Nucleosome maps of the human cytomegalovirus genome reveal a temporal switch in chromatin organization linked to a major IE protein

Einat Zalckvar[a,b], Christina Paulus[c], Desiree Tillo[a,b], Alexandra Asbach-Nitzsche[c], Yaniv Lubling[a], Carla Winterling[c,1], Nicholas Strieder[c,2], Katrin Mücke[c], Felicia Goodrum[d], Eran Segal[a,b,3], and Michael Nevels[c,3]

Departments of [a]Computer Science and Applied Mathematics and [b]Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel; [c]Institute for Medical Microbiology and Hygiene, University of Regensburg, 93053 Regensburg, Germany; and [d]Department of Immunobiology, BIO5 Institute, University of Arizona, Tucson, AZ 65721

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Human CMV (hCMV) establishes lifelong infections in most of us, causing developmental defects in human embryos and life-threatening disease in immunocompromised individuals. During productive infection, the viral >230,000-bp dsDNA genome is expressed widely and in a temporal cascade. The hCMV genome does not carry histones when encapsidated but has been proposed to form nucleosomes after release into the host cell nucleus. Here, we present hCMV genome-wide nucleosome occupancy and nascent transcript maps during infection of permissive human primary cells. We show that nucleosomes occupy nuclear viral DNA in a nonrandom and highly predictable fashion. At early times of infection, nucleosomes associate with the hCMV genome largely according to their intrinsic DNA sequence preferences, indicating that initial nucleosome formation is genetically encoded in the virus. However, as infection proceeds to the late phase, nucleosomes redistribute extensively to establish patterns mostly determined by nongenetic factors. We propose that these factors include key regulators of viral gene expression encoded at the hCMV major immediate-early (IE) locus. Indeed, mutant virus genomes deficient for IE1 expression exhibit globally increased nucleosome loads and reduced nucleosome dynamics compared with WT genomes. The temporal nucleosome occupancy differences between IE1-deficient and WT viruses correlate inversely with changes in the pattern of viral nascent and total transcript accumulation. These results provide a framework of spatial and temporal nucleosome organization across the genome of a major human pathogen and suggest that an hCMV major IE protein governs overall viral chromatin structure and function.

herpesvirus | functional genomics | epigenetic regulation | ChIP-chip

Nuclear DNA is typically organized and maneuvered through nucleosomes that individually assemble ~147 bp of DNA in 1.65 superhelical turns around a core histone octamer composed of a central H3-H4 tetramer flanked by two H2A-H2B dimers (1, 2). Additionally, linker histone H1 binds to the nucleosome at the DNA entry–exit points outside the octamer. Nucleosomes have higher intrinsic affinity for particular DNA sequences, reflecting the ability of the sequence to bend sharply (3, 4). We have previously shown that these sequence preferences are not only relevant in vitro but also predictive of nucleosome occupancy across eukaryotic genomes in vivo (5–9). Nucleosome occupancy and positioning is also controlled by remodeling machines, which may read out DNA sequence features to establish specific nucleosome patterns (10), and structural alterations to the histone octamer, including histone variant exchange and posttranslational modifications. These alterations can directly affect nucleosome properties and/or may serve to recruit chromatin-modifying proteins (11–13). The dynamic regulation of genome accessibility through nucleosomes is thought to affect transcription and most other DNA-based nuclear processes in only partly resolved ways.

Human CMV (hCMV), one of eight human herpesviruses, establishes lifelong infections in most people worldwide. The virus is best known for causing developmental defects in human embryos and major disease or death in immunocompromised individuals, including AIDS, cancer, and transplant patients (14, 15). hCMV is among the largest and most complex viruses presently known. During productive infection of human cells, the viral ~235,000-bp dsDNA genome is expressed extensively and in a temporal cascade of immediate-early (IE), early, and late transcription. The most abundant gene products in the IE phase of hCMV infection are expressed from the viral major IE transcription unit. The predominant major IE protein species are the nuclear phosphoproteins IE1-72kDa (also known as IE72, pUL123, or IE1) and IE2-86kDa (also known as IE86, pUL122, or IE2). These viral proteins are key transcriptional regulators that are well-known for activating viral gene expression to facilitate productive infection (14, 16, 17). Notably, IE1 engages in host nuclear interactions with both the interchromatin and chromatin compartments (18), including human proteins involved in histone modification (19) and nucleosome assembly (20).

The genomes of hCMV and other herpesviruses do not carry histones when encapsidated (21–23). However, on release from virus capsids into cell nuclei, hCMV DNA associates with host-derived histones likely forming nucleosomes (24). Progression through the viral transcriptional program concurs with changes in posttranslational histone modifications as analyzed at individual viral genomic sites (25–29). However, information on the nucleosomal organization and function of hCMV chromatin during infection is limited, in part because no genome-wide studies are available. For instance, it is not known whether the hCMV genome has evolved to contain nucleosome favorable and disfavorable sequences, if the viral DNA forms nucleosomes in an organized fashion, how the virus may control its nucleosome organization, and how nucleosome occupancy may regulate the cascade of viral transcription.

To address these questions, we generated high-resolution spatial and temporal maps of nucleosome occupancy and (nascent) transcription across entire hCMV genomes after infection

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[1]Present address: Department of Molecular Biology, Max Planck Institute for Infection Biology, 10117 Berlin, Germany.

[2]Present address: Institute for Biochemistry I, University of Regensburg, 93053 Regensburg, Germany.

[3]To whom correspondence may be addressed. E-mail: eran.segal@weizmann.ac.il or michael.nevels@ukr.de.

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of permissive human primary cells with WT or IE1-deficient (*dl*IE1) viruses. Our results suggest that, despite not carrying histones when encapsidated, hCMV genomes undergo organized nucleosome formation and encode nucleosome reorganizing mechanisms to function properly within the chromatin environment of their host.

Results

Nucleosomes Initially Occupy hCMV Genomes According to Their DNA Sequence Preferences but Undergo Massive Reorganization as Infection Proceeds. Our work has suggested that hCMV DNA forms nucleosomes in the nuclei of productively infected human cells (24). This view is supported by the fact that hCMV genomes associate with all four classes of human core histones (H2A, H2B, H3, and H4) as well as the human linker histone (H1) as determined by ChIP analysis (Fig. S1*A*). Although the histone content of hCMV chromatin is generally lower compared with bulk cellular chromatin (Fig. S1*A*) (24), core histones associate with nuclear viral DNA at a ratio equaling the histone ratio of chromatinized cellular genomic sites (H2A:H2B:H3:H4 ~ 1:1:1:1) (Fig. S1*A*). Accordingly, hCMV nuclear DNA is at least partially present in (canonical) nucleosomes, which was judged from micrococcal nuclease (MNase) assays (Fig. S1*B*) (24).

To study the organization and dynamics of nucleosomes across nuclear viral genomes, we infected primary human fibroblasts (MRC-5) with the hCMV low-passage strain TB40E (WT) for 8, 48, or 96 h. Subsequently, we either performed ChIP using an antibody directed against the H3 histone fold domain or prepared mononucleosomes by MNase digestion. The resulting input and output (for ChIP-chip) or sonicated and nucleosomal (for MNase-chip) DNA sample pairs were subjected to dual-color labeling and cohybridized to custom-designed tiling arrays covering most of the hCMV genome at ~7-bp resolution. By using tiling arrays, we selectively measured the viral (but not human) DNA sequences associated with H3 or nucleosomes. Subsequently, we determined the average nucleosome occupancy per base pair. Data were obtained from two to six independent infections per each virus and time point. There was high correlation between replicate results (Fig. S2*A*), and signal intensities were not generally guanosine-cytidine (GC)-biased (Fig. S2*C*).

We found that the nucleosome patterns derived from ChIP-chip and MNase-chip analyses were highly similar, at least at the 8 h time point ($\rho = 0.8$ at 8 h and 0.41 at 48 h postinfection) (Fig. 1*B*). The modest differences observed at 48 h might be caused by detection of slightly dissimilar nucleosome populations by methods involving fixed (ChIP-chip) or nonfixed (MNase-chip) chromatin samples. More significantly, we observed marked differences between nucleosome occupancy across different viral genomic sites (Figs. 1*A* and 2*B*, WT and Fig. S3). For example, compared with the genome average, the viral terminal repeat long segment was nucleosome-depleted at 8 h (Fig. 1*A*), whereas relatively

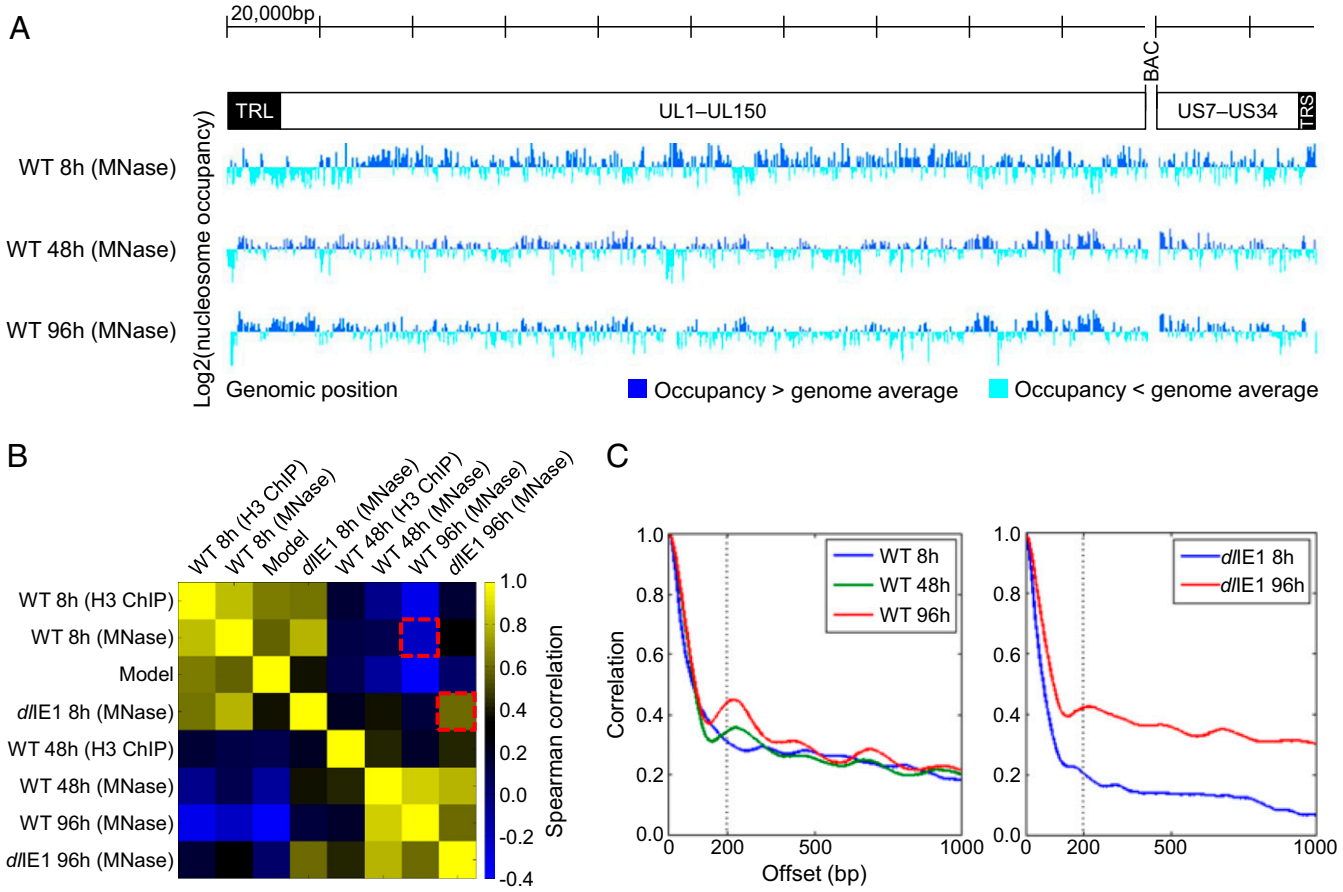

Fig. 1. Massive changes in nucleosome organization across the hCMV genome during productive infection. (*A*) Nucleosome occupancy profiles of the hCMV (TB40E) WT strain at 8, 48, and 96 h postinfection. At the top, the hCMV genome organization is schematically depicted [terminal repeat long (TRL), UL1–150, unique short ORFs 7–34 (US7–34), and terminal repeat short (TRS)]. The location of BAC sequences replacing viral ORFs IRS1, US1, US2 (partial), and US3–US6 in the TB40-BAC4 is indicated. (*B*) Summary of correlations between hCMV genome-wide nucleosome maps from this study and between these maps and the model by Kaplan et al. (6) (n = 204,747). Red squares mark the correlation between 8 and 96 h postinfection for the WT or *dl*IE1 maps. Correlations were grouped using 2D hierarchical clustering. (*C*) Autocorrelation plots (44) of nucleosome occupancy data from WT and *dl*IE1 virus genomes. The y axis on each plot shows Pearson correlation values obtained from correlating each MNase-derived nucleosome occupancy map set with shifted versions of itself from 0 to 1,000 bp (offset; x axis). Dashed vertical lines represent the typical length of one nucleosome including linker DNA.

nucleosome-rich [e.g., unique long 80 (UL80) –UL83] and nucleosome-depleted regions (e.g., UL1–UL6) were present across the UL segment at this time (Fig. 2*B*, WT). We also observed dramatic differences in nucleosome occupancy between different postinfection time points. Notably, at 8 h postinfection, nucleosome occupancy was determined to be high at regions with high intrinsic DNA sequence preference, which was predicted by a computational model derived from in vitro nucleosome occupancy data (6) ($\rho = 0.58$) (Figs. 1*B* and 2*A*). Briefly, this sequence-based probabilistic model computes a score of intrinsic nucleosome sequence preference based on the 5-mer composition and periodic dinucleotide signals contained in a given 147-bp DNA sequence. However, nucleosome occupancy did not positively correlate with the in vitro model at later time points ($\rho = -0.11$ at 48 h and -0.37 at 96 h postinfection) (Figs. 1*B* and 2*A*). In addition, we observed an increasing periodicity of the nucleosomal signal in these later time points (Fig. 1*C*, WT), which approached the in vivo nucleosomal repeat length (including linker DNA) seen in higher eukaryotes (30, 31) (Fig. 1*C*, dashed line). These findings suggest that, at early times postinfection, nucleosome occupancy is rather irregular and largely a consequence of default deposition prescribed genetically, whereas nucleosome organization is actively altered by nongenetic viral and/or cellular factors and becomes more regular during later infection times.

An hCMV Major IE Protein Mediates Nucleosome Temporal Reorganization and Reduces Global Nucleosome Occupancy Across Viral Genomes. To test whether IE1, a chromatin-associated key viral regulator of transcription and pathogenicity, affects hCMV nucleosome organization, we compared genome-wide nucleosome occupancy maps of WT and *dl*IE1 viruses. At early postinfection times, patterns of nucleosome occupancy across WT and *dl*IE1 genomes were overall similar ($\rho = 0.8$). Notably, in the absence of IE1, the nucleosome reorganization seen in later times during infection with the WT strain was greatly reduced (WT 8 vs. 96 h, $\rho = -0.21$; *dl*IE1 8 vs. 96 h, $\rho = 0.6$) (Figs. 1*B* and 2 and Fig. S4). Moreover, the early to late increase in periodicity seen in the WT was much less evident in the absence of IE1 expression (Fig. 1*C*). The fact that the gross nucleosome rearrangements observed during WT infection were drastically reduced in the mutant suggests that IE1 is a major contributing factor to the temporal dynamics of hCMV chromatin, although IE2 may contribute as well (*Discussion*).

To further study the effect of IE1 on global nucleosome occupancy across the hCMV genome, we performed MNase-Southern experiments with cells infected by WT or *dl*IE1 viruses. We used a ^{32}P-labeled hCMV BAC-derived probe to detect only nucleosomes associated with viral genomes at 8 h postinfection. At this early time point, little WT viral DNA is present as nucleosomal-sized bands (Fig. 3*A*) (24). However, substantially

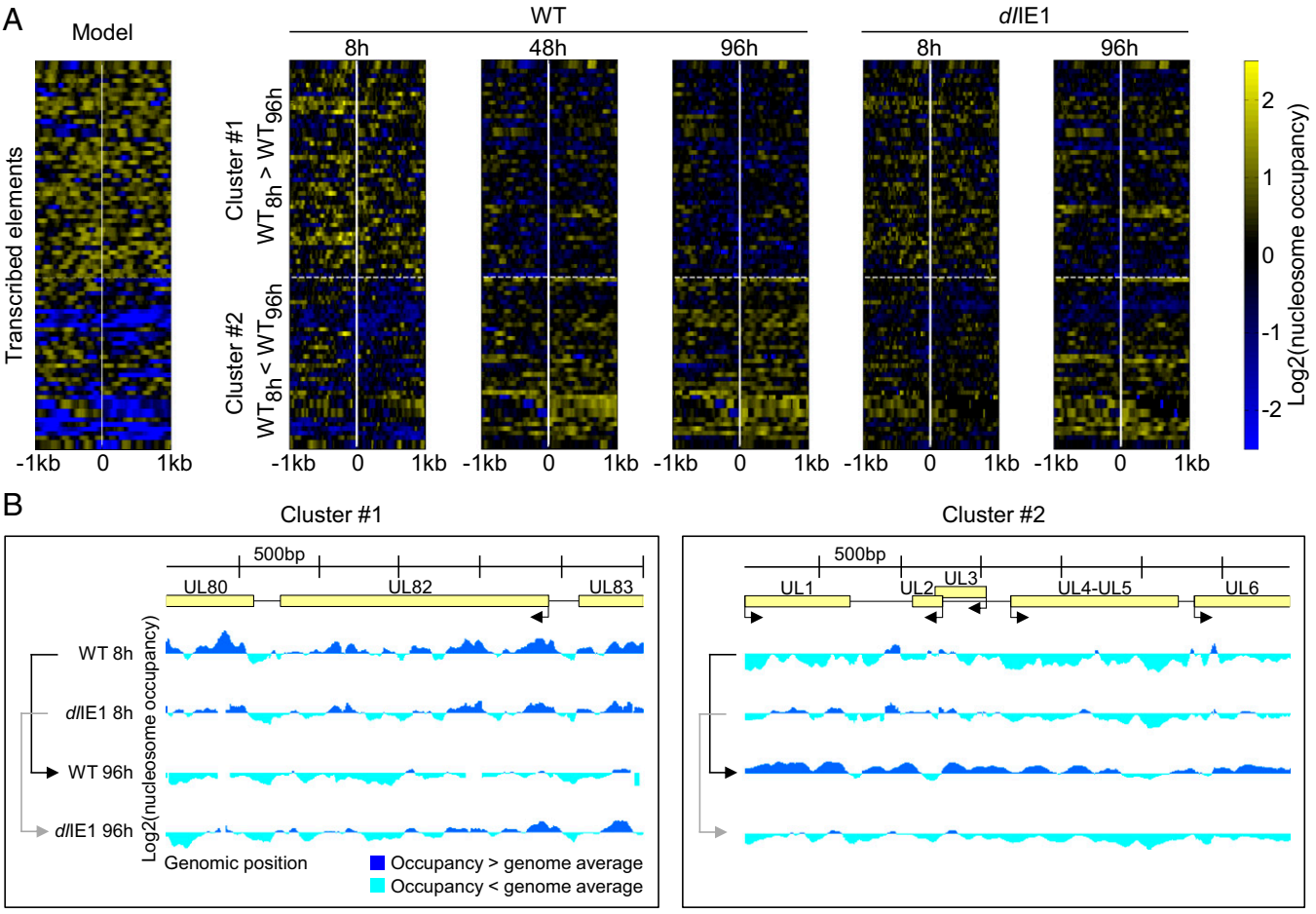

Fig. 2. IE1-dependent temporal changes in nucleosome organization across hCMV genomes. (*A*) Global views of nucleosome occupancy on WT and *dl*IE1 viral genomes across time postinfection. The model is by Kaplan et al. (6), and it predicts intrinsic nucleosome occupancy. Data were grouped based on WT nucleosome occupancy profiles (MNase data) of all time points postinfection from −1 to +1 kb relative to the manually annotated transcription start sites (k = 2, correlation distance metric), resulting in clusters #1 (higher occupancy of transcribed elements of the WT strain at 8 h postinfection) and #2 (higher occupancy of transcribed elements of the WT strain at 96 h postinfection). (*B*) Examples of nucleosome occupancy profiles from WT and *dl*IE1 at 8 and 96 h postinfection in viral genomic regions UL80–UL83 (cluster #1) and UL1–UL6 (cluster #2).

more nucleosomal viral DNA was detected in the *dl*IE1 infections relative to WT. These data indicate that IE1 generally reduces the nucleosome load on hCMV genomes. To further explore this effect, we subjected chromatin from cells infected by WT or *dl*IE1 viruses to MNase accessibility and ChIP assays linked to quantitative PCR (qPCR). Consistent with our global MNase-Southern analysis, the hCMV genome was markedly less accessible to MNase digestion at each of seven tested viral sites in the absence of IE1 (Fig. 3*B*). Likewise, all hCMV sequences under investigation were more highly associated with each core histone class in the absence of IE1 (Fig. 3*C*). We note that the observed increase in global nucleosome occupancy cannot be inferred from our microarray analyses because of the normalization strategy that we applied (*Materials and Methods*). These results indicate that IE1 expression regulates both the temporal reorganization of nucleosomes and the global nucleosome load on hCMV genomes during productive infection of human cells.

Major IE-Dependent Changes in Nucleosome Occupancy Are Linked to Differential Transcription from hCMV Genomes. Next, we set out to understand whether changes in nucleosome organization relate to transcription from the hCMV genome. To this end, we analyzed nascent and steady state transcript levels across the viral genome over time postinfection in parallel to nucleosome occupancy. Data were obtained from two independent infections per each virus (WT and *dl*IE1) and time point (8 and 96 h postinfection), and there was high correlation between replicate results (Fig. S2*B*). When comparing nucleosome occupancy with transcription across viral genomic regions (promoters and transcribed sequences) in the WT virus, we did not find significant overall correlations. This observation indicates that there is no trivial (inverse) relationship between nucleosome occupancy and gene activity. We then asked whether viral genomic regions (promoters and transcribed sequences) exhibiting changes in nucleosome occupancy between WT and *dl*IE1 viruses show altered transcription. At early times (8 h) postinfection, we observed little, if any, correlation between IE1-related differences in nucleosome occupancy and corresponding differences in gene expression (nascent and steady state mRNA) (Fig. 4*A* and Fig. S5). However, at late times (96 h) postinfection, promoters showing increased nucleosome occupancy in the absence of IE1 also exhibited reduced levels of nascent and steady state mRNA (Fig. 4 and Fig. S5). We found highly similar results irrespective of whether we used a previously published viral gene annotation (32) or our own transcript classification (Fig. 4 and Fig. S5). Inverse relationships between nucleosome occupancy and transcription are well-established for promoters in yeast and other eukaryotes (31, 33, 34). Notably, however, the negative correlation between changes in nucleosome occupancy and transcription was not restricted to hCMV promoters but also extended to viral transcribed regions (Fig. 4 and Figs. S5, S6, and S7).

Discussion

Although herpesvirus genomes are commonly considered to form regular nucleosomal arrays resembling bulk cellular chromatin in nonproductively (latently) infected cells (21–23), this type of chromatin organization has not been formally shown for hCMV or any β-herpesvirus. Moreover, there has been considerable debate over the general nature of herpesvirus chromatin during productive infection. Herpesvirus genomes are devoid of histones inside virus particles, and some earlier work provided evidence for a mostly nonnucleosomal structure of viral genomes in the nuclei of cells productively infected with various herpesviruses. However, other reports (including more recent ones) have pointed to significant histone association and nucleosome formation on nuclear DNA of herpesviruses, including hCMV (21–23). The present study strongly supports the notion that much of the hCMV genome is present as (canonical) nucleosomes composed of core and linker histones in infected human cell nuclei throughout the viral productive cycle.

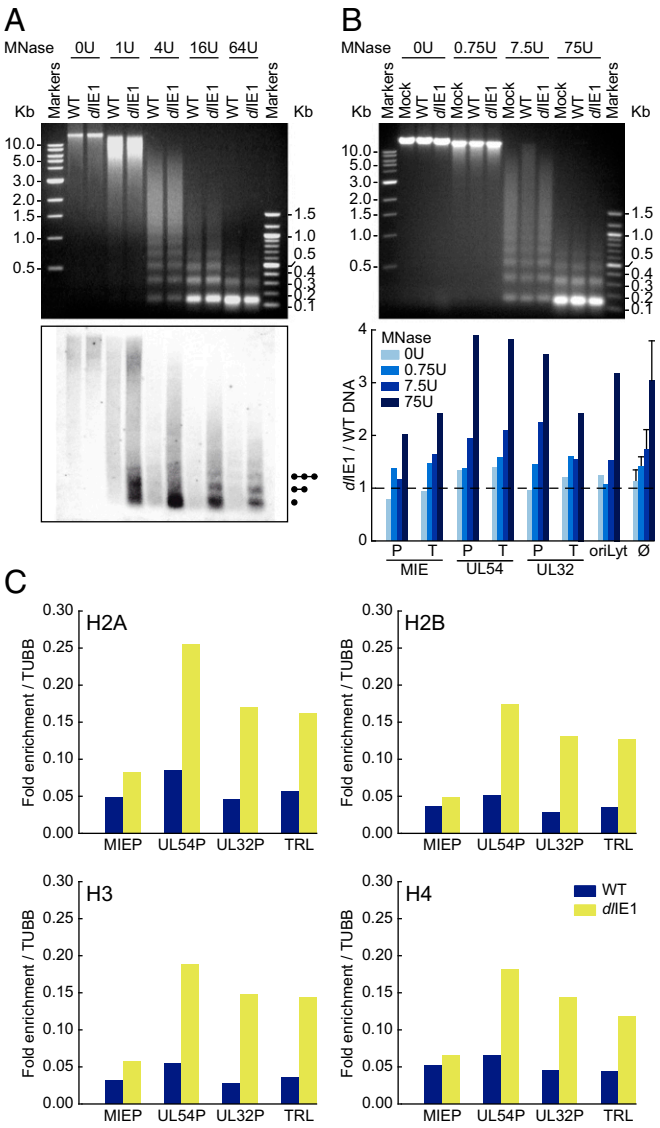

Fig. 3. IE1-dependent effects on global nucleosome occupancy across hCMV genomes. (*A*) MRC-5 cells were infected with WT or *dl*IE1 strains of hCMV TB40E (3 pfu/cell) for 8 h. (*Upper*) Nuclei were reacted with the indicated amounts of MNase, and purified DNA was separated in a 1.2% (wt/vol) agarose gel stained with ethidium bromide. (*Lower*) The same DNA samples were subjected to Southern blotting using a whole-genome probe derived from TB40-BAC4. (*B*) MRC-5 cells were infected with WT or *dl*IE1 strains of hCMV Towne (0.5 pfu/cell) for 8 h. (*Upper*) Nuclei were reacted with the indicated amounts of MNase, and DNA was separated in a 1.2% (wt/vol) agarose gel stained with ethidium bromide. The same DNA samples were subjected to chromatin accessibility real-time PCR at the indicated viral genomic sites. Columns represent *dl*IE1-to-WT DNA ratios from two measurements or ratios averaged across all tested sites (Ø), with SDs shown as error bars. (*C*) MRC-5 cells were infected with WT or *dl*IE1 strains of hCMV Towne (3 pfu/cell) for 0.5 h. ChIP was performed using antibodies for the indicated core histones, and DNA was quantified by qPCR at the indicated viral genomic sites. Columns represent mean output-to-input DNA ratios from two measurements normalized to the respective ratios at the cellular tubulin beta (TUBB) locus.

Our study presents high-resolution genome-wide nucleosome and nascent transcript maps of hCMV. To generate these maps, we chose to use tiling array technology instead of next generation sequencing. The array-based strategy allowed us to selectively interrogate the viral genome without having to contend with the host DNA present at excess amounts in infected cells, especially

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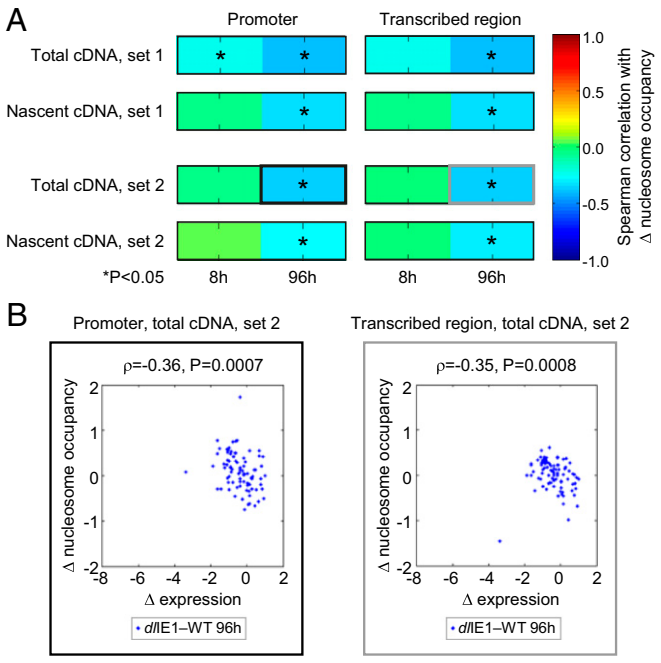

Fig. 4. Negative correlation between IE1-dependent changes in nucleosome organization and expression across hCMV genomes. (*A*) Summary of correlations between IE1-dependent changes in transcription and nucleosome occupancy across viral promoters and transcribed regions for the indicated times postinfection and gene sets [set 1: annotations from the work by Zhang et al. (32), $n = 135$; set 2: manual annotations, $n = 86$]. An average transcription (from total and nascent cDNA data) and nucleosome occupancy score (from MNase data) was calculated across promoters (defined as −200 to 0 bp relative to the transcription start site) or transcribed regions (defined as ORFs or sequences from transcription start to end), and differences between *dl*IE1 and WT were determined. Spearman correlations between differences in nucleosome occupancy and differences in transcript levels are shown. Asterisks indicate correlations with $P < 0.05$. (*B*) Scatterplots illustrating the negative relationship (ρ = Spearman correlation, $P = P$ value) between IE1-dependent changes in nucleosome occupancy (from MNase data) and gene expression (from total cDNA data, set 2) across viral promoters and transcribed regions at 96 h postinfection (Figs. S5, S6, and S7).

at early times postinfection (before viral DNA replication). From analyzing these maps, we conclude that nuclear hCMV genomes form nucleosomes in a nonrandom and highly predictable fashion (Figs. 1 and 2 and Figs. S1 and S2), which was previously not understood. However, nucleosome organization not only varies between different viral genomic sites (Figs. 1*A* and 2) but also changes with time during infection (Figs. 1 and 2 and Figs. S2, S3, and S4). In fact, early nucleosome occupancy was found to be mostly directed by the viral DNA, indicating that de novo nucleosome deposition on hCMV genomes entering the nucleus may be genetically encoded. This concept is supported by not only a model predicting nucleosome occupancy based on DNA sequence preferences (Figs. 1*B* and 2*A*) but also the fact that nucleosome occupancy at early (but not late) times of infection correlates positively with GC content of the underlying viral genomic site (Fig. S2*C*). GC content has been previously shown to dominate intrinsic preferences of nucleosome occupancy (5, 9). However, nucleosomes undergo reorganization to an unprecedented extent as infection proceeds to the late phase, and they eventually form patterns largely determined by factors other than DNA (Figs. 1 and 2 and Fig. S2*C*). These factors include one or more viral major IE gene products (Figs. 1 *B* and *C* and 2 and Fig. S4). Although most likely related to IE1, we cannot exclude that IE2 contributes to nucleosome temporal organization, because the kinetics and levels of IE2 expression are altered in *dl*IE1 infections (Fig. S8*F*). Notably, the differences in hCMV nucleosome occupancy between early and late times postinfection are larger than those differences observed between different human cell types or between human cells under different conditions [WT hCMV 8 vs. 96 h, $\rho = -0.21$; Gm12878 lymphoblastoid cells vs. K562 chronic myelogenous leukemia lymphoblasts, $\rho = 0.61$ (35); resting vs. activated CD4+ T cells, $\rho = 0.53$ (36)].

It is tempting to suspect that the profound rearrangements in hCMV chromatin during productive infection may contribute to the concurring extensive changes in viral transcription or vice versa. However, we could not find clear overall correlations between nucleosome occupancy and gene expression at any time point postinfection. Although this finding does not exclude that such correlations exist locally (e.g., at a subset of viral promoters), it implies that the relationship between expression and nucleosomes is complex. There are actually numerous examples in different organisms showing that factors that affect transcription do not necessarily affect nucleosome organization and vice versa. One illustrative example is a nucleosome-free DNA sequence to which a transcription factor may or may not bind, thus affecting expression without changing nucleosome organization. In fact, genome-wide analyses in yeast have indicated that nucleosome occupancy correlates poorly with transcription (37). Nonetheless, we found significant negative correlations between changes in nucleosome occupancy and changes in gene expression between WT and *dl*IE1 strains 96 h postinfection (Fig. 4 and Figs. S5, S6, and S7). This finding is consistent with the long-standing observation that disruption of IE1 delays transcription during productive hCMV infection (16, 17). However, we do not know if diminished transcription in the absence of IE1 results in the observed changes in nucleosome organization or if nucleosome changes are required for transcriptional activation by the viral protein. Any contributions by IE2 remain to be determined as well (see above). Notably, replication of the TB40E-derived *dl*IE1 virus described here seems to be more profoundly attenuated at both high- and low-input multiplicities than previously described similar mutants in the Towne strain (Fig. S8 *C*–*E*) (16). The replication phenotype of the *dl*IE1 mutant is consistent with an important role of nucleosome occupancy and dynamics in productive hCMV infection.

We show that major IE gene expression has a dual role in both limiting the global nucleosome load and facilitating nucleosome reorganization on hCMV genomes (Figs. 1, 2, and 3 and Figs. S2, S3, and S4). To our knowledge, the gross rearrangement of nucleosomes that we observe across hCMV genomes in the presence of IE1 (and IE2) has not been anticipated in other biological systems. There is, however, precedent for virus-encoded proteins controlling histone levels on viral DNA. Although these studies were performed at a small number of sequences, they show that virion protein 16 (VP16) and infected cell protein 0 (ICP0) of herpes simplex virus type 1 (HSV-1) have active roles in reducing the amount of core histones associated with viral genes through unresolved mechanisms (38–40). VP16, ICP0, and IE1 do not obviously share structural features, but they exhibit functional similarities. For example, all three proteins act as transcriptional activators and promote histone acetylation (19, 40, 41). Moreover, IE1 has been shown to partly compensate for ICP0 in supporting HSV-1 replication (42). Although IE1 is not considered to bind DNA directly (43), the viral protein associates with chromatin (18) and nucleosome modifying factors (19, 20). Therefore, the effect of IE1 on nucleosome occupancy and organization might be mediated through direct interaction between the viral protein and chromatin components, although we cannot rule out a more indirect mechanism possibly involving other hCMV gene products (including IE2).

Although many questions remain, our work provides a framework for understanding nucleosome organization and dynamics across the genome of a major human pathogen and suggests a unique role for a viral protein in global chromatin control. These findings put epigenome dynamics in the context of a common

infection, thereby shedding light on the molecular mechanisms that underlie viral pathogenesis.

Materials and Methods

Viral genome-wide maps of nucleosomes as well as total and nascent transcripts were prepared from growth-arrested human fibroblasts (MRC-5) infected at high multiplicity (3 pfu/cell) with WT or *dl*IE1 mutant strains of the hCMV clinical isolate TB40E. Mononucleosome preparation by MNase digestion and RNA purification was performed at 8, 48, and/or 96 h postinfection for WT and *dl*IE1 viruses. H3-ChIP assays were performed for the WT virus at 8 and 48 h postinfection. The resulting genomic DNA or cDNA was labeled and hybridized to $4 \times 72{,}000$ custom-designed arrays (NimbleGen/Roche) tiling both strands of the hCMV TB40E genome (GenBank accession no. EF999921) across each subarray. Signal intensities from all tiling arrays were extracted using NimbleScan 2.5–2.6 software. The array-based nucleosome analyses were complemented by ChIP assays at individual viral and cellular genomic sites (ChIP-qPCR) and MNase assays coupled to Southern blotting (MNase-Southern) or qPCR (chromatin accessibility real-time PCR). All raw and processed tiling array data are freely available at http://genie.weizmann.ac.il/pubs/hcmv2013. A more detailed account is provided in *SI Materials and Methods*.

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