Replication-Uncoupled Histone Deposition during Adenovirus DNA Replication

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Replication-uncoupled histone deposition during adenovirus DNA replication

Tetsuro Komatsu and Kyosuke Nagata*

Department of Infection Biology, Faculty of Medicine and Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba 305-8575, Japan

*Corresponding author. Department of Infection Biology, Faculty of Medicine and Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan. Phone and fax: (81) 298-53-3233. E-mail: knagata@md.tsukuba.ac.jp.

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ABSTRACT

In infected cells, the chromatin structure of the adenovirus genome DNA plays critical roles in its genome functions. Previously, we have reported that in early phases of infection, incoming viral DNA is associated with both viral core protein VII and cellular histones. Here we show that in late phases of infection, newly synthesized viral DNA is also associated with histones. We also found that knockdown of CAF-1, a histone chaperone that functions in replication-coupled deposition of histones, does not affect the level of histone H3 bound on viral chromatin, although CAF-1 is accumulated at viral DNA replication foci together with PCNA. Chromatin immunoprecipitation assays using epitope-tagged histone H3 demonstrated that histone variant H3.3, which is deposited onto the cellular genome in a replication-independent manner, is selectively associated with both incoming and newly synthesized viral DNAs. Microscopic analyses indicated that histones but not USF1, a transcription factor that regulates viral late gene expression, are excluded from viral DNA replication foci and this is achieved by oligomerization of DBP. Taken together, these results suggest that the histone deposition onto newly synthesized viral DNA is most likely uncoupled with viral DNA replication, and a possible role of DBP oligomerization in this replication-uncoupled histone deposition is discussed.
INTRODUCTION

In the cell nucleus, the genomic DNA is not naked, but forms chromatin structure with chromatin proteins. The fundamental unit of the chromatin structure is a nucleosome, which consists of histone octamer (two copies each of histone H2A, H2B, H3, and H4) and DNA wrapping around the octamer. Deposition of histones and/or remodeling of nucleosome arrays are critical processes for the expression of genome functions (2), since nucleosome packaging could be barrier for trans-acting factors to access their cognate sites on DNA. Thus, the nucleosome structure must be strictly and dynamically regulated in connection with several events on chromatin, such as transcription, DNA replication, and DNA repair.

Currently, it is known that histone deposition is carried out mainly by two fashions, DNA replication-dependent and independent ones, and a role of histone variants in these deposition pathways has been elucidated (14). In mammalian somatic cells, there are three major histone H3 variants, H3.1, H3.2, and H3.3, and they have only slight differences in amino acid (aa) sequences (16). The canonical histone H3, histone H3.1 and highly related variant H3.2 (which differs only 1 aa from H3.1) are expressed exclusively during S phase, while the expression of the variant H3.3 that differs 4 and 5 aa from H3.2 and H3.1, respectively, is observed throughout cell cycle. Thus, this variant is called “replication-independent” one (11). Tagami et al. demonstrated that the canonical histone H3 (H3.1) interacts with histone chaperone CAF-1 complex and is deposited onto DNA in a replication-dependent manner, while HIRA specifically binds to and deposits histone variant H3.3 onto DNA independently.
of DNA synthesis (43). CAF-1 is composed of three subunits, p150, p60, and p48, and associated with the cellular DNA replication machinery through the interaction with PCNA, a sliding clamp for DNA polymerases, allowing DNA replication-coupled deposition of histones (40, 41, 50). On the other hand, HIRA was identified as a DNA synthesis-independent histone chaperone by cell-free systems using Xenopus egg extracts (32), and histone variant H3.3 is shown to mark transcriptional active genomic regions (1). Furthermore, additional H3.3-specific chaperones are recently identified. Daxx is one of components of PML nuclear bodies and reported to deposit histone H3.3 onto the specific genomic regions such as telomeres and pericentric heterochromatin, together with an ATP-dependent chromatin remodeler, ATRX (10, 21). It is also reported that in Drosophila cells, DEK is a coactivator of a nuclear receptor and functions as an H3.3-specific chaperone (37). Thus, the mechanistic evidences for histone deposition are accumulating, in the case of cellular chromatin.

The regulatory events for chromatin structure are not limited to the cellular genome, as some viruses also have chromatin and/or chromatin-like structures with their own genomes. The adenovirus (Ad) genome is a liner double-stranded DNA (dsDNA) of ~36000 bp in length. In the virion, the Ad genome forms chromatin-like structure with viral basic core proteins, as it is revealed by electron microscopic analyses that viral core protein-DNA complexes purified from the virion show “beads-on-a-string” structure (49). Among core proteins, protein VII is a major DNA binding protein that can introduce superhelical turns into DNA as do cellular histones (4), and remains associated with viral DNA after the entry of the nucleus (7, 17).
When viral DNA-core protein complexes purified from the virion are used as a template for cell-free DNA replication/transcription systems, the reactions occur at a much lower level, compared with the case of naked DNA, indicating that viral chromatin-like structure must be remodeled to execute its genome functions (22, 23). Previously, we have identified host cell-derived remodeling factors for Ad chromatin with biochemical analyses (19, 22, 24, 26) and demonstrated that TAF-I, one of these host factors, plays an important role in the regulation of viral early gene expression in infected cells through the interaction with protein VII (15, 17, 18, 20, 27). Thus, it is indicated that remodeling of Ad chromatin is a crucial process for its genome functions (13), as is the case for the cellular genome. In addition, recently we have reported using chromatin immunoprecipitation (ChIP) assays that in early phases of infection, cellular histones are incorporated into viral DNA-protein VII complexes and histone modification occurs depending upon transcription states on viral chromatin, suggesting that cellular histones could be functional components of viral chromatin in infected cells (20).

As described above, although viral chromatin structure and its regulation in early phases of infection are being clarified, it is quite unclear how viral chromatin structure is regulated in late phases of infection. In particular, since the expression of viral late genes is largely dependent on its own DNA replication (45), the regulation of the chromatin structure during viral DNA replication could be a key step. Therefore, in this study we sought to elucidate the regulatory mechanism how the chromatin structure is formed on newly synthesized viral DNA through viral DNA replication, in particular with respect to the histone deposition. We found that after the onset of viral
DNA replication, cellular histones are also incorporated into viral chromatin. We also found that although CAF-1 is accumulated at the site of viral DNA replication, this factor seems not to be involved in the histone deposition during viral DNA replication, since knockdown of CAF-1 did not affect the binding level of histone H3 on viral chromatin and histone variant H3.3, which is deposited onto DNA in a DNA synthesis-independent manner, is specifically deposited onto viral DNA even after the onset of viral DNA replication. Microscopic analyses suggest that histones but not USF1, a transcription factor which is shown to bind to and regulate transcription from viral major late promoter (MLP) (46), are excluded from the site of viral DNA replication, possibly by oligomerization of Ad single-stranded DNA (ssDNA) binding protein (DBP), one of viral DNA replication factors. Based on these results, we would propose a model that unlike cellular chromatin, the histone deposition onto the newly synthesized viral DNA is not coupled with viral DNA replication. A feasible role of this uncoupled deposition mechanism mediated by DBP oligomerization on Ad genome functions is discussed.
MATERIALS AND METHODS

Cells and viruses.

Maintenance of HeLa cells, and purification and infection of human adenovirus type 5 (HAdV5) were carried out essentially as described previously (18, 20). Hydroxyurea (HU) was added at the final concentration of 2 mM right after infection when DNA replication was to be blocked. HeLa cells stably expressing EGFP-tagged histone H3.2 and H3.3 [a kind gift from Dr. M. Okuwaki (University of Tsukuba)] were also maintained as described above. Transfection of expression plasmids was performed using GeneJuice (Novagen) according to the manufacturer’s protocol.

Antibodies.

Antibodies used in this study are as follows: rabbit anti-histone H3 (catalog no. ab1791; abcam), rabbit anti-histone H4 (catalog no. 04-858; Millipore), rabbit anti-histone H2A (catalog no. ab18255; abcam), mouse anti-HIRA (catalog no. 04-1488; Millipore), mouse anti-FLAG M2 (catalog no. F3165; Sigma), rat anti-HA (3F10; Roche), and mouse anti-β-actin (Sigma) antibodies. Rabbit anti-histone H2A-H2B, mouse anti-CAF-1 p150, and mouse anti-DBP antibodies were kindly provided by Dr. M. Okuwaki (University of Tsukuba), Dr. A. Verreault (University of Montreal), and Dr. W. C. Russel, respectively. Rat anti-protein VII antibody was described elsewhere (17).
Vector construction.

To construct the expression vectors for USF1, full-length DBP, and its deletion mutant (DBPΔC, which lacks the C-terminal 17 aa), cDNA fragments of USF1, DBP, and DBPΔC were amplified by PCR, digested with BamHI and EcoRI, and cloned in-frame into pCHA vector containing a hemagglutinin (HA) epitope tag and the puromycin-resistance gene [pCHA-puro vector, kindly provided from K. Kajitani (University of Tsukuba)]. The resulting vectors are designated pCHA-puro-USF1, pCHA-puro-DBP, and pCHA-puro-DBPΔC, respectively. Similarly, for the expression vector of PCNA, amplified cDNA fragment was digested with BamHI and cloned into pCHA-puro vector digested with BamHI and EcoRV (pCHA-puro-PCNA). The primers used here were as follows:

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5’-GTGGAGGATCCCATATGAAGGGGCAGCAG-3’ and
5’-GGGCCGAATTCTTAGTTGCTGTCATTCTTG-3’ for USF1 cDNA,
5’-AAAGGATCCATGGCCAGTCGGG-3’ and
5’-GCGGAATTCTTAAAAATCAAAGGGGTTCTG-3’ for DBP cDNA,
5’-AAAGGATCCATGGCCAGTCGGG-3’ and
5’-CCCGAATTCTTAGTTGCGATACTGG-3’ for DBPΔC cDNA, and
5’-AAAGGATCCATGGCCAGTCGGG-3’ and
5’-ATCGTGACCTAAGATCCTTCTTCC-3’ for PCNA cDNA.
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For preparation of cells stably expressing HA-PCNA, HeLa cells were transfected with pCHA-puro-PCNA and cultured in the presence of 2 μg/mL puromycin for 2 weeks.
For construction of the expression vector of histone H3.1, cDNA fragment of histone H3.1 was amplified by PCR, digested with NcoI, and cloned into pBS-FLAG vector (pBS-H3.1-FLAG). Then, the DNA fragment containing cDNA of H3.1 and the C-terminal FLAG tag was obtained from pBS-H3.1-FLAG by digestion with BamHI and EcoRI, and cloned into pcDNA3 vector (pcDNA3-H3.1-FLAG). The primers used here were as follows: 5’-AAAACCATGCGCGTACTAAGCAG-3’ and 5’-TTATTCCATGGGCCGCTCTCCCCA-3’.

The expression vectors for FLAG-tagged histone H3.2 and H3.3 (pcDNA3-H3.2-FLAG and pcDNA3-H3.3-FLAG) and HA-tagged DEK (pCHA-DEK) were generously provided by Dr. M. Okuwaki and Dr. S. Saito, respectively (University of Tsukuba).

**Indirect immunofluorescence assays.**

Indirect immunofluorescence (IF) assays were carried out essentially as previously described (18). Localization of the protein was visualized with the secondary antibodies (anti-mouse IgG conjugated with AlexaFluor 488, anti-mouse IgG conjugated with AlexaFluor 568, and anti-rabbit IgG conjugated with AlexaFluor 568; Invitrogen). DNA was visualized by staining with TO-PRO-3 iodide (Invitrogen). Labeled cells were observed with confocal laser scanning microscopy (LSM5 Exciter; Carl Zeiss) using argon laser (488 nm) and He/Ne laser (546 and 633 mm) lines.

**ChIP, RT-PCR, siRNA-mediated knockdown, and western blot assays.**
These experiments were carried out essentially as described previously (20). siRNA targeted for CAF-1 p150 was commercially purchased (Stealth siRNA; Invitrogen). Primers for CAF-1 p150 mRNA are as follows: 5’-GGAGCAGGACAGTTGGAGTG-3’ and 5’-GACGAATGGCTGAGTACAGA-3’. Other primers for ChIP and RT-PCR assays were described elsewhere (20). In all the experiments by quantitative PCR (qPCR), mean values with SD were obtained from three independent experiments.
RESULTS

Cellular histones are bound with viral chromatin both in early and late phases of infection.

Previously, we reported that in early phases of infection (before the onset of viral DNA replication), viral chromatin is composed of both viral core protein VII and cellular histones and this “chimeric” chromatin functions as template for transcription (20). To examine whether histones are also bound with viral chromatin after the onset of viral DNA replication, we performed ChIP assays using antibodies against histones and protein VII (Fig. 1). The viral DNA replication starts around 8 hpi (hours post infection) in our condition (17, 20). In order to reveal the viral chromatin state during/right after viral DNA replication, HeLa cells infected at an MOI (multiplicity of infection) of 100 were harvested at 6 and 12 hpi for ChIP assays. We chose five regions for ChIP assays, four viral genome regions (E1A pro, MLP, Hexon, E4 pro, Fig. 1A) and one cellular genomic region (ribosomal RNA gene, rDNA) as a control (20). In this condition, the amount of viral DNA was increased by ~20 fold through viral DNA replication (Fig. 1B). At 6 hpi, all core histones are bound with viral chromatin, but at the low binding level compared with cellular chromatin (Fig. 1C, histone H3, H4, and H2A-H2B). This was in good agreement with our previous observation (20). At 12 hpi, core histones were also found to be associated with viral chromatin. The binding level of histones on viral chromatin at 12 hpi was more than those at 6 hpi, but slightly less than those on cellular chromatin. This is consistent with the previous report of electron microscopic analyses showing that viral genome DNA purified from
infected cells at late phases of infection has the nucleosome-like particles, which are less dense compared with cellular nucleosome arrays (3). In contrast, the binding level of protein VII was drastically decreased after the onset of viral DNA replication (Fig. 1C, protein VII), suggesting that newly synthesized viral DNA is mainly associated with cellular histones. We do not exclude the possibility that protein VII remains associated with small population of viral chromatin because the binding level of protein VII on viral chromatin was still higher than that on cellular chromatin even at 12 hpi. The ratio among core histones bound on viral chromatin was almost the same as that on cellular chromatin both at 6 (data not shown, 20) and 12 hpi (Fig. 1D), indicating that viral chromatin contains the canonical nucleosome structure.

CAF-1 and PCNA are not involved in the histone deposition onto newly synthesized viral DNA.

It is known that during DNA replication of the cellular genome and some DNA virus genomes, histones are deposited by CAF-1, a replication-dependent histone chaperone (41, 43). CAF-1 is associated with the DNA replication machinery through the interaction with PCNA, thereby enabling replication-coupled deposition of histone H3-H4 complexes (40). Thus, it is worthwhile to examine whether CAF-1 and PCNA are also involved in the histone deposition onto newly synthesized Ad DNA, although there is no definitive evidence that those two are involved in the Ad DNA replication. To test this, we first performed IF assays using cells stably expressing HA-tagged PCNA (HA-PCNA) to examine the relationship among viral DNA replication, PCNA,
and CAF-1 (Fig. 2A). Using antibody against DBP, Ad ssDNA binding protein involved in viral DNA replication, the place for viral DNA replication designated “viral DNA replication foci” (hereafter referred to as “VDRF”) can be visualized (31). HeLa cells or cells stably HA-PCNA were infected at an MOI of 50, and at 18 hpi subjected to IF assays using anti-DBP and anti-HA antibodies. In mock-infected cells, HA-PCNA was localized throughout the nucleus and shows the punctate localization in some cell population, as reported previously (29). At 18 hpi, VDRF was observed as a donut-like signal using anti-DBP antibody, and we found that HA-PCNA showing punctate localization was accumulated inside VDRF. We also observed the similar localization pattern of CAF-1 inside VDRF (see below in Fig. 4A). These results suggest that PCNA and CAF-1 are recruited together into the site of viral DNA replication.

Next, to investigate a role of CAF-1 in the histone deposition onto viral DNA, siRNA-mediated knockdown of CAF-1 p150, a largest subunit of CAF-1 complex, was carried out (Fig. 2B, C, and D). HeLa cells were treated with control siRNA (siCont) or siRNA for CAF-1 p150 (siCAF-1), infected with HAdV5 at an MOI of 100, and harvested at 6 and 12 hpi. First, we examined the knockdown efficiency of CAF-1 p150 by RT-qPCR assays (Fig. 2B). The mRNA level of CAF-1 p150 in siRNA-treated cells was about 20% of those in control cells, although at 12 hpi the level was slightly increased possibly due to S-phase like environment induced by Ad infection (30). In contrast, the mRNA level of GAPDH was almost unaffected by siRNA treatment and Ad infection. In this condition, the binding level of histone H3
on viral chromatin was examined by ChIP assays (Fig. 2C). The binding level of H3 on viral chromatin was not decreased by CAF-1 knockdown, and rather slightly increased (but not statistically significant) at 12 hpi (Fig. 2C, E1A pro and MLP). It is noted that the binding level of H3 on cellular chromatin was also unaffected by CAF-1 knockdown (Fig. 2C, rDNA, see Discussion). In addition, we could not observe any effect of CAF-1 knockdown on viral DNA replication levels (Fig. 2D). Taken together, these results suggest that it is not likely that CAF-1 is involved in the histone deposition onto viral chromatin during viral DNA replication, although CAF-1 is accumulated at VDRF together with PCNA.

Replication-independent histone H3.3 is selectively incorporated into viral chromatin.

It is known that among histone H3 variants, histone H3.1 and H3.2 are deposited onto DNA by CAF-1 during DNA replication, while H3.3 is deposited independently of DNA replication (11, 14). If CAF-1 is not involved in the histone deposition during viral DNA replication, histone H3.3 rather than H3.1 and H3.2 could be incorporated into newly synthesized viral DNA. Therefore, to examine this possibility, we performed ChIP assays using FLAG-tagged histone H3.2 and H3.3 (Fig. 3). HeLa cells were transfected with expression vectors for H3.2 and H3.3 and at 24 hpt (hours post transfection) infected at an MOI of 100. We first studied using cells at early phases of infection (before the onset of viral DNA replication) (Fig. 3A). Infected cells were harvested at 2 and 6 hpi, and at 10 hpi in presence of HU to block
viral DNA replication (20), and subjected to ChIP assays with anti-FLAG antibody. As shown in Figure 3A, the exclusive binding of H3.3 on viral chromatin was observed at all the regions we tested, with a gradual increment as infection proceeded. This is consistent with the recent report on helper-dependent Ad vector (HdAd) indicating that histone H3.3 are specifically deposited onto HdAd DNA by HIRA, an H3.3-specific histone chaperone (34). Since HdAd alone does not undergo its DNA replication, the chromatin state of HdAd may reflect that of wildtype Ad in early phases of infection (13, 34).

Next we performed ChIP assays at 12 hpi to examine which H3 variant is deposited onto newly synthesized viral DNA (Fig. 3B). We found that histone H3.3 but not H3.2 was associated with viral chromatin at this time point, as observed in early phases of infection. The expression levels of both H3 variants were comparable (Fig. 3C), and both variants were associated with cellular chromatin with a similar binding level (Fig. 3A and B, rDNA). These strongly suggest that this result was not due to some technical issues. Further, we obtained the same results by using FLAG-tagged histone H3.1 instead of H3.2 (Fig. 3D and E). Thus, these results suggest that replication-independent histone variant H3.3 is selectively deposited onto not only incoming but also newly synthesized viral DNA in infected cells.

Histones but not transcription factor USF1 are excluded from the site of viral DNA replication.

To further investigate the histone fluctuation during viral DNA replication, we
performed IF analyses using HeLa cells stably expressing EGFP-tagged histone H3.3 (Fig. 4A). Cells were infected at an MOI of 50, and at 18 hpi subjected to IF assays using anti-DBP antibody (Fig. 4A, upper panels). VDRF was observed at 18 hpi as described above, and H3.3-EGFP was found to be excluded from VDRF. Similar results were obtained by using cells stably expressing EGFP-tagged histone H3.2 (Fig. 4B). This was due to neither exogenous expression nor EGFP tag of H3 variants as we observed the similar exclusion of the endogenous histone using anti-histone H2A antibody (Fig. 4C). This localization pattern was specific for late phases of infection since the localization of EGFP-tagged H3 variants was not changed in early phases of infection (Fig. 4D). We also performed IF analyses using anti-CAF-1 p150 antibody and observed that CAF-1 was accumulated at “histone-less” region, that is, VDRF (Fig. 4A, lower panels), as was observed with HA-PCNA (Fig. 2A). In summary, these results indicated that histones are localized reciprocally to VDRF (and CAF-1/PCNA) in late phases of infection.

To gain more insights into the accessibility of other nuclear proteins to VDRF, we again performed IF analyses (Fig. 5). First, IF analyses were carried out using antibody against HIRA, an H3.3-specific histone chaperone, and it was observed that the localization of HIRA was not drastically changed in both early and late phases of infection (Fig. 5A). We also performed IF analyses using cells transiently transfected with expression vectors for HA-tagged DEK and USF1 (Fig. 5B and C). DEK is a cellular chromatin protein with potential histone chaperone activity (37), and USF1 is an E-box-binding transcription factor and reported to bind to and regulate transcription
from the MLP region (46). HeLa cells were transfected with expression vectors and at 24 hpt subjected to western blot analyses (Fig. 5B) or infected at an MOI of 50. At 18 hpi, localization of DBP, HA-DEK, and HA-USF1 was visualized by IF analyses using anti-DBP and anti-HA antibodies (Fig. 5C). In mock-infected cells, both HA-tagged proteins showed nuclear localization, and in the case of HA-DEK, a strong signal was observed at the nuclear periphery. At 18 hpi, HA-DEK appeared to be excluded from VDRF (Fig. 5C, HA-DEK). However, in contrast to HA-DEK, we observed that HA-USF1 could be localized inside VDRF (Fig. 5C, HA-USF1). Taken together, our IF analyses suggest that VDRF may allow selective access of cellular nuclear proteins, and at least one of transcription factors, USF1, is able to access the inside of VDRF.

Oligomerization of DBP is critical for the histone exclusion from VDRF.

To investigate the mechanism of the histone exclusion from VDRF, we hypothesized that DBP may play a role, since an abundant amount of DBP is associated with Ad DNA in VDRF. The crystal structure of DBP revealed that this protein has a 17 aa extension at its C-terminus (see Fig. 6A), and this C-terminal “arm” hooks onto the next DBP molecule, resulting in oligomerization of DBP (47). It was also reported that oligomerization of DBP mediated by the C-terminal “arm” enables ATP-independent unwinding of dsDNA, and thus full-length DBP, but not the deletion mutant that lacks the C-terminal “arm” (DBPΔC), could support viral DNA replication in vitro (9). Therefore, to examine a role of DBP and its oligomerization on the histone localization, HeLa cells expressing histone H3.3-EGFP were transfected with
the expression vectors for HA-tagged full-length DBP or DBPΔC and at 36 hpt subjected to western blotting and IF assays using anti-HA and anti-DBP antibodies (Fig. 6B and C). The expression levels of both DBP proteins were almost the same as indicated by western blotting (Fig. 6B). In IF analyses, we observed that full-length DBP forms the foci like VDRF in the absence of any viral proteins/DNA, and histone H3.3-EGFP was excluded from these foci as observed in infected cells (Fig. 6C, HA-DBP). In sharp contrast, DBPΔC was localized throughout the nucleus and did not form such foci (Fig. 6C, HA-DBPΔC). Taken together, these results suggest that the oligomerization of DBP has a critical role in the histone exclusion from VDRF.
DISCUSSION

In this study we showed that replication-independent histone variant H3.3 is deposited onto both incoming and newly synthesized Ad DNA (Fig. 3). These results, together with the results from knockdown experiments of CAF-1 (Fig. 2) and microscopic analyses (Figs. 4, 5, and 6), indicated that the histone deposition onto the replicated virus genome is most likely uncoupled with viral DNA replication. Based on these results, together with the previous our work (20), we hypothesize a model with respect to the fluctuation of viral chromatin structure during infection cycle (Fig. 7).

In virions, viral DNA is tightly packed with viral core proteins (13). After the entry to the cell, cellular histones are incorporated into incoming viral DNA-protein VII complexes in the nucleus, and viral chromatin composed of both protein VII and histones functions as template for viral early gene expression (20). In this process, histone H3.3 is specifically deposited onto viral DNA, possibly by a histone chaperone HIRA (34). As infection proceeds and then viral DNA replication is initiated, oligomerization of DBP establishes the “histone-free” environment for viral DNA replication. Newly synthesized viral DNA is then associated with histone H3.3 in a replication-uncoupling fashion and might be acting as template for viral late gene expression outside VDRF (31). In later phases of infection (24- hpi), both histones and newly synthesized core proteins VII and V are associated with viral DNA, which likely reflects the processes during the progeny virion assembly (35). Since histones are not included in virions, histones must be removed and replaced with newly synthesized core proteins for progeny virions. Although the packaging mechanism of
progeny viral DNA during virion assembly remains unclear, we have reported the involvement of a nucleolar protein B23/nucleophosmin in the regulation of viral chromatin structure during progeny virion assembly (35, 36).

The mechanistic details of the histone deposition after viral DNA replication remain still unclear. First, what factor(s) is involved in the histone deposition at late phases of infection? HIRA is a potential candidate for this process, likewise in early phases of infection (34). However, we did not perform knockdown experiments for HIRA, since even if we could observe some effect of HIRA knockdown on viral chromatin in late phases of infection, we could hardly distinguish whether the knockdown directly affects the chromatin structure of progeny viral DNA or the effect is derived indirectly from earlier events on incoming viral chromatin. IF analyses showed that the localization of HIRA was not drastically changed during infection cycle (Fig. 5A). Recent reports indicated that Daxx, a component of PML bodies, is also an H3.3-specific histone chaperone (10, 21). However, Daxx seems not to function the H3.3 deposition onto viral DNA because during Ad infection, some components of PML bodies including Daxx are re-localized by viral protein E4orf3, possibly for inactivation of the components (6, 42). Indeed, it is shown that Daxx-mediated antiviral response is antagonized by E4orf3 (48). It is also revealed that Daxx negatively functions and undergoes E1B-55K- and proteasome-dependent degradation during Ad infection (39). Furthermore, most recently Schreiner et al. reported that during/immediately after nuclear import of incoming virus genome, protein VI, one of capsid proteins, binds to and counteracts Daxx, at least partly by displacing it from PML bodies (38). These
reports strongly suggest that Daxx is inactivated entirely throughout infection cycle by viral proteins. DEK is also recently reported as a chaperone for histone H3.3 in *Drosophila* cells (37), but it is unknown whether human DEK also functions as a variant-specific chaperone or not. Our IF analyses indicated that exogenously expressed DEK is excluded from VDRF (Fig. 5B and C). Further studies are needed to elucidate the functions of these factors in late phases of infection.

In this study, we could not observe a role of CAF-1 in the histone deposition onto viral DNA, while the accumulation of CAF-1 at VDRF was observed (Figs. 2 and 4A). CAF-1 knockdown did not affect the binding levels of histone H3 on viral chromatin (Fig. 2C). Although we could not exclude the possibility that the knockdown efficiency of CAF-1 is not sufficient in the condition employed here, we concluded that the function of CAF-1 is largely inhibited under our condition: We observed that siCAF-1-treated cells exhibit aberrant cell shapes (data not show) and the knockdown affects viral gene expression (see below). Second, histone H3.3 is selectively incorporated into viral chromatin (Fig. 3), while CAF-1 generally functions as a chaperone for H3.1 and H3.2 (43). Third, although CAF-1 is reported to be able to be associated with H3.3 under some specific conditions (10, 21), we could not observe any interaction between CAF-1 and H3.3 during Ad infection, at least, in our experimental conditions (data not shown). In addition to their roles during DNA replication, CAF-1 and PCNA are also reported to be involved in the DNA damage response pathway (29). Carson *et al.* reported that the DNA damage response pathway is only partially activated during Ad infection, and some related factors, such as ATRIP
and TopBP1, are accumulated at VDRF (5). Therefore, CAF-1 (and PCNA) might localize at VDRF in the course of this limited DNA damage response. Recently, it was reported that FACD2, one of factors involved in the DNA damage response, is accumulated at VDRF, and loss of this protein results in less expression of viral late, but not early, genes (8). Similarly, we observed that CAF-1 knockdown affects mRNA levels of viral late genes without any effect on viral DNA replication (unpublished observation). Thus, factors related to the DNA damage response such as FANCD2 and CAF-1 might be required for viral late gene expression, although the underlying mechanisms are unknown. In our condition, CAF-1 knockdown did not affect the binding level of histone H3 on cellular chromatin (Fig. 2C, rDNA). This is consistent with the report that loss of CAF-1 impairs replication-coupled deposition of histones but the formation of nucleosome arrays on genomic DNA is still observed in the absence of CAF-1 (44). In addition, a recent report demonstrated that a defect of histone H3.1 deposition by CAF-1 depletion could be rescued by HIRA-mediated H3.3 deposition (33). Thus, in the case of cellular chromatin, alternative histone deposition pathway(s) could rescue the loss of CAF-1 function.

It remains to be clarified what is the biological/virological significance of histone deposition uncoupled with viral DNA replication. On cellular chromatin, a replication-dependent histone chaperone CAF-1 is associated with the DNA replication machinery and deposits histone H3.1-H4 (and H3.2-H4) complexes during DNA replication (14, 40, 43). This DNA replication-coupled system of the histone deposition is thought to be also utilized by some DNA viruses. For instance, DNA
replication of SV40 is largely depending on the cellular replication machinery, and indeed CAF-1 was originally identified using cell-free DNA replication systems of SV40 (41). In cytomegalovirus infection, it is reported that cellular histones, CAF-1, and PCNA are accumulated at viral replication compartments (25). In the case of herpes simplex virus type 1, it is shown that histone H3.3 is first deposited onto incoming viral DNA by HIRA, and then H3.1 becomes associated with viral DNA accompanied with viral DNA replication (28). It is suggested that this functional link between DNA replication and the histone deposition enables to transfer “epigenetic memory” such as histone modifications to the daughter DNA strands (43). Thus, some DNA viruses might take advantage of this system for late gene expression, which generally occurs after viral DNA replication. On the other hand, Ad seems to utilize another strategy, that is, the uncoupling mechanism, as shown here. Like other DNA viruses, Ad late genes are expressed only after the onset of viral DNA replication. Thomas and Mathews demonstrated that Ad late gene expression requires its DNA replication in cis (45), although the molecular mechanism remains to be determined. This report leads us to hypothesize that the regulation of viral chromatin structure during DNA replication could be an important process for the late gene expression. In general, histone/nucleosome structure on DNA restricts the access of trans-acting factors, such as transcription factors. In this view, DBP is an attractive candidate of the key regulatory factor for DNA replication-dependent expression of viral late genes. By oligomerization, DBP is able to not only support viral DNA replication, but also establish the “histone-free” environment, which could be an opportunity window for
transcription factors to access the viral DNA for the activation of viral late genes. Our IF analyses showed that transcription factor USF1, which binds to the MLP region after viral DNA replication (46), are not excluded from VDRF (Fig. 5B and C), supporting this notion. Further, this is in agreement with the report that DBP enhances the binding of USF1 to the MLP region in vitro (51). Overall, we speculate that uncoupling of the histone deposition with viral DNA replication is mediated by DBP oligomerization, at least partly, and plays a role in DNA replication-dependent activation of viral late gene expression.

The expression of certain cellular genes, such as HoxB gene, is shown to require DNA replication (12). However, the regulation mechanism of “DNA replication-dependent gene expression” remains to be determined. As Ad has late genes, the expression of which are DNA replication-dependent (45), this virus could be a good model for the analyses of such regulations. Therefore, this study might give a clue for understanding the functional relationship between DNA replication and transcription on cellular and/or viral chromatin.
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FIGURE LEGENDS

FIG. 1. Viral chromatin structure in early and late phases of infection.  (A) The structure of Ad genome. Arrows represent promoters of viral genes. Target regions for ChIP assays are indicated by arrowheads.  (B) The amounts of viral DNA. HeLa cells were infected with HAdV5 at an MOI of 100, and DNA samples were purified from infected cells at 6 and 12 hpi. The amount of viral DNA was quantitatively measured by qPCR using primers for the E1A promoter region. The amount of viral DNA was graphed as the ratio relative to that at 6 hpi. (C) ChIP assays. HeLa cells were infected with HAdV5 at an MOI of 100 and subjected to ChIP assays using infected cells at 6 and 12 hpi. Immunoprecipitation was carried out using indicated antibodies and anti-FLAG antibody (as a negative control). The obtained DNAs were quantitatively measured by qPCR using indicated primer sets. The binding levels of each protein were calculated as relative enrichment against that obtained in a negative control (anti-FLAG antibody). (D) The binding levels of core histones. Base on the results of ChIP assays shown in (C), the binding level of histone H4 and H2A-H2B was normalized by that of histone H3.

FIG. 2. Localization and role of PCNA and CAF-1 during viral DNA replication. (A) IF assays. HeLa cells and cells stably expressing HA-PCNA grown on cover slips were mock-infected or infected with HAdV5 at an MOI of 50. At 18 hpi the localization patterns of HA-PCNA and DBP were analyzed by IF using anti-HA and anti-DBP antibodies. DNA was visualized by TO-PRO-3 iodide staining. Merged
images are also indicated. Higher-magnified images of the regions marked by squares are shown below. (B) RT-qPCR assays. HeLa cells were treated with siControl or siCAF-1 and then either mock-infected or infected with HAdV5 at an MOI of 100, and total RNAs were purified at 6 and 12 hpi. cDNAs were synthesized with reverse transcription and subjected to qPCR using primer sets for \textit{CAF-1 p150} and \textit{GAPDH} mRNAs. The mRNA levels relative to those in control cells at 12 hpi were graphed. (C) ChIP assays. siRNA-treated cells were infected with HAdV5 at an MOI of 100 and at 6 and 12 hpi subjected to ChIP assays using anti-histone H3 and anti-FLAG antibodies as described above. (D) Relative amounts of viral DNA. Viral DNA was purified from lysates for ChIP assays in (C) and subjected to qPCR using primer set for the E1A promoter. The DNA amounts at 12 hpi relative to those at 6 hpi were shown.

\textbf{FIG. 3. Incorporation of histone H3 variants into viral chromatin.} (A, B) ChIP assays with FLAG-tagged histone H3 variants. HeLa cells were transfected with pcDNA3 empty vector, pcDNA3-H3.2-FLAG, or pcDNA3-H3.3-FLAG, and at 24 hpt (hours post transfection) infected with HAdV5 at an MOI of 100. At 2, 6, and 10 hpi (A) or 12 hpi (B), ChIP assays were carried out using anti-FLAG and anti-HA (as a negative control) antibodies, as described above. Note that in the case of 10 hpi, HU was added to block viral DNA replication. The results were graphed as relative enrichment as described above. (C) Western blot analyses. At 24 hpt, lysates were prepared from cells transfected with pcDNA3 empty (lane 1), pcDNA3-H3.2-FLAG
(lane 2), and pcDNA3-H3.3-FLAG (lane 3) and subjected to 15% SDS-PAGE, followed by western blot analyses using anti-FLAG (upper panel) and anti-β-actin (lower panel) antibodies. (D) Western blot analyses. HeLa cells were transfected with pcDNA3 empty vector (lane 1), pcDNA3-H3.1-FLAG (Lane 2), or pcDNA3-H3.3-FLAG (lane 3), and at 24 hpt lysates were prepared and subjected to 15% SDS-PAGE, followed by western blot analyses using anti-FLAG (upper panel) and anti-β-actin (lower panel) antibodies. (E) ChIP assays. HeLa cells transfected with pcDNA3 empty vector (lanes 2, 3, 9, and 10), pcDNA3-H3.1-FLAG (lanes 4, 5, 11, and 12), or pcDNA3-H3.3-FLAG (lanes 6, 7, 13, and 14) were infected with HAdV5 at an MOI of 100. At 10 hpi (left panels, lanes 1-7) or 12 hpi (right panels, lanes 8-14), ChIP assays were carried out using anti-FLAG (lanes 3, 5, 7, 10, 12, and 14) and anti-HA (as a negative control, lanes 2, 4, 6, 9, 11, and 13) antibodies. In the case of 10 hpi, HU was added to block viral DNA replication. The immunoprecipitated DNAs were amplified by semi-quantitative PCR using the indicated primer sets. PCR products were separated on a 7% polyacrylamide gel and visualized by staining with EtBr. Input DNAs (lanes 1 and 8) were purified from 0.5% of lysates of cells transfected with the empty vector.

**FIG. 4. Localization of histones in late phases of infection. (A) IF analyses using cells stably expressing histone H3.3-EGFP.** HeLa cells stably expressing histone H3.3-EGFP grown on cover slips were mock-infected or infected at an MOI of 50, and at 18 hpi subjected to IF assays using anti-DBP (upper panels) and anti-CAF-1 p150
(lower panels) antibodies, as described above. Higher-magnified images of the regions marked by squares are shown below. (B) Localization of histone H3.2 in late phases of infection. HeLa cells stably expressing histone H3.2-EGFP were mock-infected or infected with HAdV5 at an MOI of 50, and at 18 hpi subjected to IF analyses using anti-DBP antibody. Higher-magnified images of the regions marked by squares are shown. (C) Localization of endogenous histone H2A in late phases of infection. HeLa cells were mock-infected or infected with HAdV5 at an MOI of 50, and at 18 hpi subjected to IF assays using anti-histone H2A and anti-DBP antibodies. Higher-magnified images of the regions marked by squares are shown below. (D) Histone localization in early phases of infection. HeLa cells stably expressing histone H3.2-EGFP and H3.3-EGFP were mock-infected or infected with HAdV5 at an MOI of 250, and at 4 hpi subjected to IF analyses using anti-protein VII antibody.

FIG. 5. Localization of nuclear proteins in late phases of infection. (A) IF analyses using anti-HIRA antibody. HeLa cells were mock-infected or infected with HAdV5 at an MOI of 250 (for 4 hpi) or 50 (for 18 hpi) and subjected to IF analyses using anti-protein VII and anti-HIRA antibodies. (B) Western blot analyses. Lysates were prepared from HeLa cells transfected with pCHA-puro empty vector (lane 1), pCHA-DEK (Lane 2), or pCHA-puro-USF1 (lane 3) at 24 hpt, and subjected to 10% SDS-PAGE, followed by western blot analyses using anti-HA (upper panel) and anti-β-actin (lower panel) antibodies. (C) Localization of HA-DEK and HA-USF1. HeLa cells were transfected pCHA-puro empty vector, pCHA-DEK, or
pCHA-puro-USF1. At 24 hpt, cells were mock-infected or infected with HAdV5 at an
MOI of 50, and at 18 hpi subjected to IF assays using anti-HA and anti-DBP antibodies.

**FIG. 6. Role of DBP oligomerization on histone localization.** (A) Schematic
diagrams of full-length DBP and C-terminally deleted mutant DBPΔC. DBP of
HAdV5 consists of 529 aa, and the C-terminal 17 aa (513-529) functions as an “arm”
for oligomerization. DBPΔC lacks the C-terminal 17 aa. (B) Western blot analyses.
HeLa cells stably expressing histone H3.3-EGFP were transfected with pCHA-puro
empty vector (lane 1), pCHA-puro-DBP (lane 2), or pCHA-puro-DBPΔC (lane 3), and
lysates prepared at 36 hpt were subjected to 10% SDS-PAGE, followed by western blot
analyses using anti-HA (top), anti-DBP (middle), and anti-β-actin (bottom panel)
antibodies. (C) IF analyses. At 36 hpt, cells as described in (B) grown on cover
slips were subjected to IF analyses using anti-HA (left panels) and anti-DBP (right
panels) antibodies as described above. Higher-magnified images of the regions
marked by squares are shown below.

**FIG. 7. A hypothetical model for viral chromatin structure during infection cycle.**
For detail, see Discussion section.
Figure 1 Komatsu et al.

A

E1A pro → E1B pro → IX pro → MLP → Hexon → E3 pro → 2 kbp → E2late pro → E2early pro → E4 pro

B

Viral genome

Relative amount

6 hpi 12 hpi

D

Histone ratio

[12 hpi]

H3

H4

H2A

H2B

E1A pro

MLP

Hexon

E4 pro

rDNA

C

Histone H3

Histone H4

Histone H2A-H2B

Protein VII

Relative enrichment

6 hpi 12 hpi

E1A pro

MLP

Hexon

E4 pro

rDNA

E1A pro

MLP

Hexon

E4 pro

rDNA

E1A pro

MLP

Hexon

E4 pro

rDNA
Figure 2  Komatsu et al.

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Viral genome

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Figure 4  Komatsu et al.

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Figure 6  Komatsu et al.

A

HA tag

HA-DBP

C-terminal “arm”

1 512 529

HA-DBPΔC

B

Empty HA-DBP HA-DBPΔC

HA DBP β-actin

C

TO-PRO-3 HA H3.3-EGFP TO-PRO-3 DBP H3.3-EGFP

Empty HA-DBP HA-DBPΔC

HA-DBP HA-DBPΔC

HA-DBP ΔC

Empty HA-DBP HA-DBPΔC

HA-DBP HA-DBPΔC

HA-DBP HA-DBPΔC
Figure 7  Komatsu et al.

i) Virion

H3.3
HIRA

“Chimeric” chromatin

ii) Entry of nucleus

“VII” chromatin

iii) Early phases

iv) DNA replication

“Histone-free” replication foci formed by DBP oligomerization

v) Late phases

“Nucleosomal” chromatin