

Adenovirus Protein VII Functions throughout Early Phase and Interacts with Cellular Proteins SET and pp32

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Adenovirus protein VII is the major component of the viral nucleoprotein core. It is a highly basic nonspecific DNA-binding protein that condenses viral DNA inside the capsid. We have investigated the fate and function of protein VII during infection. “Input” protein VII persisted in the nucleus throughout early phase and the beginning of DNA replication. Chromatin immunoprecipitation revealed that input protein VII remained associated with viral DNA during this period. Two cellular proteins, SET and pp32, also associated with viral DNA during early phase. They are components of two multiprotein complexes, the SET and INHAT complexes, implicated in chromatin-related activities. Protein VII associated with SET and pp32 in vitro and distinct domains of protein VII were responsible for binding to the two proteins. Interestingly, protein VII was found in novel nuclear dot structures as visualized by immunofluorescence. The dots likely represent individual infectious genomes in association with protein VII. They appeared within 30 min after infection and localized in the nucleus with a peak of intensity between 4 and 10 h postinfection. After this, their intensity decreased and they disappeared between 16 and 24 h postinfection. Interestingly, disappearance of the dots required ongoing RNA synthesis but not DNA synthesis. Taken together these data indicate that protein VII has an ongoing role during early phase and the beginning of DNA replication.

The adenovirus nucleoprotein core consists of double-stranded genomic DNA, the highly basic viral proteins VII, V, and μ (mu), as well as protein IVa2 and the 55-kDa terminal protein (1, 5, 19, 25, 31, 33, 34, 42). Protein VII is the major protein component of the core with an estimated 1,070 copies present per virion (9) and is primarily responsible for establishing viral chromatin structure. It can potentially condense DNA in vitro and in vivo and also repress transcription (3, 6, 21, 35). This is consistent with the highly condensed configuration of viral chromatin found within the virion. When delivered to the nucleus, the chromatin is silent prior to stimulation by the viral transcriptional activator E1A (13).

We and others have reported that protein VII from infectious viral particles enters the nucleus along with viral DNA and remains associated with it, suggesting that the protein VII-DNA complex is the substrate for transcriptional activation by E1A during early phase (8, 15, 18, 21). Moreover we found that protein VII can associate with E1A protein in vitro (21).

In this report we have studied the fate and function of protein VII during the early phase of infection. We demonstrate that virus-derived “input” protein VII persists in the nucleus throughout early phase and the beginning of viral DNA replication, suggesting that it has an ongoing role in gene regulation and perhaps DNA replication. During this period protein VII is found in discrete nuclear dot structures and viral DNA continues to associate with protein VII.

We have also investigated the association of protein VII with cellular proteins SET and pp32. SET, also known as TAF1 β , has recently been shown to associate with adenovirus chromatin during infection (18). SET is homologous to evolutionarily conserved nucleosome assembly proteins (NAPs) found in yeast and higher eukaryotes (2, 14, 24, 27, 39, 41). Moreover, SET satisfies the requirements of a NAP experimentally: when added to reactions containing histones and relaxed circular plasmid, SET mediates the assembly of histones and DNA into a supercoiled structure (2, 14, 23, 28). Interestingly, when SET is incubated with purified adenovirus core, some structural rearrangement occurs. SET can stimulate in vitro DNA replication and transcription reactions containing adenovirus core as the template and can also stimulate transcription from nucleosomal templates composed of histones and DNA (28). It also has been shown to trigger decondensation of *Xenopus* sperm chromatin (26, 28).

SET has been found by several groups to be a component of at least two distinct multisubunit complexes termed SET and INHAT (2, 4, 10–12, 36, 37). We find that SET and pp32, components of the SET and INHAT complexes, associate with viral chromatin in vivo and bind to protein VII specifically in vitro. These data suggest that SET and pp32 associate with viral chromatin as components of the SET and INHAT complexes and, along with protein VII, likely have important roles in viral chromatin function during early phase.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% newborn calf serum (NCS), penicillin, and streptomycin (all from GIBCO). Phenotypically wild-type adenovirus type 5 *dl309* (22) was propagated in HeLa cells to produce virus stocks.

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Plasmids. pGSTe1A289 and pGSTVII were described previously (21). pGST CAS YXXP, a gift from Amy Bouton, contains the YXXP domain of CAS in pGEX-2TK (7). To produce pGSTVII, pGSTVII 1–57, pGSTVII 45–94, pGSTVII 94–148, and pGSTVII 149–174, inserts were generated by PCR with adenovirus type 5 DNA. Primers contained 5' BamHI and 3' EcoRI sites and the PCR products were cloned into pGEX3X (Amersham Biosciences).

Western blotting. Western blot analysis was performed essentially as described previously (17). Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with antibodies directed against protein VII (21), E1A (16), the 72-kDa DNA-binding protein (DBP) (32), SET (2), pp32 (2), or Ku86 (a gift from Gary Kupfer). Anti-rabbit horseradish peroxidase (Amersham Pharmacia) or anti-mouse horseradish peroxidase (Amersham Pharmacia) was used as secondary antibody and blots were visualized by enhanced chemiluminescence.

Production of GST fusion proteins. Plasmids were transformed into *Escherichia coli* BL21 CodonPlus RP (Stratagene). Cultures (3 ml) were grown overnight at 37°C in Luria-Bertani broth with ampicillin (100 µg/ml) and chloramphenicol (50 µg/ml). They were expanded in 500 ml of Luria-Bertani broth to an optical density at 600 nm of 0.2, the temperature was then reduced to 30°C and IPTG (isopropyl-β-D-thiogalactopyranoside) (Gibco BRL) was added to a final concentration of 0.25 mM. After 3 h, the cells were pelleted by centrifugation and resuspended in binding buffer (540 mM NaCl, 2.7 mM KCl, 10.15 mM Na₂HPO₄, 1.75 mM KH₂PO₄, 10 mM MgCl₂, 1% [vol/vol] Triton X-100, 50 µg of DNase I [Sigma] per ml, 30 µg of phenylmethylsulfonyl fluoride/ml, 1 µg of aprotinin/ml, 1 µg of pepstatin A/ml, 10 µg of leupeptin/ml, pH 7.4). Lysis was by French press at 18,000 lb/in² at 4°C, and the lysate was cleared by centrifugation in a Sorvall GSA rotor (5,000 rpm, 15 min, 4°C). To purify GST fusion proteins, 1 ml of a 1:1 slurry of glutathione agarose beads (Sigma) in phosphate-buffered saline (PBS) was added to 10 ml of cleared lysate at 1 mg of protein/ml and incubated for 4 h at 4°C. The bead-bound proteins were washed three times in 15 ml of binding buffer.

GST pull-down assays. To make lysates, HeLa cells were scraped from plates and placed in lysis/binding buffer (50 mM HEPES, 250 mM NaCl, 10 mM MgCl₂, 1 mM CaCl₂, 0.2 mM ZnCl₂, 0.1% Nonidet P-40, 50 µg of DNase I [Sigma] per ml, 30 µg of phenylmethylsulfonyl fluoride/ml, 1 µg of aprotinin/ml, 1 µg of pepstatin A/ml, 10 µg of leupeptin/ml, pH 7.8) on ice for 90 min. Lysates (250 µl; approximately 1.5 mg of total protein/ml) were then cleared by centrifugation in a Sorvall MC-12V at 12,000 rpm and 4°C for 20 min. Lysate was added to 5 µg of bead-bound GST fusion proteins (1:1 slurry in PBS) in 300 µl of lysis/binding buffer and incubated 2 h at 4°C while rotating, and the beads were washed four times in 1.4 ml of binding buffer. Bound proteins were separated by SDS-PAGE and analyzed by Western blotting.

Chromatin immunoprecipitation. HeLa cells (4×10^7) were infected with adenovirus type 5 *d*309 at a multiplicity of infection (MOI) of 50 and incubated for 4 h. Cells were recovered, washed, and resuspended in cross-linking buffer (5 mM HEPES, pH 8.0, 50 µM EGTA, 10 mM NaCl, 100 µM EDTA, 1% formaldehyde, 1% methanol) for 30 min at room temperature. Glycine (125 mM) was added to stop the reaction, and the cells were pelleted as described above. The cells were washed once in PBS, resuspended in radioimmunoprecipitation buffer, and sonicated twice for 15 s at 4°C. The lysate was then cleared by centrifugation (13,000 rpm in a Sorvall MC12V microcentrifuge, 10 min, 4°C). Antibody or control antiserum was prebound to protein A-Sepharose beads (Sigma), washed two times in PBS, and incubated overnight at 4°C with nuclear lysate. Beads were washed five times with PBS, NaCl was added to a final concentration of 200 mM, and the beads were incubated for 6 h at 65°C to reverse cross-linking. Proteinase K (50 µg) and RNase A (5 µg) were added and the sample was incubated for 2 h at 37°C followed by extraction two times with phenol-chloroform-isoamyl alcohol. DNA was ethanol precipitated overnight at –20°C, air dried, and resuspended in water. PCR was performed using Deep Vent polymerase (New England Biolabs) under standard conditions for 35 cycles of 1 min at 97°C, 1.5 min at 55°C, and 2 min at 72°C, with adenovirus-specific primers (sequences available on request).

Immunofluorescence. HeLa cells were grown on 22-by-22-mm glass coverslips (Fisher) at 37°C in DMEM supplemented with 10% NCS for 24 h prior to infection. Cells were infected for 1 h as previously described (21). Cells were then washed two times with 37°C PBS to remove unadsorbed virus, followed by incubation at 37°C in DMEM–10% NCS for the indicated times. After incubation, the cells were washed with 37°C PBS and fixed by a 10-min incubation in 100% methanol at –20°C followed by air drying.

Cell staining was performed essentially as described previously (29). Cells were washed two times for 5 min in PBS supplemented with 1.5 mM MgCl₂ (PBS+) followed by blocking in TBS-BGT blocking solution (25 mM Tris, pH 8.0, 137 mM NaCl, 3 mM KCl, 1.5 mM MgCl₂, 2.5% bovine serum albumin, 13 mM

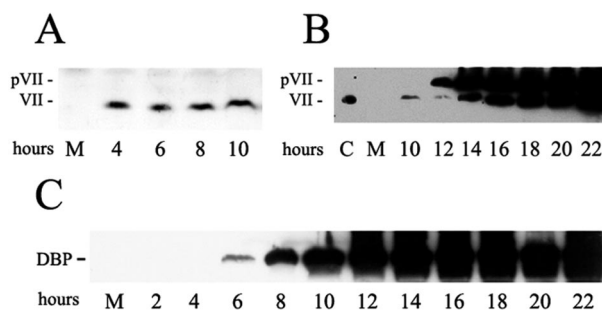


FIG. 1. Appearance of input protein VII and new synthesis of pre-VII and mature protein VII during infection. HeLa cells were infected for the indicated times and analyzed by Western blot for protein VII (A and B) or the 72-kDa DBP (C). The anti-protein VII antibody recognizes both pre-VII (pVII) and protein VII. M, mock infection; C, control lane containing virus-derived protein VII.

glycine, 0.05% Tween 20, 20% goat serum) for 1 h. Cells were then incubated with affinity-purified anti-protein VII rabbit polyclonal and in some instances anti-DBP mouse monoclonal primary antibodies in TBS-BGT blocking solution for 1.5 h followed by washing four times for 5 min in TBS-BGT (25 mM Tris, pH 8.0, 137 mM NaCl, 3 mM KCl, 1.5 mM MgCl₂, 0.5% bovine serum albumin, 13 mM glycine, 0.05% Tween 20). Cells were then incubated with anti-rabbit rhodamine and anti-mouse fluorescein isothiocyanate (FITC) (Boehringer Mannheim) secondary antibody conjugates in TBS-BGT blocking solution for 45 min under dark conditions, followed by washing four times for 5 min in TBS-BGT and once in PBS+ for 1 min. Cells were counterstained with 200 ng of DAPI (4',6'-diamidino-2-phenylindole)/ml in PBS+ for 8 min under dark conditions and washed four times for 5 min in PBS+. Cells were then dipped in H₂O to remove salt and mounted on glass slides in Vectashield mounting media (Vector Laboratories, Inc.). All steps were at room temperature unless otherwise noted. Slides were examined using a Nikon Eclipse E800 fluorescence microscope and a Princeton Instruments charged-coupled-device camera. Exposures were standardized for each experiment.

RESULTS

Virus-derived protein VII persists during infection. We and others have reported that protein VII from infectious viral particles enters the cell along with viral DNA (8, 15, 18, 21). We wanted to examine the appearance of this input protein VII and also the new production of pre-protein VII and mature protein VII during infection. These studies were expected to reveal when the various forms of protein VII function during infection. HeLa cells were infected with phenotypically wild-type *d*309 at a MOI of 50. This MOI was used to more easily observe amounts of input protein VII. Western blot analysis was performed using our previously described anti-protein VII antibody (21). Previously we showed that 100% of cell-associated protein VII enters the nucleus by 4 h postinfection (21). As shown in Fig. 1A, input protein VII was apparent within 4 h of infection and remained constant through the first 10 h. In other experiments input protein VII could be seen entering the nucleus as early as 1 h postinfection (data not shown; see also Fig. 7). For Fig. 1B, later time points were examined. Production of pre-protein VII began around 12 h postinfection. This is consistent with the fact that the mRNA for pre-protein VII is transcribed from the L2 gene, which is controlled by the major late promoter. Mature protein VII began to accumulate between 12 and 14 h postinfection and both the precursor and mature forms of the protein then accumulated to high levels. To establish the timing of early-phase events, expression DBP was also monitored. DBP is encoded

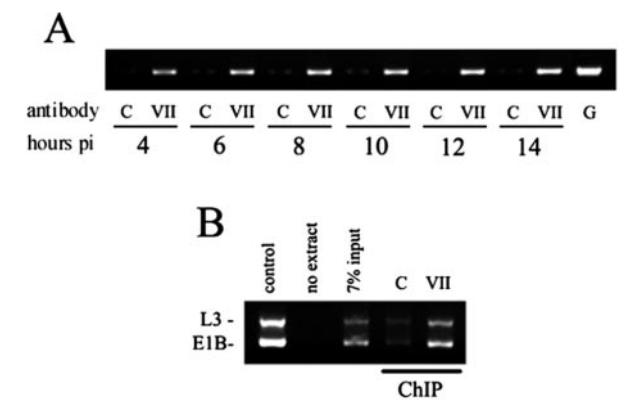


FIG. 2. Protein VII association with viral chromatin. (A) HeLa cells were infected for the indicated times and analyzed by ChIP as described in Materials and Methods. Cells were treated with formaldehyde to cross-link protein to nucleic acid. Cell extracts were prepared, sonicated to shear the DNA to roughly 200 bp, and then incubated with anti-protein VII antibody (VII) or control preimmune serum (C). Precipitates were heated to reverse the cross-links, deproteinized, and assayed by PCR with primers specific for the E1B gene. G, genomic virus DNA control. pi, postinfection. (B) ChIP efficiency. Cells were infected for 4 h and analyzed by ChIP.

by the E2A early gene. As shown in Fig. 1C, DBP was first detected 6 h postinfection and accumulated to high levels thereafter. To relate these protein expression results to viral DNA replication, PCR analysis of extracts from infected cells revealed that DNA replication commenced between 6 and 8 h postinfection (data not shown), as has been reported previously (38). These data demonstrate that input protein VII is present in cells during the early phase of infection and that it persists during the beginning of the DNA replication phase. They also show that accumulation of pre-protein VII starts after the onset of DNA replication and that production of mature protein VII commences shortly thereafter.

Protein VII associates with viral DNA throughout the early phase and also during DNA replication. Recently we used chromatin immunoprecipitation (ChIP) to demonstrate that input protein VII can be found associated with viral DNA at an early-phase time point, 4 h postinfection (21). Furthermore, we observed that the input protein VII was restricted to viral sequences and did not migrate to cellular sequences (21). We next wanted to extend this analysis to determine if the protein VII-DNA association persists through later phases of infection. HeLa cells were again infected with *dl309* at a MOI of 50 and subjected to ChIP analysis as described previously. As shown in Fig. 2A, specific association of protein VII with viral DNA was detected throughout a 14-h time course of infection. Figure 2B shows that greater than 7% of the input DNA was immunoprecipitated from an extract of cells infected for 4 h. This is the same as the percentage of protein VII brought down in a parallel immunoprecipitation (data not shown), indicating that the ChIP was very efficient. Since newly synthesized pre-protein VII does not begin to accumulate significantly until around 12 h postinfection, these data demonstrate that input protein VII remains associated with the viral DNA throughout the early phase and also well into the DNA replication phase, which starts between 6 and 8 h postinfection. This suggests a functional role for protein VII throughout early transcription

and possibly DNA replication. In repeated experiments the extent of protein VII-DNA association increased somewhat over time (Fig. 2A and see also Fig. 4A). It is likely that this increase was due in part to an accumulation of newly synthesized viral DNA and/or pre-protein VII as the infection proceeds. A similar time course ChIP experiment was recently reported by Haruki et al. (18). However, they reported a decrease in protein VII-DNA association as infection proceeds. This discrepancy with our results, which may stem from procedural differences, will be addressed in Discussion.

The majority of protein VII is not released from the viral DNA during early phase. Our ChIP data indicate a stable, ongoing association between protein VII and the DNA. However, we considered the possibility that during the first 4 h of infection the establishment of early viral transcription might lead to the loss of a majority of input protein VII from the viral chromatin. We used the potent transcription inhibitor α -amanitin to examine this possibility. If early transcription normally causes a significant release of protein VII from the DNA, then a general inhibition of transcription by α -amanitin would allow the bulk of protein VII to remain chromatin associated. This would result in an increase in the observed protein VII-DNA association at 4 h. Accordingly, infected cells were treated with 10 μ g of α -amanitin/ml starting at 1 h postinfection and harvested for ChIP analysis 3 h later. As shown in Fig. 3A, there was no effect of α -amanitin on the protein VII-DNA association. As a control, infected cells treated with α -amanitin were analyzed by Western blotting for the presence of E1A protein and also for input protein VII (Fig. 3B). Complete inhibition of E1A production occurred, indicating that early transcription was efficiently shut off by α -amanitin. Since the E1A gene is the first viral gene to be transcribed during infection, this result

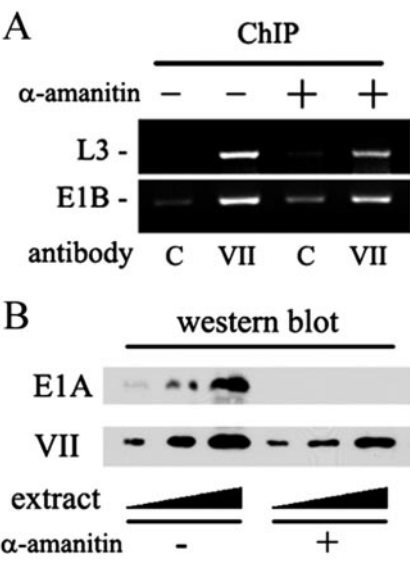


FIG. 3. α -Amanitin treatment of infected cells. (A) Cells were infected with *dl309* for 4 h and treated with 10 μ g of α -amanitin/ml starting 1 h postinfection. (A) ChIP analysis was performed as described in Materials and Methods by using primers representing the L3 and E1B coding regions. C, control preimmune serum; VII, anti-protein VII antiserum. (B) Western blot analysis of E1A and input protein VII. Ramps indicate that increasing amounts of extract were analyzed.

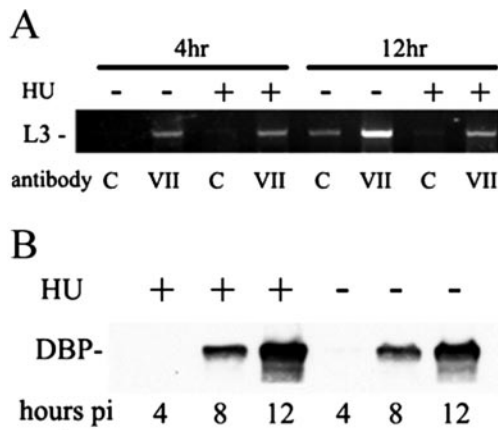


FIG. 4. (A) ChIP of infected cells treated with HU. Cells were infected with *dl309* for the indicated times and treated with 3 μ g of HU/ml starting 1 h post infection (pi). ChIP was performed as described in Materials and Methods by using primers specific for the L3 gene. C, control preimmune serum; VII, anti-protein VII antibody. (B) Cells were infected for the indicated times and treated with HU as described for panel A. Extracts were prepared and analyzed by SDS-PAGE and Western blotting for DBP.

also confirms previous studies demonstrating that early transcription was indeed ongoing during the initial 4-h time period in our experiments. As expected there was little or no effect of α -amanitin on the presence of input protein VII because this protein is not synthesized during the early phase (Fig. 3B). We conclude that the majority of input protein VII that is bound to viral DNA in the infecting virion remains associated with the DNA during the early phase of infection. These data are further supported by immunofluorescence experiments (below).

The data presented above strongly suggested that protein VII remains associated with incoming viral DNA at least through the onset of viral DNA replication. To test directly if the presence of protein VII on the DNA is consistent with all steps that precede the onset of viral DNA replication, the DNA synthesis inhibitor hydroxyurea (HU) was employed. Cells were infected and then treated with 3 μ g of HU/ml starting 1 h postinfection. The cells were harvested at 4 and 12 h postinfection and analyzed by ChIP. As shown in Fig. 4A, there was no effect of HU on protein VII-DNA association at the 4-h time point. This was expected because no DNA replication takes place during this time. In the absence of HU there was a detectable increase in protein VII-DNA association at 12 h postinfection. This was due to the accumulation of newly synthesized DNA and possibly newly synthesized pre-protein VII. In the presence of HU this increase was completely inhibited, as expected. Significantly, the remaining level of protein VII-DNA association at the 12-h time point in the presence of HU was equivalent to that seen in the absence of HU at 4 h postinfection (compare lane 2 to lane 8). At 12 h postinfection, a background signal was reproducibly observed due to nonspecific trapping of newly synthesized viral DNA in the immunoprecipitation with preimmune serum (lane 5). This background signal was also inhibited by HU (lane 7).

These data indicate that protein VII remains associated with viral DNA at least up to the point at which DNA replication commences. As a control to confirm that early-phase events were indeed intact in HU-treated cells, the accumulation of the

72-kDa DBP was measured. As shown in Fig. 4B, HU had no effect on production of DBP, demonstrating that early-phase transcription was normal in this experiment. These data demonstrate that the presence of protein VII in viral chromatin is consistent with all steps that precede the onset of DNA replication.

Viral chromatin and protein VII associate with SET and pp32. The SET protein has been shown to interact with viral chromatin in vivo and with protein VII in vitro (18). SET is a component of two multiprotein complexes called SET and INHAT (2, 4, 10–12, 36, 37). We wondered if only the SET protein could associate with adenovirus chromatin, as reported earlier (18), or if additional members of the SET and INHAT complexes are also present. To test this we performed a ChIP experiment by using an antibody directed against pp32, which is a component of both the SET and INHAT complexes. As shown in Fig. 5A, at 6 h postinfection pp32 was indeed found associated with adenovirus chromatin. Identical results were obtained for the SET protein, confirming previous data (18).

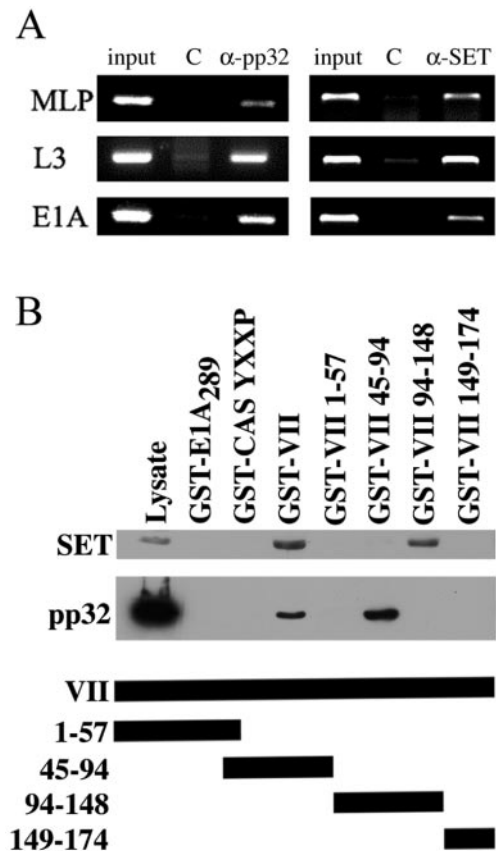


FIG. 5. Viral chromatin and protein VII association with pp32 and SET. (A) ChIP analysis. Cells were infected with *dl309* for 6 h and analyzed by ChIP with anti-pp32 (α -pp32), anti-SET (α -SET), or control (C) antibodies. PCR products: MLP, major late promoter; L3, late region 3; E1A, early region 1A. (B) HeLa cell lysates were used in binding reactions with GST-protein VII, GST-E1A₂₈₉, or the unrelated protein control GST-CAS YXXP (7). Bound proteins were separated by SDS-PAGE and analyzed by Western blotting for SET or pp32. Lower panel, structure of GST-protein VII mutants. Bars indicate regions of protein VII expressed in the mutants. The GST moiety is not illustrated.

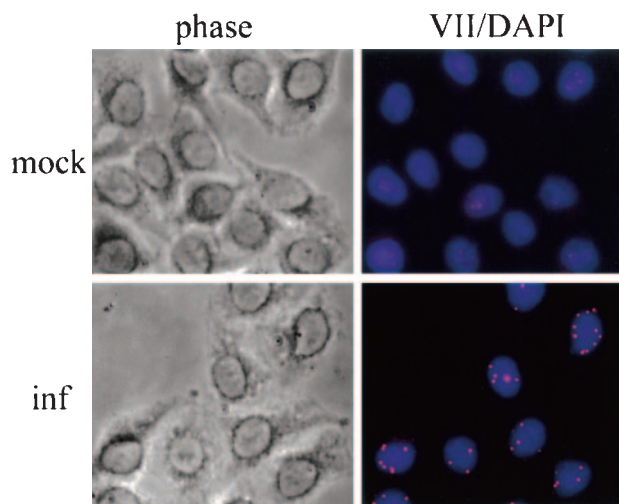


FIG. 6. Protein VII is in nuclear dots in early phase. HeLa cells were grown on glass coverslips and infected with phenotypically wild-type *dl309* for 4 h. Cells were fixed by cold methanol dehydration and probed with rabbit polyclonal anti-protein VII antibody. For visualization, cells were incubated with anti-rabbit rhodamine secondary antibody conjugate. Prior to mounting, cells were counterstained with DAPI. Phase, phase-contrast microscopy; mock, uninfected control; inf, infected cells.

Additionally we asked if cellular pp32 and SET could interact with protein VII in a GST pull-down assay. HeLa cell extracts were incubated with agarose-bound GST-protein VII. The beads were washed and then analyzed by SDS-PAGE. As shown in Fig. 5B, GST-protein VII could specifically pull down pp32 and also SET. As a negative control, GST-protein VII was unable to associate with the nuclear DNA-binding protein Ku86 (21; also data not shown). GST-E1A also did not show any association with either SET or pp32.

We mapped the domains of protein VII that associate with SET and pp32. Interestingly, these two proteins bound to adjacent domains on protein VII. pp32 bound to the 45–94 domain and SET associated with the 94–148 domain (Fig. 5B). Since pp32 and SET are components of multiprotein complexes we might have expected that they would be pulled down together by either of the protein VII binding domains, but this was not the case. The 45–94 domain associated efficiently only with pp32 and the 94–148 domain associated only with SET. However, the interaction between pp32 and SET is very weak (J. Lieberman, unpublished observation) so this result is not surprising. These data suggest that a multiprotein complex containing SET and pp32 associates with viral chromatin *in vivo*. Furthermore, they suggest that this association takes place by way of multiple contacts with protein VII. Protein VII can serve to nucleate the formation of a higher-order complex containing SET and pp32.

Protein VII localizes as discrete dots in the nucleus. Further evidence for an important role for input protein VII was developed using immunofluorescence. Cells were infected for 4 h at a MOI of 5, followed by methanol fixation and staining with anti-protein VII antibody. As shown in Fig. 6, input protein VII localized as discrete dots within the nucleus. No staining was observed in mock-infected control cells (Fig. 6, top) or when only a fluorescent secondary antibody was used (data not shown). Comparison with phase-contrast images and DAPI-

stained nuclei indicated that at 4 h postinfection, 100% of the protein VII staining was located within the nuclei. These data, taken together with the ChIP and protein expression data presented above, suggest that each dot represents an individual viral genome associated with protein VII. The success of these assays was probably due to the presence of up to 1,000 copies of protein VII per genome and the polymeric nature of the protein VII-DNA complex, which resulted in an intense signal upon binding of antibody.

Early-to-late shift in protein VII localization. The localization of protein VII dots was examined over a time course of infection. As shown in Fig. 7, specific staining was visible as early as 30 min postinfection. At the 30-min and 1-h time points a majority of dots were located outside the nucleus. Comparison with phase-contrast images indicated that at these very early times all protein VII dots were cell associated; none were located outside of the cells (data not shown). A few nucleus-associated dots were present at these times, and these generally showed more-intense staining than those outside the nucleus. There was no staining of mock-infected cells. Nuclear accumulation continued during the next 3 h such that by 4 h postinfection, staining was almost entirely nuclear and the intensity of staining was maximal. These data also indicate that 100% of the cells were infected. Considering the ChIP data presented above, these data strongly suggest that there is an accumulation of protein VII-DNA complexes within the nucleus during the initial few hours of infection. We also note that the intensity of the dots increased as they accumulated in the nucleus during the initial 4 h of infection. This suggests that there is an unmasking of protein VII epitopes perhaps due to changes in the molecular architecture of the viral chromatin.

Interestingly, nuclear dots persisted through 14 h postinfection, but their intensity weakened significantly between 8 and 14 h. By 16 h postinfection (Fig. 8) some cells began to exhibit general (diffuse) nuclear protein VII staining. Considering the protein expression data in Fig. 1, it is likely that the general nuclear staining is due to new production of pre-protein VII and mature protein VII and that the dots represent persistent input protein VII in association with input viral DNA. The diffuse nature of the nuclear staining suggests that newly synthesized pre-protein VII is dispersed in its localization. This is in sharp contrast to input protein VII, which probably localizes in a condensed form as a component of viral chromatin. At 16 h only a minority of cells contained dots, and their intensity was weak compared with dots in early phase (Fig. 8, compare 16 h to 6 h). By 24 h postinfection there was intense generalized protein VII staining in a majority of the cells. Because of the intensity of staining in those cells, it was not possible to know if dots were present. Some cells exhibited no general staining and no nuclear dots even though they were clearly infected as judged by the presence of the 72-kDa DNA-binding protein. These data indicate that there was a peak of nuclear dot intensity (input protein VII) between 2 and 10 h postinfection and that these structures subsequently dissipated, were masked, or lost their association with protein VII. The immunofluorescence data for the early time points (through 8 h postinfection) support our conclusion from ChIP assays that the majority of protein VII is not released from the DNA during early phase. They also suggest that there is an indepen-

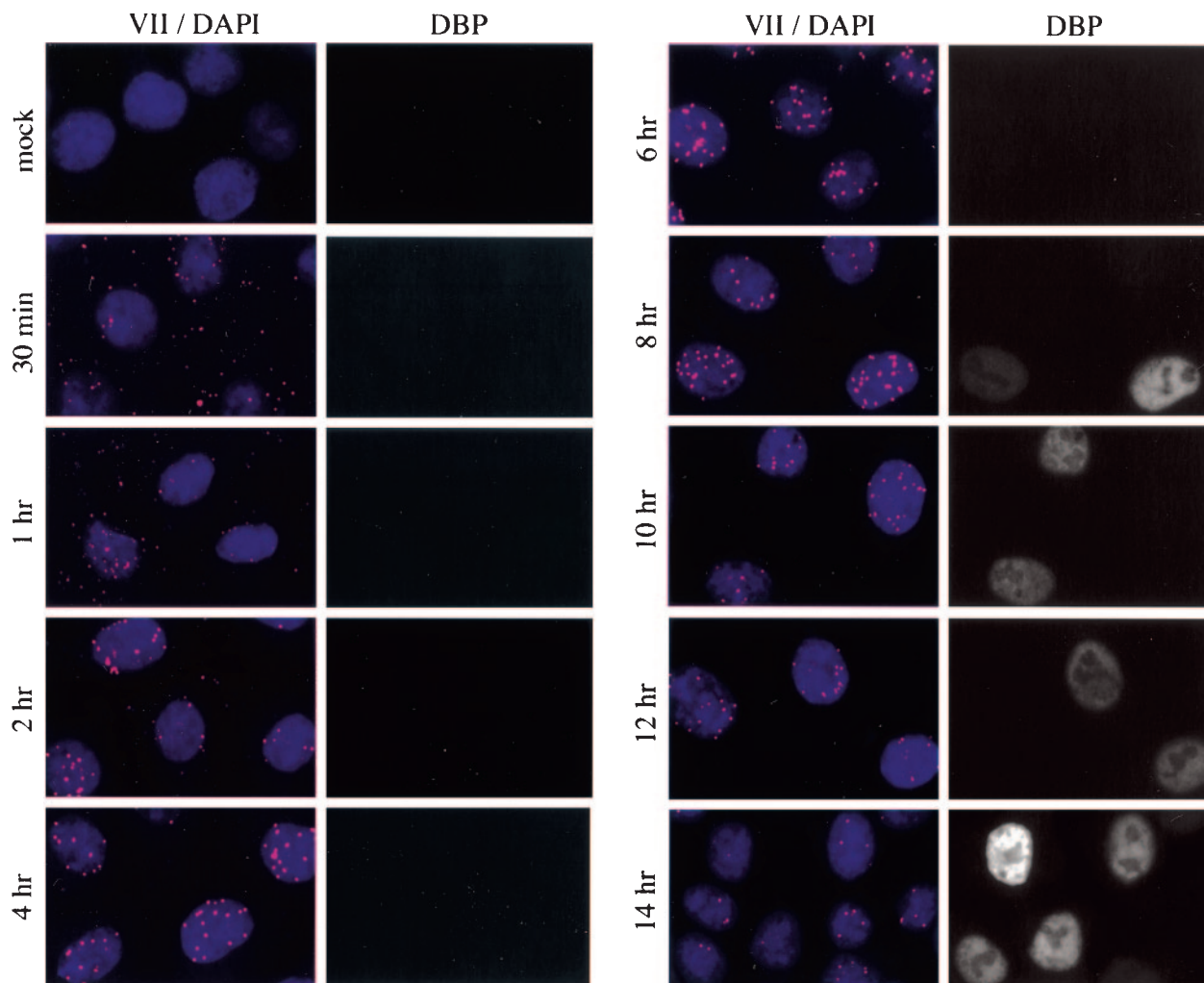


FIG. 7. Protein VII dot localization throughout infection. HeLa cells were infected for the indicated times at a MOI of 20. Cells were fixed by cold methanol dehydration and probed with mouse monoclonal anti-DBP and rabbit polyclonal anti-protein VII antibodies. For visualization, cells were probed with anti-mouse FITC and anti-rabbit rhodamine secondary antibody conjugates. Prior to mounting, cells were counterstained with DAPI.

dent accumulation of pre-protein VII and mature protein VII at late times that results in general nuclear staining.

Localization of 72-kDa DNA-binding protein. To relate the appearance and localization of protein VII to other events during infection, the cells were costained for the 72-kDa DBP, whose localization has been previously described. Starting at 8 h postinfection individual cells could be seen to express DBP, and their number increased dramatically after this (Fig. 7 and 8). As the infection progressed to 16 h, DBP localized as expected in replication centers, as has been reported (30, 40) (Fig. 8). By 24 h postinfection virtually all cells expressed DBP, which showed punctate staining. As expected, the overall morphology of these late infected cells was drastically altered compared to uninfected cells (data not shown).

Clearly, cells that showed general DBP staining (e.g., at 12 h postinfection) were further along in the infection cycle than cells that had yet to express DBP. Furthermore, cells in which DBP was localized to replication centers (e.g., at 16 h postinfection) had progressed further than cells with only general

DBP staining. Examination of the 16-h time point revealed a trend: cells with DBP staining showed a decrease in dot intensity compared with the 6-h time point, and cells containing prominent replication centers contained no or very few dots. Conversely, cells that had no DBP staining contained more-intense nuclear dots and little if any general protein VII staining (e.g., at 6 h). These data strongly suggest a progression in protein VII localization that correlates with the progress of infection. At very early times (no DBP staining) only dots are present. At later times (general DBP staining or localization to replication centers) there is production of pre-protein VII which results in general nuclear staining and a concomitant decrease in dot intensity due perhaps to release of input protein VII from the viral DNA. This indicates that the cells that express new protein VII are those that have progressed further in the infection. Taken together, these data provide further support for the protein expression and ChIP data presented above. During the early phase (0 to 6 h) and at least partially into the DNA replication phase, input protein VII persists in

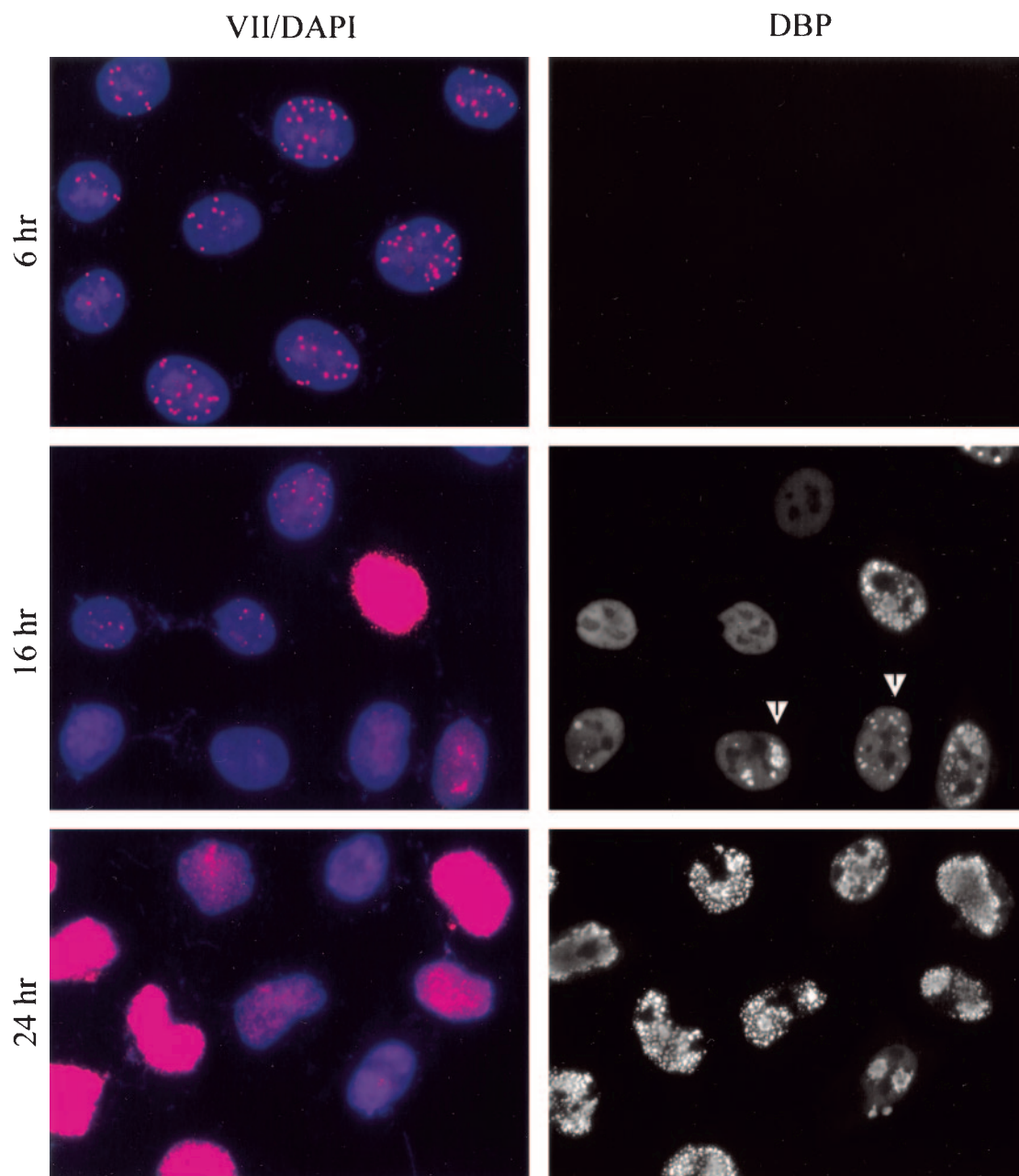


FIG. 8. Protein VII dot localization at late times. HeLa cells were infected for the indicated times at a MOI of 20. Cells were fixed by cold methanol dehydration and probed with mouse monoclonal anti-DBP and rabbit polyclonal anti-protein VII antibodies. For visualization, cells were probed with anti-mouse FITC and anti-rabbit rhodamine secondary antibody conjugates. Prior to mounting, cells were counterstained with DAPI. Arrowheads indicate examples of cells containing replication centers.

discrete structures that are likely to include input viral genomes. As the infection progresses, production of pre-protein VII and mature protein VII correlates with other late-phase events. At the same time there is a decrease in dot intensity that may indicate a release of input protein VII from the DNA at late times or a specific reorganization of the viral genome that precludes recognition by the anti-protein VII antibody.

Disappearance of input protein VII dots is due to an active process but does not require ongoing DNA replication. As mentioned above, input protein VII dots decreased signifi-

cantly in number and intensity at late times (Fig. 7 and 8). We considered the possibility that this change was due to loss of protein VII from chromatin during DNA replication. To test this, infected cells were treated with Ara-C, an inhibitor of DNA replication. Infected cells were treated starting 1 h postinfection with 25 μ g of Ara-C/ml and then prepared for immunofluorescence at 4 and 16 h postinfection. Figure 9 shows that cells treated with Ara-C behaved identically to untreated cells: they contained dots at 4 h postinfection but not at 16 h postinfection. This demonstrates that ongoing DNA replica-

tion does not trigger disappearance of the protein VII dots. Identical results were obtained with the DNA synthesis inhibitor hydroxyurea (data not shown). We also challenged cells with 10 μ g of α -amanitin/ml to determine the effect of inhibition of RNA synthesis on disappearance of the dots. Interestingly, α -amanitin largely prevented loss of the protein VII dots. This indicates that loss of the dots was not due to passive diffusion of protein VII away from their concentrated centers. Rather, these data argue that an active process was involved in altering the signal of input protein VII in the cell.

DISCUSSION

We have examined the fate and function of adenovirus protein VII during infection. Five lines of investigation support an ongoing role for protein VII throughout early phase and perhaps during DNA replication as well. First, as judged by Western blotting, input protein VII persists in the nucleus at least through the beginning of DNA replication. Second, ChIP assays reveal an extended association between protein VII and viral DNA. Third, protein VII can be found in nuclear dot structures during the early phase and beyond. Fourth, SET and pp32, both of which associate with protein VII in GST pull-down assays, are found in association with viral DNA during early phase. Finally, our previous finding that protein VII can bind to E1A supports a role for protein VII in early transcriptional activation (21).

We wondered if the nuclear dots seen at early times might be precursors to previously characterized viral replication centers, which are the major sites of DNA replication. Input viral genomes by definition must be the templates for early viral transcription and replication. Both input adenoviral genomes at early times and adenoviral replication centers at late times have been found adjacent to promyelocytic leukemia nuclear bodies (PML-NBs or ND10) (20), suggesting that adenoviral replication centers develop from input viral genomes. We observed no concordance between protein VII dots and viral replication centers as judged by costaining of protein VII and DBP, a marker for replication centers. However, cells that contained visible replication centers had generally lost their dots (Fig. 8). A small minority of cells were observed in which some protein VII dots were visible along with replication centers, though these signals did not colocalize (data not shown). It is possible, however, that protein VII dot structures progress into replication centers after protein VII fluorescence is no longer detectable. It is also possible that, after the earliest rounds of DNA replication, there could be a shift in localization of DNA replication to the replication centers. Both hypotheses are consistent with our data. We showed that the nuclear dots disappear at late times and that their disappearance occurs even in the absence of ongoing DNA synthesis. Similarly, Haruki et al. used ChIP to show that a decrease in protein VII-DNA association at late times does not require DNA replication (18). These data indicate that the process of DNA replication does not cause release of protein VII from the DNA. Rather, we find that RNA synthesis is required for disappearance of the dots to occur. Since loss of the dots occurs at late times but not at early times, our data are consistent with the idea that late transcription itself could be responsible for this effect. Other possibilities exist however.

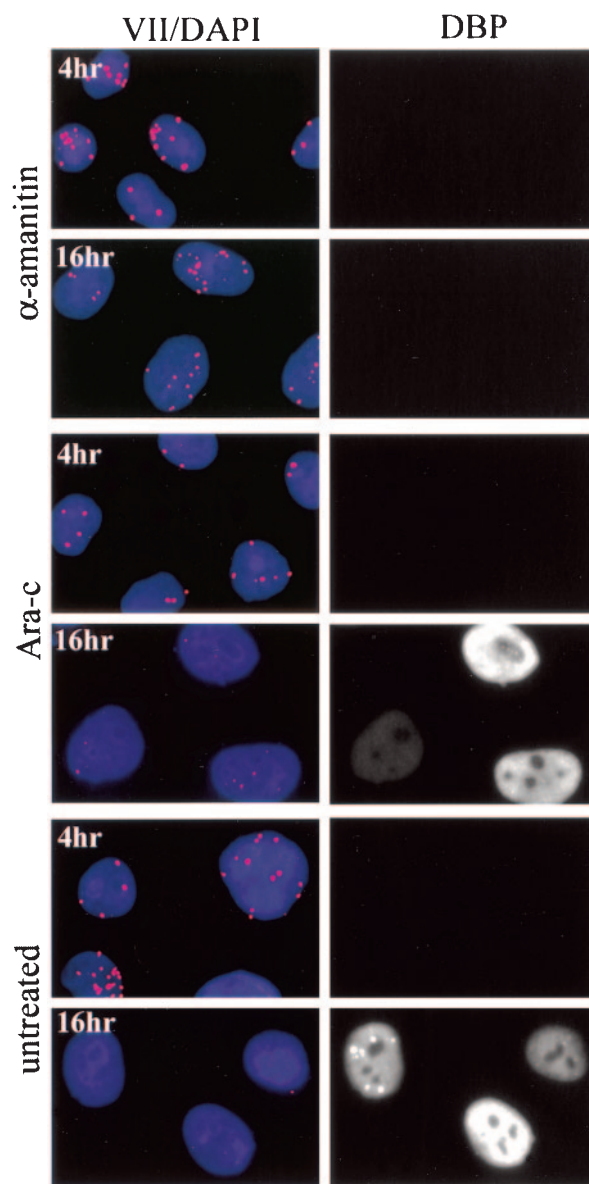


FIG. 9. Disappearance of nuclear dots requires RNA synthesis but not DNA synthesis. HeLa cells were infected for the indicated times and were treated at 1 h postinfection with either 25 μ g of Ara-C/ml or 10 μ g of α -amanitin/ml. Cells were fixed by cold methanol dehydration and probed with mouse monoclonal anti-DBP and rabbit polyclonal anti-protein VII antibodies. For visualization, cells were probed with anti-mouse FITC and anti-rabbit rhodamine secondary antibody conjugates. Prior to mounting, cells were counterstained with DAPI.

For instance, it is conceivable that DBP could displace protein VII from the DNA either in the presence or absence of ongoing DNA replication. Alternatively, the observed decrease in intensity could be due to a change in chromatin structure that leads to an altered accessibility of the viral chromatin to the antibody. This possibility is consistent with a stable or increasing level of protein VII in association with the viral DNA. Finally, it is possible that at late times the nuclear dot structures experience a weakening in the protein VII-DNA associ-

ation and that our methanol fixation method results in removal of protein VII. We are currently working to characterize the association between protein VII and viral DNA at late times. The observation that protein VII remains associated with viral DNA during the beginning of the DNA replication phase suggests a role for protein VII in this process.

We note a minor discrepancy between our results and those of Haruki et al. In their ChIP experiments the association between protein VII and viral DNA diminished over the course of a 12-h infection (18). In our ChIP experiments we showed an increase in association as the infection proceeded through 12 to 14 h (Fig. 2 and 4). As described earlier, the increase we observed was due to an increase in available DNA due to new DNA replication. This conclusion is based on the fact that the observed increase in protein VII-DNA association was completely inhibited by addition of HU to the infected cells (Fig. 4). The difference between our results and those of Haruki et al. are likely due to our longer cross-linking times used in the ChIP assay, which would result in the detection of both strong and weak DNA-protein interactions. If newly synthesized DNA at late times is not fully condensed and therefore is weakly associated with protein VII, these interactions might be detected only with longer cross-linking times. In our immunofluorescence experiments we did observe a decrease in the protein VII signal in nuclear dots as the infection proceeded. We used methanol for fixing the infected cells, which might cause disruption of weak protein VII-DNA interactions at late times, leading to a less-intense signal. On the other hand, our ChIP experiments were performed with formaldehyde cross-linking, which would preserve those weak interactions.

Our data concerning the SET and pp32 proteins clearly suggest that a multiprotein complex, possibly the SET and/or INHAT complex, is functioning at the viral chromatin. The identity, integrity, and function of such a complex will be the subject of additional studies. Both the SET complex and INHAT have been implicated in chromatin metabolism. INHAT can inhibit histone acetyltransferases by binding directly to histones and masking their sites of acetylation (36, 37). The SET complex has been shown to mediate granzyme A-dependent apoptosis, in part due to a DNase activity of the complex (2, 10–12). It is interesting to speculate that this complex has additional functions including the regulation of viral chromatin. The SET protein, when studied alone by using in vitro approaches, has NAP, chromatin remodeling, and chromosome decondensing activities (2, 26, 28). It remains to be determined how these activities relate to one another biochemically and whether the SET protein acts alone at adenovirus chromatin or in combination with other components of the INHAT and SET complexes. The fact that both SET and pp32 can bind to protein VII, and that distinct domains of protein VII mediate binding to these proteins, suggests that a multiprotein complex containing protein VII, SET, and pp32 is likely to be functioning during the early stages of adenovirus replication.

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