times after infection (31).

Approximately 50% of nuclear HSV-1 DNA was detected in nucleosome-like complexes as early as 2 hpi (Fig. 2). A percentage of the HSV-1 DNA is likely still encapsidated at these early times (Fig. 2 and 4, 2 hpi, Insoluble, and Fig. 3, 2-hpi band at the top of the gel), which should result in an MCN-resistant DNA population highly dependent on the rate of the initial stages of infection. Interestingly, however, a large fraction of even the least accessible HSV-1 nucleosome-like complexes shows properties of unstable nucleosomes. For example, the HSV-1 DNA within these complexes was degraded if MCN was not inactivated immediately after the release of the soluble poly(nucleosomes (compare Fig. 2 and 4, 2 hpi). Nuclear HSV-1 DNA is therefore already in unstable nucleosome-like complexes as early as 2 hpi. These chromatin-like complexes containing HSV-1 DNA at such early times postinfection are consistent with the results of the ChIP assays presented by Placek et al., showing association of the replication-independent H3 variant, H3.3, with HSV-1 DNA at 1 hpi (65). The assembly of unstable HSV-1 DNA nucleosome-like complexes at 2 hpi is most likely DNA replication independent.

As the infection progressed from 2 to 5 hpi, there was a 10-fold increase in the accessibility of the HSV-1 DNA (Fig. 3 and Table 2). The majority (75%) of the recovered nuclear HSV-1 DNA was in nucleosome-like complexes at 5 hpi (Fig. 2), as already shown (42). The changes in accessibility generally coincide with the times of activation of E and L gene transcription and HSV-1 DNA replication (Fig. 5 and reference 75). As the infection progresses, the...
rates of HSV-1 transcription and DNA replication increase, and so does the accessibility of HSV-1 DNA. These results are consistent with the results of Kulaeva et al., who showed that whereas a single passage of the RNA polymerase II (RNAPII) complex does not disrupt nucleosomes, the passage of many results in full nucleosome eviction (36). Nucleosome eviction during transcription would be expected to be pronounced for HSV-1 genomes, which are transcribed on both strands and have a high gene density (68,
The changes in accessibility also temporarily correlate, in general terms, with the transition from prereplication to replication complexes (10). However, the patterns of protection of the HSV-1 DNA are far more consistent with its protection by assembly into unstable nucleosome-like complexes than by larger subnuclear structures, such as replication factories. Moreover, whereas PAA disrupts the replication complexes (18, 77), it does not disrupt the protection of the HSV-1 DNA (Fig. 7). Consistent with the continuous binding and unbinding of histones associated with unstable HSV-1 nucleosomes, our group has independently shown that both linker (7) and core (6) histones are mobilized during lytic HSV-1 infection.

Although most of the nuclear HSV-1 DNA was in highly accessible nucleosome-like complexes at 9 hpi, there was also an increase in the less accessible HSV-1 DNA. For example, the percentage of HSV-1 DNA migrating at the top of the gel following time course MCN digestions increased at these late times. This increase likely results from the encapsidation of progeny virions (Fig. 3, 9 hpi).

Physiologically, nucleosomes assemble by DNA replication-dependent or -independent processes. The initial chromatinization of HSV-1 DNA did not appear to depend on HSV-1 DNA replication (Fig. 2 and 3). Using PAA, we evaluated directly the role of HSV-1 DNA replication in the chromatinization of HSV-1 DNA. Time course MCN digestions showed a significant decrease in the accessibility of nuclear HSV-1 DNA in cells treated with PAA (Fig. 6). The pattern of the HSV-1 DNA fragments under these conditions was generally similar to that in nuclei of cells harvested at 2 hpi (compare Fig. 3A, 2 hpi, and Fig. 6, PAA), although the HSV-1 DNA in PAA-treated cells was 2.3-fold more accessible. The HSV-1 DNA in PAA-treated cells was also recovered in nucleosome-like complexes migrating as cellular mono- to polynucleosomes following serial MCN digestions (Fig. 7A) or as smaller complexes resolving mostly to the same fractions as cellular mono- and dinucleosomes after continuous digestion (Fig. 7C). In contrast, the chromatin-like complexes recovered from nuclei of cells infected for 2 h were so rapidly degraded that they were undetectable following continuous MCN digestions (Fig. 4). There are therefore differences in the accessibility of nuclear HSV-1 DNA present in the nuclei of cells infected for 2 h and left untreated or treated with PAA for 7 h. Although there is only minimal HSV-1 DNA replication under either condition, there are major changes in HSV-1 transcription between these two conditions.

Time course MCN digestions on nuclei from cells in which there is little to no HSV-1 DNA replication (2 hpi or PAA) never resulted in detectable nucleosome-sized HSV-1 DNA fragments (Fig. 3, 2 hpi, and Fig. 6, PAA). These results suggest that the HSV-1 chromatin-like complexes that exist prior to DNA replication are highly unstable and unable to protect the DNA during long MCN digestions. As an intrinsic limitation, the sensitivity of our assays would not detect very few genomes stably chromatinized at these earlier times. In contrast, the HSV-1 DNA was protected to sizes consistent with mono- (5, 7, and 9 hpi), or dinucleosome (7 and 9 hpi) DNA when HSV-1 DNA replication was ongoing. These findings are consistent with histone H3.3, which forms less-stable nucleosomes (28), associating with HSV-1 DNA before DNA replication, and H3.1, which forms more-stable nucleosomes (28), only during or after DNA replication (65). The increase in the relative amounts of nucleosome-size fragments (with respect to fragments of all other sizes) may also simply reflect an increase in total nuclear HSV-1 DNA, allowing the detection of the small amounts of nucleosome-size fragments.

Although HSV-1 DNA replication is not required for the chromatinization of HSV-1 DNA during lytic infection, it does appear to play a role in its accessibility. Prior to the onset of HSV-1 DNA replication, transcription is limited to immediate-early (IE) and early (E) loci, which comprise only ~30% of the HSV-1 genome, whereas the entire genome is transcribed after HSV-1 DNA replication. Our results are therefore consistent with a model in which the accessibility of nuclear HSV-1 genomes is directly affected by two DNA-dependent processes, transcription and DNA replication. However, it remains unclear whether the transcription and DNA replication of the viral genomes result in the destabilization of HSV-1 chromatin, subsequently increasing the accessibility of HSV-1 DNA, or the nucleosome destabilization increases the accessibility to HSV-1 DNA for its transcription and DNA replication. Likewise, the histone composition of the unstable HSV-1 nucleosomes and the roles of posttranslational modifications in nucleosome destabilization remain unknown (and are the focus of current studies). Histone composition and posttranslational modifications, such as acetylation, methylation, and phosphorylation, regulate nucleosome stability. For example, nucleosomes containing both H3.3 and H2AZ are highly unstable (27, 28). Likewise, acetylation of the amino-terminal tails of the core histones decreases nucleosome stability, as does certain phosphorylations of linker histone H1 and core H3 (16, 23, 32, 41, 44, 72). Histone variants and posttranslational modifications also correlate with transcriptional activity. For example, highly transcribed genes are enriched in H3.3 and in lysine-9- and -14-acetylated and lysine-4-methylated H3, whereas nontranscribed genes are enriched in H3.1 and lysine-9-trimethylated H3 (3, 17, 37, 53, 66, 71, 78).

The temporal dynamics of histone and nucleosome occupancy have also been evaluated with other herpesviruses (54, 60). Nitschke et al. showed that the chromatinization of HCMV DNA during lytic infections shows many similarities to that of HSV-1 (60). The accessibility of HCMV DNA was also evaluated by time course MCN digestions (60). Although the Southern blots were not quantitated, the results showed many qualitative similarities to those obtained with HSV-1. For example, a percentage of HCMV DNA was protected to nucleosome sizes (60), ranging from a very minor percentage at 2 hpi to a much larger one at later times (48 and 96 hpi). Like that of HSV-1, the HCMV DNA digested to nucleosome sizes could be detected only as fragments ranging from mono- to dinucleosome in size but not trinucleosomal or any longer. This pattern suggests that like HSV-1 DNA, HCMV DNA is in unstable nucleosome-like complexes. Also consistent with the results presented here, most HCMV DNA was poorly accessible to MCN at early times postinfection, and its accessibility increased as the infection progressed (60).

A percentage of DNA of HSV-1 or HCMV is poorly accessible to MCN. Such poorly accessible HSV-1 DNA is typically referred to as “resistant” and is interpreted as encapsidated (11, 43, 56, 60). A percentage of this resistant DNA at 2 hpi or at late times after infection is indeed likely encapsidated. The percentage of the “resistant” HSV-1 DNA is higher at early and late times postinfection, when the percentages of encapsidated HSV-1 genomes are higher. However, not all “resistant” HSV-1 DNA is actually encapsidated. The resistant DNA is detected through infection, even at 5 hpi,
when most HSV-1 DNA is in replication intermediates (26), or at 7 hpi in cells treated with PAA, when it is decapsidated and transcriptionally active. This resistant HSV-1 DNA is still accessible in that it is eventually digested by MCN (Fig. 3 and 4) and reference 42). Moreover, HSV-1 DNA is recovered as soluble chromatin in polynucleosome-like complexes even when it is poorly accessible to MCN (such as at 2 hpi or with PAA [Fig. 2 and 7]).

In summary, most HSV-1 DNA is in unstable nucleosome-like complexes throughout the lytic replication cycle. Furthermore, the presence and instability of the HSV-1 nucleosome-like nucleoprotein complexes are independent of HSV-1 DNA replication, although the specific accessibility varied with time after infection or when HSV-1 DNA replication was inhibited.

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