CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Elsevier - Publisher Connector

Available online at www.sciencedirect.com
ScienceDirect
Virology 369 (2007) 411–422
VIROLOGY
www.elsevier.com/locate/yviro

Transcription releases protein VII from adenovirus chromatin

Jiangning Chen [a], Nuria Morral [b], Daniel A. Engel [a,*]

[a] *Department of Microbiology, University of Virginia Health System, P.O. Box 800734, Charlottesville, VA 22908-0734, USA*
[b] *Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202, USA*

Received 27 June 2007; returned to author for revision 6 August 2007; accepted 13 August 2007
Available online 20 September 2007

Abstract

Adenovirus protein VII is the major protein component of the viral nucleoprotein core. It is a nonspecific DNA-binding protein that condenses viral DNA inside the capsid. Protein VII remains associated with viral chromatin throughout early phase, indicating its continuing role during infection. Here we characterize the release of protein VII from infectious genomes during a time period that corresponds to the late phase of infection. Interestingly, the early viral transactivator E1A, but not other early gene products, is responsible for releasing protein VII by a mechanism that requires ongoing transcription but not viral DNA replication. Moreover transcription *per se*, in the absence of E1A, is also sufficient to trigger release. Accordingly, a recombinant genome containing only non-coding "stuffer" DNA is unable to support release of protein VII. Our data support a model in which early gene transcription results in a change in the structure of the viral chromatin.
© 2007 Elsevier Inc. All rights reserved.

Keywords: Adenovirus; Transcription; Chromatin; Gene therapy; Protein VII

Introduction

Transcriptional regulation during virus infection ensures the proper timing and amount of gene expression. In adenovirus infection, early phase is controlled in large part by the transcriptional activator E1A. Transcription of the early genes leads to production of viral regulatory and replicative functions that are required for DNA replication to commence (Shenk, 2001). Likewise viral DNA replication is a necessary precursor to the initiation of late phase transcription (the early-to-late switch) and the associated production of viral structural proteins (Flint, 1986; Iftode and Flint, 2004; Thomas and Mathews, 1980).

The physical state of adenovirus chromatin is likely important in the regulation of transcription and DNA replication during infection. The earliest transcriptional events upon introduction of viral DNA into the nucleus are governed by the interplay between cellular factors and the viral chromatin. Within the virion, the viral nucleoprotein core is composed of highly basic proteins μ (mu), protein V and protein VII, and also protein IVa2 and the 55 kDa terminal protein (Amin et al., 1977; Brown et al., 1975; Hosokawa and Sung, 1976; Maizel et al., 1968; Prage and Pettersson, 1971; Rekosh et al., 1977; Russell et al., 1968; Weber et al., 1983). Of these, protein VII is the most abundant and is primarily responsible for condensation of the viral DNA inside the virion. Upon infection protein VII enters the nucleus along with viral DNA and remains associated with it throughout early phase and the beginning of DNA replication (Chatterjee et al., 1986; Greber et al., 1996; Haruki et al., 2003; Johnson et al., 2004; Xue et al., 2005). It is capable of highly efficient DNA condensation *in vitro* and *in vivo* and can efficiently repress cellular transcription *in vivo* when introduced by microinjection (Black and Center, 1979; Johnson et al., 2004). The association of protein VII with viral DNA during infection suggests an ongoing regulatory requirement for this protein. However it is not known what precise roles protein VII might play in mechanisms of transcriptional repression or transcriptional activation of the viral genome.

Regulation of the protein VII–DNA complex by host factors during infection is important in the regulation of the viral transcriptional program. The host protein SET/template activating factor-Iβ (TAF-Iβ) can associate with protein VII *in vitro* and *in vivo* (Haruki et al., 2003, 2006; Xue et al., 2005). SET/

* Corresponding author. Fax: +1 434 982 1071.
E-mail address: dae2s@virginia.edu (D.A. Engel).

0042-6822/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2007.08.012

TAF-Iβ has nucleosome assembly activity *in vitro* and can efficiently stimulate transcription and DNA replication from adenovirus nucleoprotein core preparations (Kawase et al., 1996; Okuwaki and Nagata, 1998). RNAi knockdown of SET/TAF-Iβ resulted in reduced expression of the E1A and E2 mRNAs during infection, indicating a role for this protein in transcription from viral templates (Haruki et al., 2006). The cellular protein pp32 can also associate with protein VII *in vitro*, possesses nucleosome assembly activity and associates with adenovirus chromatin during infection (Haruki et al., 2006; Xue et al., 2005). SET/TAF-Iβ and pp32 are both components of the cellular SET and INHAT complexes (Beresford et al., 2001; Brennan et al., 2000; Fan et al., 2002; 2003a,b; Seo et al., 2002, 2001), suggesting that these complexes may have a role in viral transcriptional regulation. However RNAi knockdown of pp32 did not affect viral early transcription in a recent study (Haruki et al., 2006).

Previously we reported that protein VII localizes as nuclear dots within 1–2 h of infection of HeLa cells and that these dots remain intact through the first 10–12 h of infection (Xue et al., 2005). During this time period protein VII associates with viral DNA as shown by chromatin immunoprecipitation (ChIP) assay, indicating that the dots represent individual infectious genomes (Haruki et al., 2003; Johnson et al., 2004; Xue et al., 2005). Furthermore, the number of protein VII dots in the nucleus corresponds to the multiplicity of infection (MOI) and protein VII nuclear dots co-localize with SET/TAF1β, which has been demonstrated to regulate viral transcription (Haruki et al., 2006; Xue et al., 2005). Since protein VII associates with viral DNA throughout early phase we concluded that it has an ongoing role in establishing and/or regulating viral chromatin structure during early phase transcription. Interestingly, protein VII dots disappear from the nucleus between 10 and 16 h post-infection. Moreover α-amanitin, a potent inhibitor of transcription, prevents disappearance of the dots, suggesting that the transcriptional state of the viral template is coupled to protein VII function. In contrast, inhibitors of viral DNA replication such as cytosine arabinoside (AraC) and hydroxyurea (HU) do not prevent loss of the protein VII dots (Xue et al., 2005). These data suggest that a transcriptional mechanism is responsible for loss of protein VII from the dot structures and that an uncharacterized mode of regulation is at play here. In this report we characterize the mechanism by which association of protein VII with nuclear dot structures is disrupted between 10 and 16 h post-infection. Our results point to a novel transcriptional regulatory mechanism that serves the adenovirus gene expression program.

Results

Timed loss of protein VII from nuclear dots

The loss of protein VII nuclear staining between 10 and 16 h post-infection implies a regulatory step at this stage of infection, during which DNA replication and late phase are proceeding. Therefore we investigated the mechanism of loss of protein VII dots during infection. Fig. 1 shows the presence and location of protein VII during the period 4–12 h post-infection. HeLa cells were infected with phenotypically wild-type *dl*309. At various times after infection the cells were fixed and stained with an anti-protein VII polyclonal antibody. The infections were performed in the absence or presence of HU to block viral DNA replication. Since DNA replication is required for initiation of late phase transcription (Thomas and Mathews, 1980), treatment with HU prevents new synthesis of protein VII, a late gene product of the L2 mRNA. Therefore all protein VII staining in cells treated with HU represented material that entered along with the infecting virus. Fig. 1 shows that nuclear protein VII staining remained strong through 8–10 h post-infection. By 10 h the protein VII dots became somewhat fewer in number, and at 12 h post-infection the dots were mostly absent; those that remained were weak in intensity. Fig. 3 shows that by 16 h post-infection the dots disappeared entirely (*dl*309 + AraC panel). These data demonstrate that there is a switch in protein VII regulation that occurs around 10–12 h post-infection.

Release of protein VII

Interestingly, for cells infected for 12 h or longer we noticed the appearance of faint but reproducible diffuse nuclear staining for protein VII that correlated with the decrease in intensity of the dots, suggesting that protein VII was released from the dot structures but was retained in the nucleus. To see this diffuse staining more clearly, cells were infected at a higher MOI to provide additional material for observation and analyzed as before. Importantly, cells were treated with AraC starting at 1 hour post-infection to block DNA synthesis and prevent production of new protein VII. Fig. 2A shows that, concomitant with loss of the dots, protein VII redistributed generally within the nucleus. For clarity, nuclear DAPI staining is not shown in Fig. 2A. Western blot analysis confirmed that the bulk of protein VII was still present at the 12 hour time point, indicating that released protein VII remains in the cell. Since we previously demonstrated that virtually all cell-associated protein VII is found in the nucleus (Johnson et al., 2004) this indicates that the majority of the released protein VII is nuclear and is not degraded. In addition, treatment of cells with AraC completely blocked the synthesis of new pre-protein VII, demonstrating that the appearance of protein VII as general nuclear staining shown here was not due to new synthesis (Fig. 2B). Finally, prior treatment with α-amanitin completely prevented the appearance of the diffuse nuclear staining, demonstrating that active transcription is required for this redistribution (data not shown). These data indicate that loss of protein VII dots is due to release of the protein from viral chromatin. Consistent with this, it has been reported that there is a decrease in protein VII–DNA association during this time period as measured by ChIP (Haruki et al., 2003).

E1A protein triggers release of protein VII

As reported earlier, the ability of α-amanitin to prevent loss of protein VII dots from the nucleus indicated that there is an

active, transcription-dependent process that mediates this loss. Three possible mechanisms of protein VII loss that rely on transcription are: (1) production of E1A protein and direct action of E1A on protein VII or the viral chromatin, (2) production and action of an E1A-induced early viral gene product(s) such as E1B, E2 or E4, and (3) transcriptional elongation by RNA polymerase II, or a related process, which could directly displace protein VII from the chromatin.

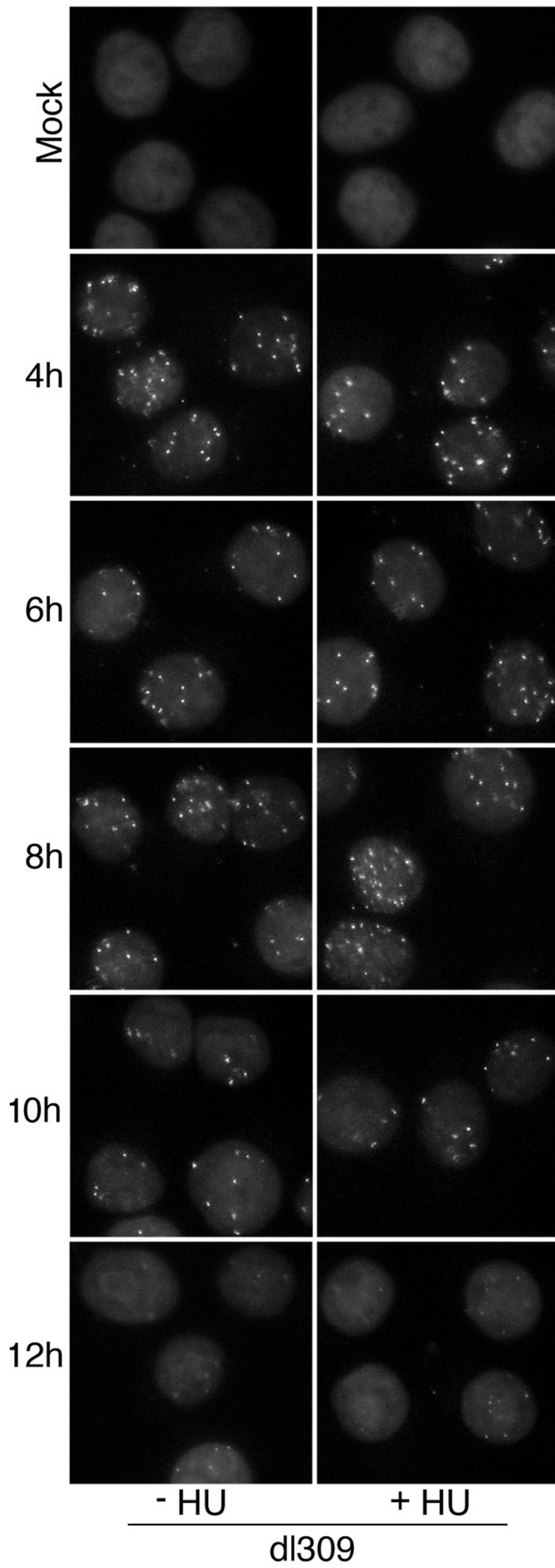

Fig. 1. Time course of protein VII localization. HeLa cells were plated on glass cover slips and infected for the indicated times with wild-type *dl*309. The cells were fixed by cold methanol dehydration and probed with rabbit polyclonal anti-protein VII antibody. For visualization, the cells were incubated with goat anti-rabbit Alexa Fluor 594 secondary antibody conjugate. Prior to mounting, the cells were counterstained with DAPI. For this and all subsequent figures, the CCD camera sensitivity was kept constant for the multiple images presented in the composite figures to allow accurate comparison. Protein VII is shown in white. DAPI is shown in gray. Mock, uninfected control; HU, hydroxyurea.

First, to investigate the role of E1A, HeLa cells were infected with mutant *dl*312, which contains a large in-frame deletion of the E1A gene and fails to produce any of the E1A gene products (Jones and Shenk, 1979). For comparison, cells were also infected with wild-type *dl*309 in the presence of either AraC or α-amanitin to block DNA replication or transcription, respectively. Cells were infected at an MOI of 5–15 and stained for protein VII at 4, 12 and 16 h post-infection. As shown in Fig. 3, *dl*309 infected cells contained intensely stained protein VII dots at 4 h post-infection. By 12 h post-infection the dots exhibited less intense staining and were fewer in number, and at 16 h post-infection they were completely absent (top panel). In the absence of AraC, by 16 h post-infection some cells showed intense general protein VII nuclear staining, indicative of new protein VII synthesis (top panel, arrows). Cells treated with AraC behaved identically to untreated cells with respect to loss of the dots, thus demonstrating that ongoing viral DNA synthesis is not required to trigger this loss. In addition, AraC completely blocked new protein VII synthesis due to the requirement of DNA replication for late gene expression. As reported previously, cells treated with α-amanitin retained their protein VII dots, indicating that transcription is required for disappearance of the dots. Interestingly, the *dl*312 mutant also contained protein VII dots throughout the entire time course of infection, suggesting that E1A protein is required for loss of the dots.

To test the involvement of additional early gene products, mutants with defects in the E1B, E2, E3 and E4 genes were examined. As shown in Fig. 4 all early gene mutants with the exception of *dl*312 exhibited a wild-type phenotype: each mutant exhibited loss of protein VII dots by 16 h post-infection.

It seemed likely that the defect in *dl*312 was due to the lack of production of E1A protein, but the possibility existed that an unanticipated alteration in *dl*312 chromatin structure prevented proper regulation of protein VII. To distinguish between these possibilities, *dl*312 was used to infect human 293 cells, which produce the viral E1A and E1B proteins endogenously. As shown in Fig. 5A, *dl*312 infection of 293 cells resulted in complete disappearance of protein VII dots. These data demonstrate that the *dl*312 chromatin is fully capable of supporting protein VII regulation and that the effect on protein VII regulation is in *trans*.

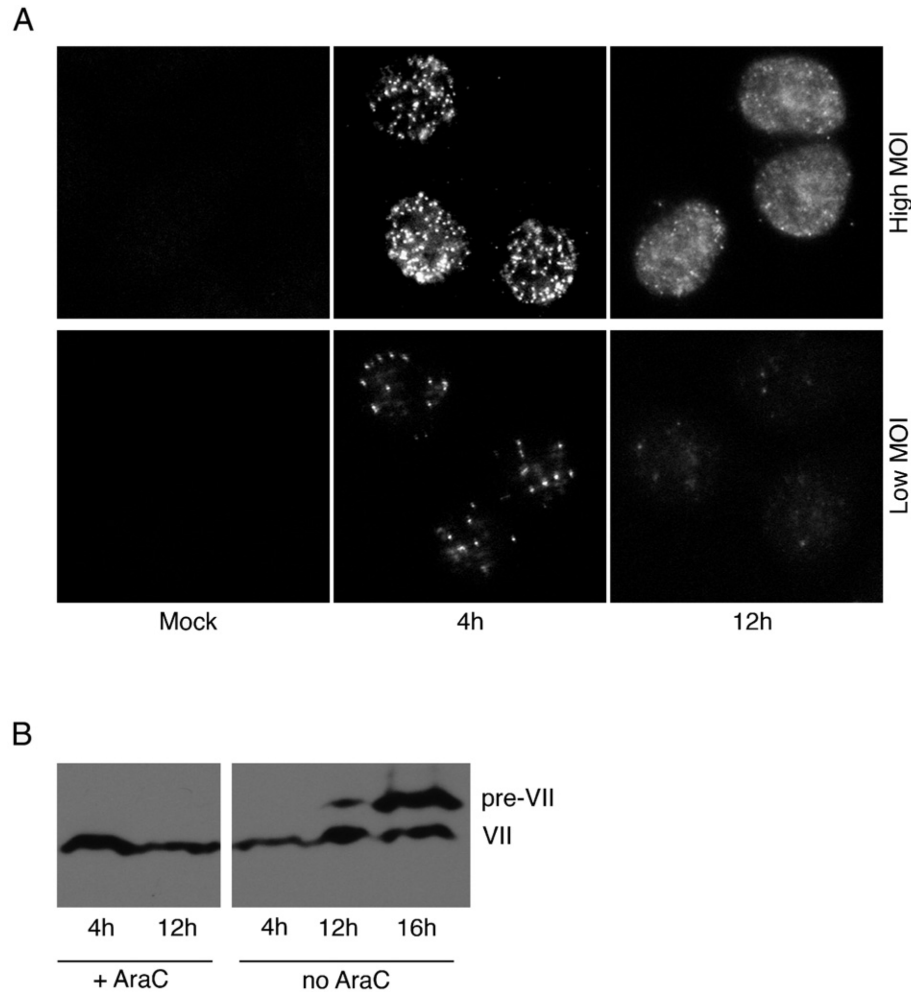

Fig. 2. (A) Nuclear release of protein VII. HeLa cells were infected with *dl*309 at an MOI of 10 (low) or 50 (high) for the indicated times, in the presence of AraC. The cells were fixed by cold methanol dehydration and probed with rabbit polyclonal anti-protein VII antibody. For visualization, the cells were incubated with goat anti-rabbit Alexa Fluor 594 secondary antibody conjugate. Mock, uninfected control. (B) Western blot analysis of protein VII. Cells were infected for the indicated times in the presence or absence of AraC and analyzed by Western blot using a polyclonal anti-VII antibody. Pre-VII, precursor to protein VII.

The two major E1A proteins, E1A243 and E1A289, differ only by an internal 46 amino acid domain found in E1A289 that confers a potent transcriptional activation function (Berk, 1986). To determine which E1A protein is responsible for triggering disappearance of protein VII dots, cells were infected with *pm*975, which produces only E1A289, or *dl*520, which synthesizes only E1A243. As shown in Fig. 5B *dl*520 failed to cause dot disappearance whereas *pm*975 had a wild-type phenotype. Since E1A289 is the major viral transcriptional activator this result indicates that disappearance of protein VII dots correlates with the ability of E1A289 to activate viral early transcription. Interestingly, it should be noted that, prior to 10–12 h post-infection, a period during which E1A289 is active and early viral transcription is ongoing (Shenk, 2001; Xue et al., 2005), no loss of protein VII from the dot structures was observed. This indicates a specific change in the regulation of protein VII after 10 h post-infection that is due to the action of E1A.

As a final test to rule out the involvement of any additional adenovirus gene products in loss of protein VII dots during infection, a recombinant "helper-dependent" (HD) adenovirus was employed that encodes no adenovirus proteins. Virus gAd. HSU/GFP contains approximately 28 kb of non-coding human stuffer DNA (Sandig et al., 2000) flanked by the adenovirus inverted terminal repeats from the extreme left and right ends of the viral genome. Embedded within the stuffer DNA is the gene for green fluorescent protein (GFP), under control of the CMV promoter. To produce gAd.HSU/GFP, cells were co-transfected with helper-dependent and helper adenovirus DNA, which encoded the necessary replication functions and structural components. During propagation, the helper sequences were discarded by way of a *cre-lox* mechanism that specifically excised the adenovirus packaging sequences from the helper genome, rendering it incapable of being packaged into virions (Chen et al., 1996; Parks et al., 1996). Therefore the gAd. HSU/GFP virus is constituted with all adenovirus capsid and nucleocapsid core proteins, including protein VII, however it is unable to produce viral proteins *de novo*. Fig. 6A (topmost panel) shows the results of infection of gAd.HSU/GFP in HeLa cells. Protein VII dots remained even after 18 h of infection.

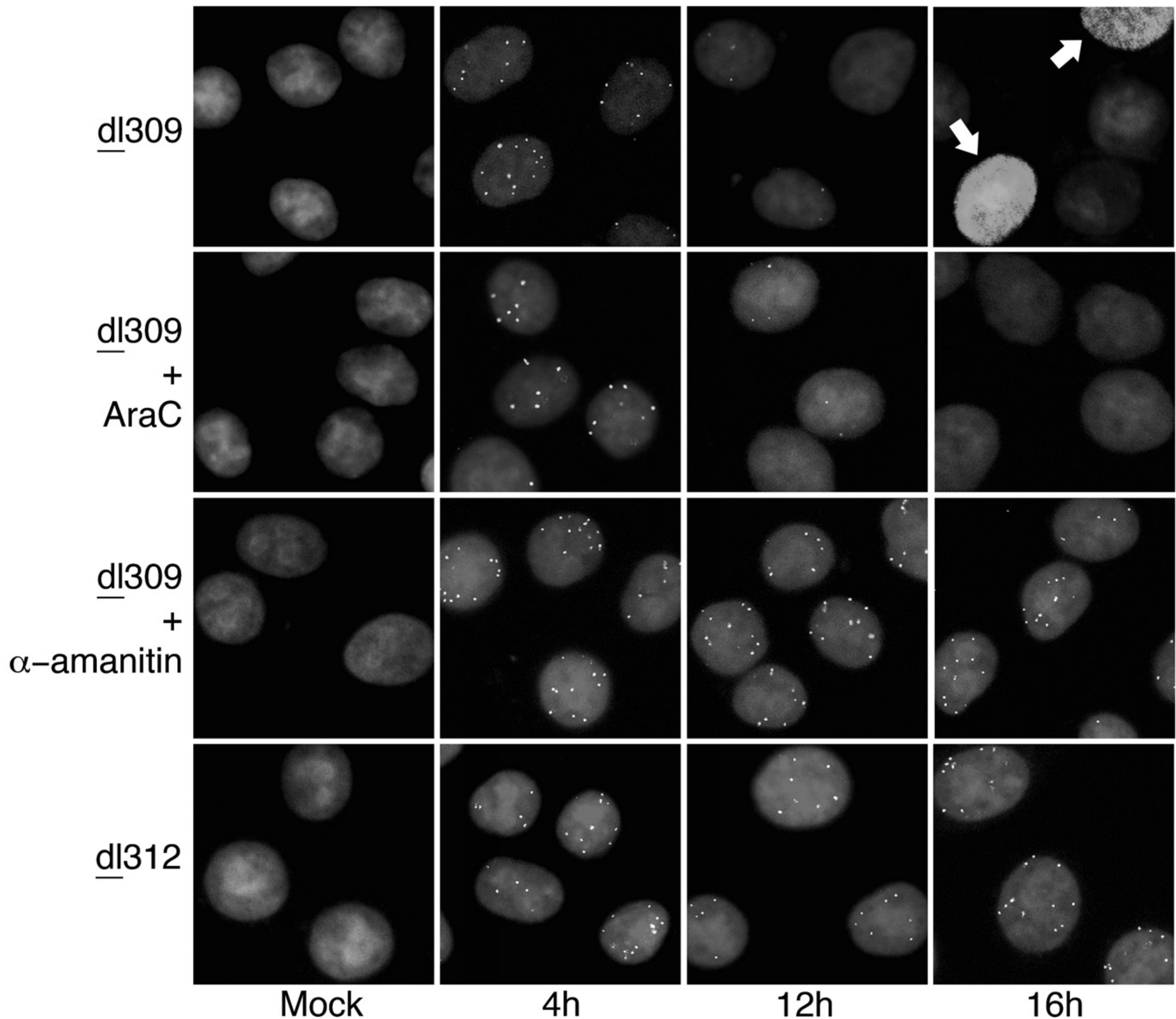

Fig. 3. E1A is required for loss of dots. HeLa cells were infected for the indicated times with *dl*309 or E1A mutant *dl*312, in the presence or absence of AraC (25 μg/ml) or α-amanitin (20 μg/ml). The cells were fixed and stained for protein VII and cellular DNA as described in Fig. 1 and Materials and methods. Arrows indicate cells that are synthesizing new protein VII. Mock, uninfected control.

These data demonstrate that in the absence of all viral gene products proper regulation of protein VII is disrupted. In some experiments, a minor reduction in the intensity of staining was observed at the 18 hour time point with gAd.HSU/GFP (see also Fig. 8, topmost panel). This reduction is due to the presence of the CMV promoter and GFP gene sequences and will be addressed below.

Again, the ability of E1A proteins to act in *trans* was tested. gAd.HSU/GFP was used to infect 293 cells (Fig. 6B). Also, gAd.HSU/GFP was co-infected along with wild-type *dl*309 in HeLa cells (Fig. 6A, bottommost panel). In both experiments protein VII dots were lost by 18 h post-infection. Concomitant with the loss of protein VII dots, an approximately three-fold increase in GFP expression was observed in cells co-infected with *dl*309 (data not shown). This activation of the CMV-driven GFP gene was due to the action of E1A, which has been shown to activate the CMV promoter (Cockett et al., 1991; Gorman et al., 1989; Olive et al., 1990). Since the experiments presented in Fig. 4 eliminated early genes E1B, E2, E3 and E4 in causing loss of protein VII dots we again conclude that loss of protein VII staining in gAD.HSU/GFP infected cells was due to the action of E1A.

Transcription per se is sufficient to release protein VII

Two mechanisms could account for the role of E1A in protein VII regulation. First, E1A could interact with protein VII or other components of the viral chromatin and thereby directly trigger loss of protein VII from the chromatin. Alternatively, transcriptional activation by E1A could cause displacement of protein VII from the chromatin due to transcriptional elongation by RNA polymerase II or a related process such as chromatin remodeling. To investigate this we took advantage of a second helper-dependent vector that is

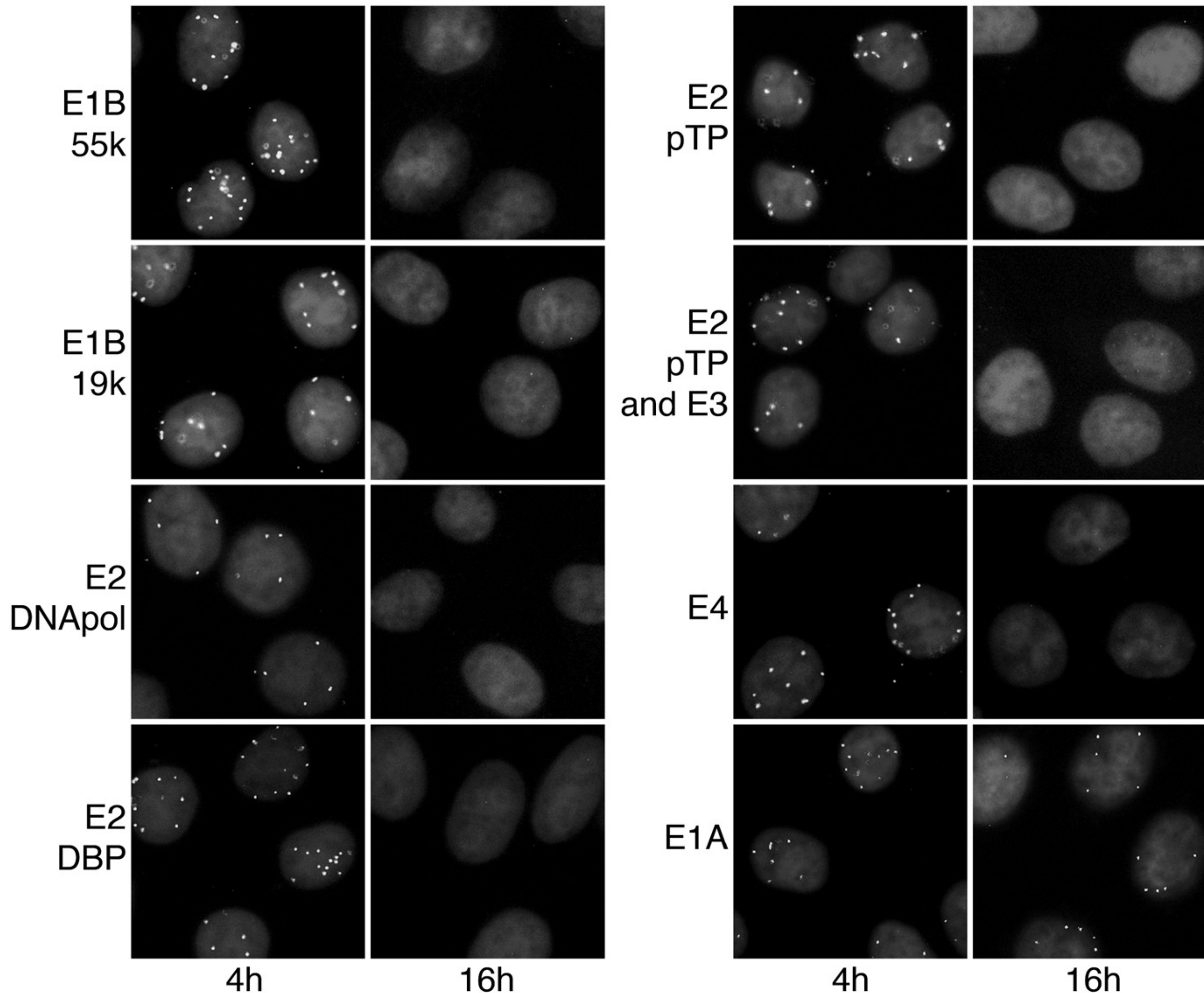

Fig. 4. Effect of early gene mutations on protein VII localization. HeLa cells were infected for the indicated times with viruses that fail to express E1B 55k protein (*dl*338), E1B 19k protein (*dl*337), E2B DNA polymerase (ts149), E2A 72k DNA-binding protein (ts125), E2B pre-terminal protein pTP (Ad5*dl*300ΔpTP), double mutant E2B pTP plus E3 (Ad5*dl*327ΔpTP), E4 (*dl*366) and E1A (*dl*312). For the ts125 and ts149 experiments, cells were maintained at 40 °C for the entire time course to inactivate the 72k DNA-binding protein and DNA polymerase, respectively. The cells were fixed and stained for protein VII and cellular DNA as described in Fig. 1 and Materials and methods.

devoid of all gene sequences. Vector gAd.HSU (Sandig et al., 2000) contains 28 kb of non-coding human sequences (identical to those in gAd.HSU/GFP) flanked by the adenoviral left and right-end inverted terminal repeats. If E1A is sufficient directly to displace protein VII, it would be expected to do so in the context of gAd.HSU chromatin. However, if the presence of gene sequences is required for E1A to act, the gAd.HSU chromatin would be resistant to the action of E1A. As shown in Fig. 7 (topmost panel) there was no loss of protein VII dots when gAd.HSU was used to infect HeLa cells over an 18 hour time course. This result was similar to that shown in Fig. 6A for gAd.HSU/GFP. Interestingly, when gAd.HSU was co-infected along with *dl*309 (Fig. 7A), about 50% of protein VII dots remained (compare 4 hour with 18 hour time point). Since loss of dots from the co-infecting *dl*309 genomes is expected in this experiment, this result suggests that there was no loss from the gAd.HSU genomes, thus resulting in an overall 50% loss of the dots. To confirm this behavior of gAd.HSU, it was used to infect 293 cells expressing E1A (Fig. 7B). No loss of the dots was observed. This is in contrast to the results obtained with gAd. HSU/GFP presented in Fig. 6, where co-infection with *dl*309 or infection of 293 cells resulted in complete loss of the dots. Since the only difference between gAd.HSU/GFP and gAd.HSU is the CMV promoter and GFP gene sequences in gAd.HSU/GFP, we conclude that the presence of active gene sequences is required for release of protein VII from the viral chromatin.

Next we tested the hypothesis that transcription itself, in the absence of E1A, is sufficient to trigger loss of the protein VII dots. The CMV promoter contains binding sites for NFκB and CREB/ATF, which can be regulated by PMA and forskolin, respectively (Meier and Stinski, 1996). Accordingly, HeLa cells were infected with gAd.HSU/GFP and then treated with a

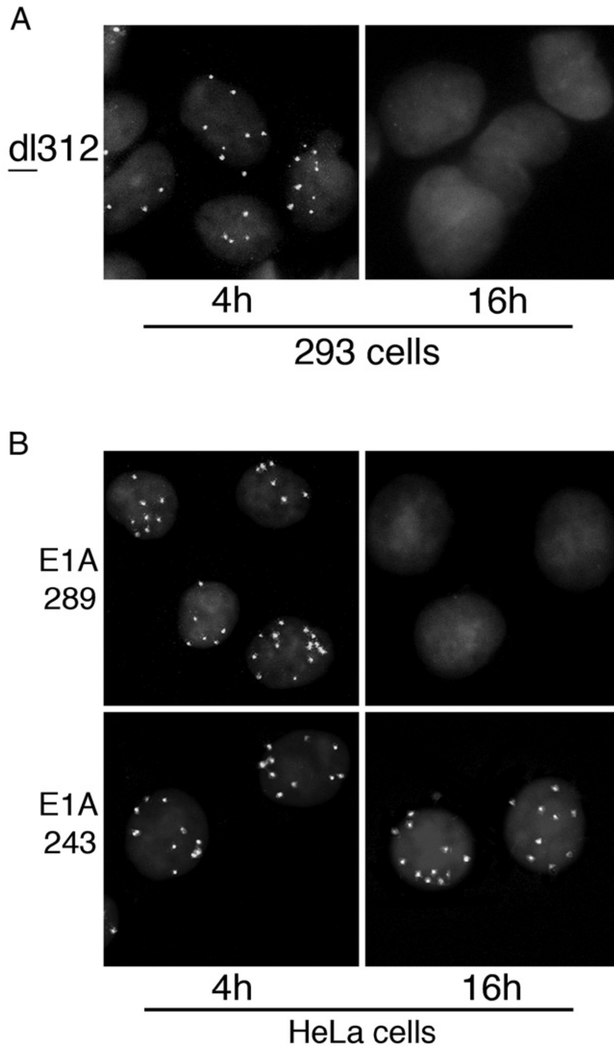

Fig. 5. E1A289 but not E1A243 triggers loss of protein VII dots. (A) 293 cells were infected for the indicated times with mutant *dl*312. (B) HeLa cells were infected with mutant viruses that express either E1A289 (*pm*975) or E1A243 (*dl*520) for the indicated times. The cells were fixed and stained for protein VII and cellular DNA as described in Fig. 1 and Materials and methods.

combination of PMA and forskolin to activate transcription of the GFP gene. As shown in Fig. 8B, in the absence of PMA and forskolin a relatively low amount of GFP protein could be detected by fluorescence microscopy. When the cells were stained for protein VII (Fig. 8A, topmost panel) dots were visible at 4 and 18 h post-infection, although there was some weakening of their intensity by 18 h. This indicates that the presence of protein VII is compatible with a low level of transcription. Upon induction with PMA and forskolin, a significant increase in GFP expression was observed (Fig. 8B). Interestingly, this resulted in complete loss of protein VII dots after 18 h of incubation. As a control, cells were also infected with gAd.HSU and treated identically with the combination of PMA and forskolin. No loss of protein VII dots was observed, again demonstrating that gene sequences are required for loss of the dots to take place. This control also demonstrated that treatment of cells with PMA and forskolin did not lead to loss of protein VII dots by way of a transcription-independent mechanism. We conclude that a high level of transcription *per se* is sufficient to displace protein VII from the viral chromatin in the absence of E1A.

Discussion

This work addresses several questions regarding the regulation of viral chromatin and the handling of viral templates during infection. First, the regulation of early phase transcription, which has been well studied, must now be considered in the context of chromatin constituted with protein VII. The presence of protein VII throughout early phase and its abrupt exit during the beginning of late phase argue for important regulatory events during both stages. Since protein VII is a powerful transcriptional repressor and is associated with viral DNA throughout early phase (Haruki et al., 2003; Johnson et al., 2004; Xue et al., 2005), a reasonable model is that it acts to dampen transcription during early phase. The reasons for this in terms of viral biology are not known, but it may be that an inappropriately high level of early protein synthesis is mechanistically counterproductive to viral replication. Alternatively high protein levels could stimulate the host immune response in a way that is detrimental to virus replication. Simian Virus 40 (SV40) prevents protein overexpression using a different mechanism, by the production of microRNAs that dampen expression of large T antigen, leading to lowered sensitivity to CTL killing (Sullivan et al., 2005). Yet another possibility is that overactive early transcription could physically prevent the initiation of DNA synthesis, which must originate from a template that has already produced early transcripts. If such a template were associated with a highly dense array of RNA polymerase II and associated factors, it might not be capable of initiating DNA synthesis.

We found that transcription intrinsically is sufficient to effect release of protein VII. Release could be triggered by the passage of elongating RNA polymerase or alternatively could be mediated by transcription-associated functions such as host chromatin remodeling complexes. As mentioned above, SET/TAF-Iβ and pp32 have been implicated in early gene control and are therefore potential candidates for carrying out this function. However, these proteins associate with viral chromatin as early as 4 h post-infection whereas the bulk of protein VII is released much later (Haruki et al., 2003; Xue et al., 2005). Therefore another step is likely required to effect release, although SET/TAF-Iβ and/or pp32 may be involved. Recently, evidence for an E1A-independent release of a population of protein VII molecules was reported to occur within 4 h post-infection (Spector, 2007). This release is proposed to occur very early after the viral DNA enters the nucleus and may be carried out by SET/TAF-Iβ. Perhaps this initial, E1A-independent release of protein VII is involved in setting up the viral chromatin for early transcription, and the late phase release we have observed is for the purpose of sustaining or modifying it. In line with our findings, several studies have observed increases in early gene mRNAs during the time period of protein VII release. Also in line with our results, chromatin

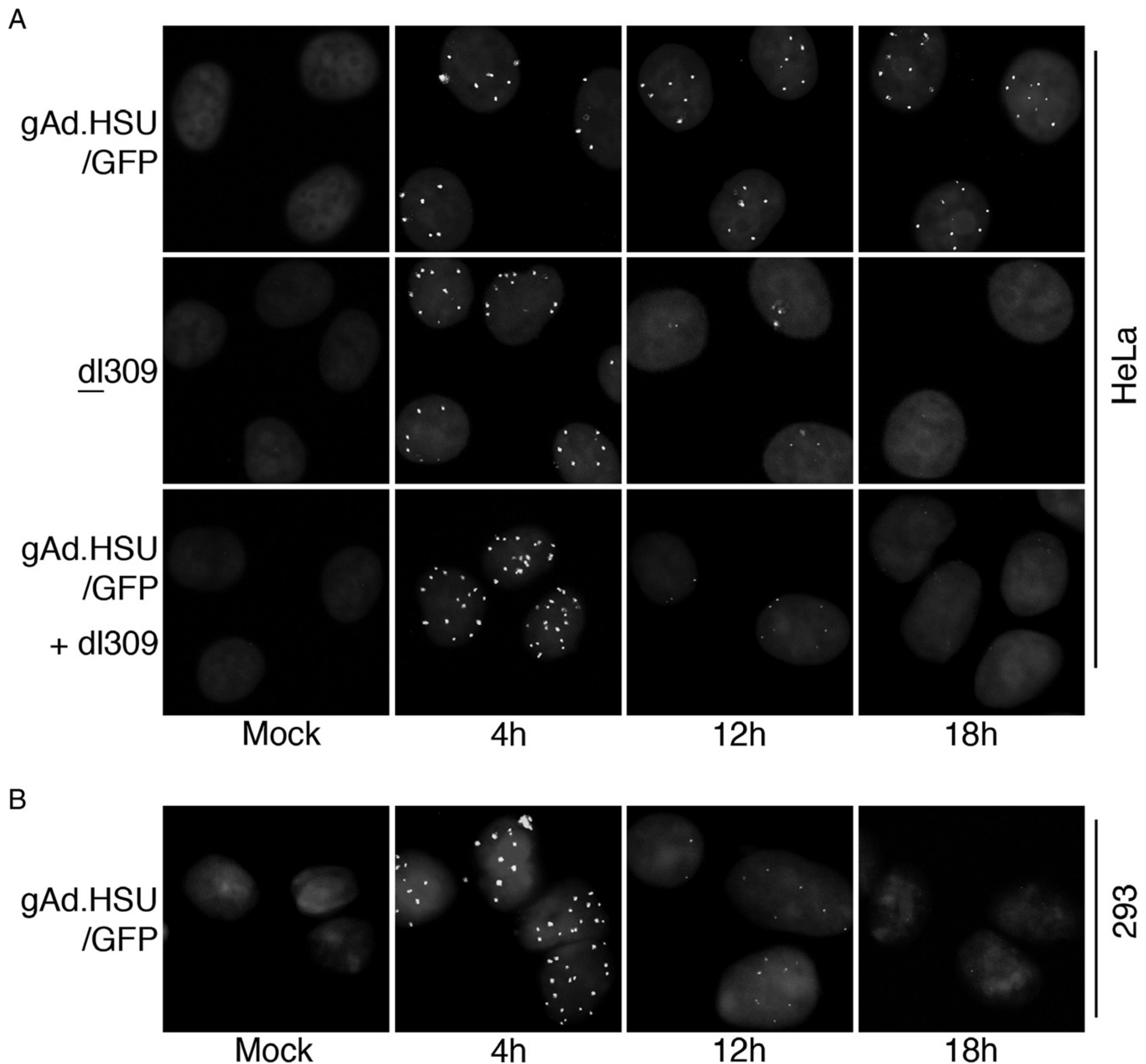

Fig. 6. Release of protein VII from a helper-dependent adenovirus vector that expresses GFP from the CMV promoter. (A) HeLa cells or (B) 293 cells were infected with the indicated viruses. The amount of helper-dependent virus particles used in the experiments was determined as described in Materials and methods. The cells were fixed and stained for protein VII and cellular DNA as described in Fig. 1 and Materials and methods.

immunoprecipitation showed that protein VII–DNA association decreases over this same period of infection (Haruki et al., 2003).

We found that release of protein VII occurs during a time window after the completion of early phase. Interestingly, we showed that this regulation does not require DNA replication or the early-to-late switch: it was not prevented by inhibitors of DNA replication or replication-defective mutants ts125 or ts149. It has been demonstrated by many laboratories that the early genes, which collectively span almost the entire viral genome, are actively transcribed during the first 8–10 h of infection (Shenk, 2001), yet there is no evidence for protein VII release in our assay during this time period. This indicates that multiple rounds of transcription through protein VII–DNA complexes fail to release the protein. This situation is similar to that of transcriptionally active cellular chromatin. Typically histones are not irreversibly displaced from coding sequences in an extensive manner. Instead it has been proposed that components of the elongation machinery actively replace H2A–H2B dimers or entire nucleosomes as the elongating polymerase passes through. In some cases however, histones are displaced from active promoters or from entire coding regions (Workman, 2006). Also, a ChIP-on-chip study provided evidence for large-scale release of histones from highly transcribed gene coding regions (Lee et al., 2004). This situation may be similar to that observed in this report, as follows. The gAd.HSU/GFP virus was transcriptionally active at a low level in the absence of inducers PMA and forskolin (Fig. 8B). Under these conditions protein VII

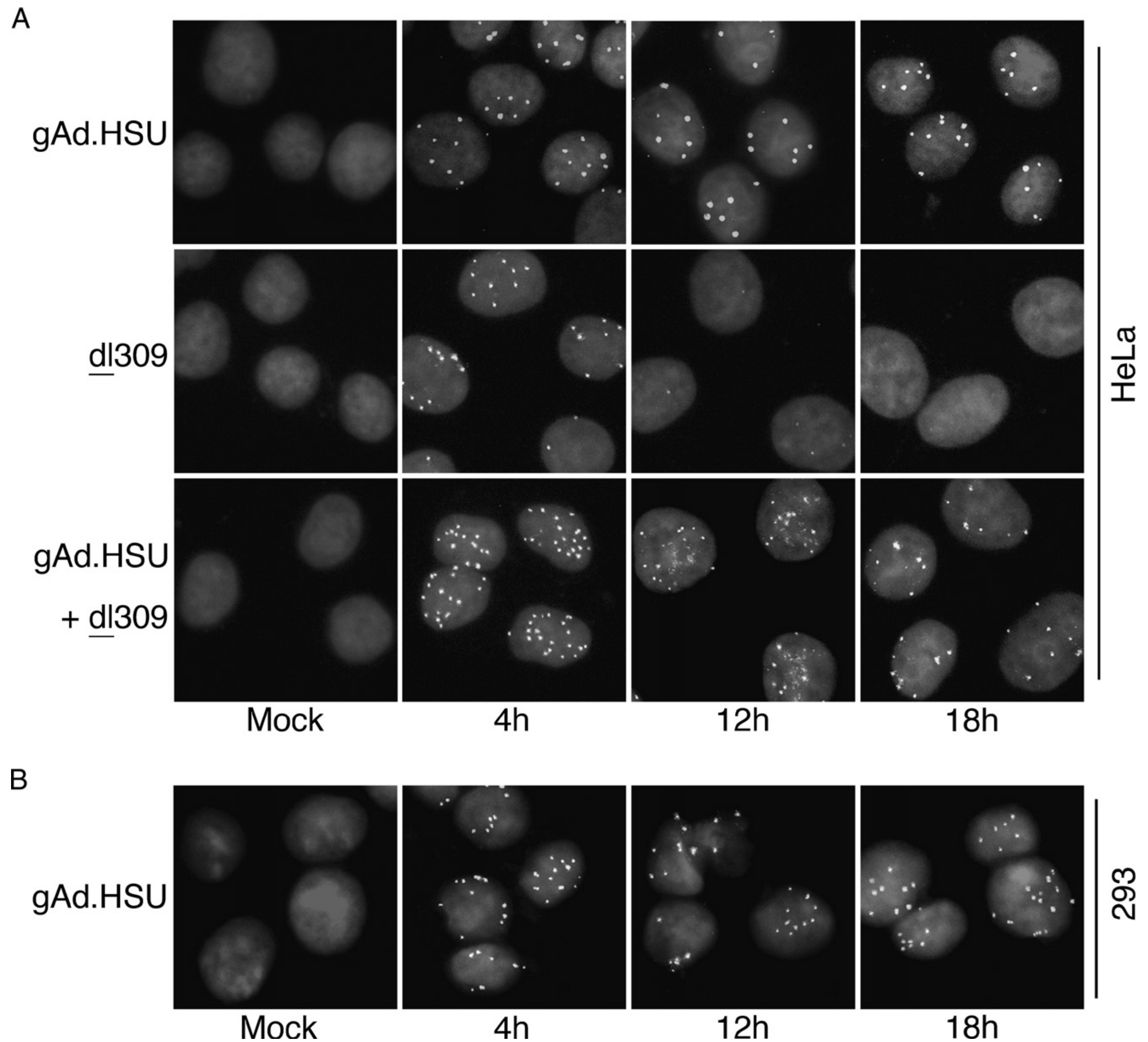

Fig. 7. Failure to release protein VII from a helper-dependent adenovirus vector containing only non-coding sequences. (A) HeLa cells or (B) 293 cells were infected with the indicated viruses. For the co-infection experiment (bottom panel of A) the cells were treated with HU to prevent DNA replication. The cells were fixed and stained for protein VII and cellular DNA as described in Fig. 1 and Materials and methods.

dots remained present at least through 18 h post-infection. At this time point, some diminishment in intensity of the protein VII signal was observed in some experiments (Fig. 8A). This suggests that a low level of transcription is much less efficient at displacing protein VII than the high level seen when the cells were treated with PMA and forskolin. Taken together our data support a model in which early transcription is set at a low level during infection, due to the presence of protein VII. At later times there may be an E1A-dependent increase in transcription of the input templates that forces protein VII release and alters the viral chromatin. A variation of this mechanism is that E1A could activate transcription and also trigger release of protein VII by a concerted mechanism that allows transcription to increase maximally. This would be consistent with our previous finding that E1A and protein VII can associate *in vitro* (Johnson et al., 2004). As mentioned above cellular mechanisms seem to exist for carrying out nucleosome displacement on highly active cellular genes. Such mechanisms may be at work on adenovirus chromatin as well.

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). Where indicated, cells were treated with AraC (25 μg/ml) or HU (10 mM). 293 cells were grown in the same medium except fetal bovine serum was used. Adenovirus type 5 *dl*309 (Jones and Shenk, 1979) was propagated in HeLa cells to produce virus stocks. E1A mutants *dl*312 (Jones and Shenk,

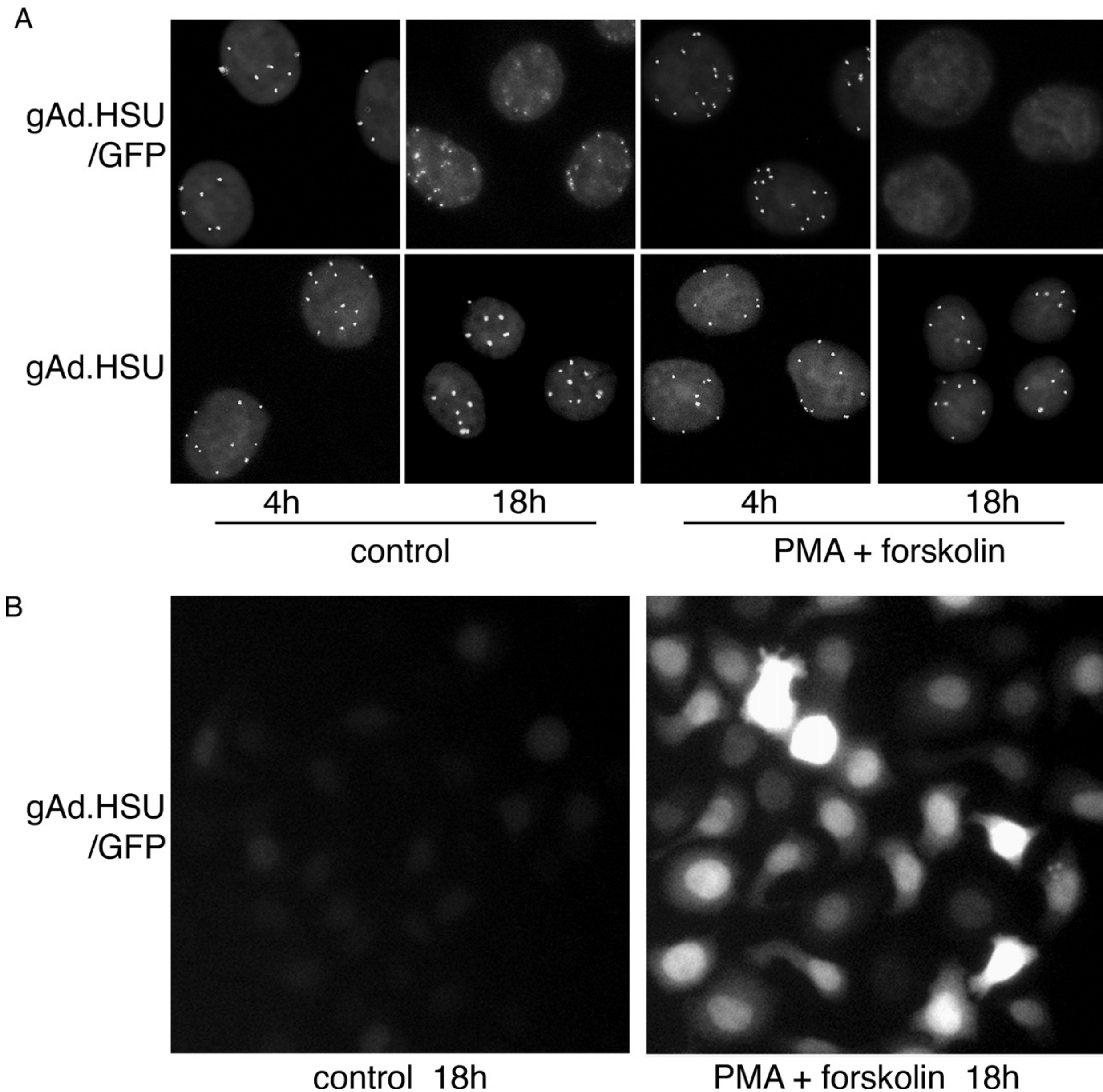

Fig. 8. Induction of transcription releases protein VII in the absence of E1A. HeLa cells were infected with the indicated viruses in the presence or absence of a combination of PMA and forskolin. (A) The cells were fixed and stained for protein VII and cellular DNA as described in Fig. 1 and Materials and methods. (B) Prior to fixing the cells were photographed using a fluorescence microscope. Mock-infected cells showed no background fluorescence (not shown).

1979), pm975 (Montell et al., 1982) and *dl*520 (Haley et al., 1984) were propagated in 293 cells. E1B mutants *dl*337 (Pilder et al., 1984) and *dl*338 (Pilder et al., 1986) were gifts from D. Ornelles. Pre-terminal protein (pTP) mutant Ad5*dl*300ΔpTP, pTP-E3 double mutant Ad5*dl*327ΔpTP (Schaack et al., 2004) and E2B mutant ts149 (Straus et al., 1975) were gifts from J. Schaack. E4 mutant *dl*366 (Halbert et al., 1985) and E2A mutant ts125 (Straus et al., 1975) were gifts from P. Hearing. Helper-dependent adenoviral vectors were generated using a Cre-loxP system obtained from Microbix (Chen et al., 1996; Parks et al., 1996; Sandig et al., 2000). This consists of plasmid pC4HSU, in which expression cassettes are cloned, Cre recombinase-expressing 293Cre4 cells and helper virus H14, which is an E1-deleted adenovirus containing *lox*P sites flanking the packaging signal (Sandig et al., 2000). Plasmids pC4HSU and pC4HSU/GFP were digested with *Pme* I, transfected into 293Cre4 cells and helper H14 was added to generate helper-dependent vectors gAd.HSU and gAd.HSU/GFP, respectively. Details of the rescue and amplification process are described elsewhere (Witting et al., submitted for publication). The virus was purified by CsCl step gradient centrifugation followed by CsCl isopycnic separation. The helper-dependent adenovirus band was collected and dialyzed against 10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 150 mM NaCl and 10% glycerol. The level of contamination with helper was determined by plaque assay and shown to be lower than 0.03%. For experiments using helper-dependent viruses, initial titrations were performed assuming a particle to infectious unit

ratio of between 20:1 and 50:1 (Kreppel et al., 2002). Cells were stained for protein VII and dots per nuclei were counted. An amount of helper-dependent virus corresponding to 5–10 dots per nucleus was used for subsequent experiments.

Immunofluorescence

HeLa cells were grown on 22- by 22-mm glass coverslips (Fisher) for 24 h prior to infection. Cells were infected for 1 h as previously described (Johnson et al., 2004). Cells were then washed two times in PBS to remove unadsorbed virus followed by incubation in DMEM plus 10% NCS at 37 °C. After incubation the cells were washed with PBS and fixed with 100% methanol for 10 min at −20 °C followed by air drying. Cell staining was performed essentially as described previously (Ornelles and Shenk, 1991). Cells were rehydrated in PBS supplemented with 1.5 mM $MgCl_2$ (PBS+) three times for 5 min followed by incubation in blocking buffer (25 mM Tris [pH 8.0], 137 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 2.5% bovine serum albumin, 13 mM glycine, 0.05% Tween 20 and 20% goat serum) for 1 h. Cells were then incubated in blocking buffer containing affinity-purified anti-protein VII rabbit polyclonal antibody for 100 min followed by washing four times in PBS+ supplemented with 0.1% Tween 20 for 10 min. Cells were then incubated with goat anti-rabbit Alexa Fluor 594 (Invitrogen) secondary antibody conjugate in blocking buffer for 45 min in the dark, followed by washing four times in PBS+ supplemented with 0.1% Tween 20 for 10 min, and were then washed three times in PBS+ for 5 min. Cells were then dipped briefly in H_2O and mounted on glass slides in Vectashield mounting media with DAPI (Vector Laboratories, Inc.). All steps were at room temperature unless otherwise noted. Slides were sealed with nail polish and examined using a Nikon Eclipse E800 fluorescence microscope and a Princeton Instruments charged-coupled-device camera.

Acknowledgments

We thank Ron Taylor, Ann Beyer, David J. Spector and Marcin Walkiewicz for helpful discussions, and Mariano Garcia-Blanco for critically reading the manuscript. This work was supported by NIH Public Health Service grant CA60675 to D.A.E.

References

Amin, M., Mirza, A., Weber, J., 1977. Genetic analysis of adenovirus type 2. VII. Cleavage-modified affinity for DNA of internal virion proteins. Virology 80, 83–97.

Beresford, P.J., Zhang, D., Oh, D.Y., Fan, Z., Greer, E.L., Russo, M.L., Jaju, M., Lieberman, J., 2001. Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. J. Biol. Chem. 276, 43285–43293.

Berk, A.J., 1986. Adenovirus promoters and E1A transactivation. Annu. Rev. Genet. 20, 45–79.

Black, B.C., Center, M.S., 1979. DNA-binding properties of the major core protein of adenovirus 2. Nucleic Acids Res. 6, 2339–2353.

Brennan, C.M., Gallouzi, I.E., Steitz, J.A., 2000. Protein ligands to HuR modulate its interaction with target mRNAs in vivo. J. Cell Biol. 151, 1–14.

Brown, D.T., Westphal, M., Burlingham, B.T., Winterhoff, U., Doerfler, W., 1975. Structure and composition of the adenovirus type 2 core. J. Virol. 16, 366–387.

Chatterjee, P.K., Vayda, M.E., Flint, S.J., 1986. Adenoviral protein VII packages intracellular viral DNA throughout the early phase of infection. EMBO J. 5, 1633–1644.

Chen, L., Anton, M., Graham, F.L., 1996. Production and characterization of human 293 cell lines expressing the site-specific recombinase Cre. Somat. Cell. Mol. Genet. 22, 477–488.

Cockett, M.I., Bebbington, C.R., Yarranton, G.T., 1991. The use of engineered E1A genes to transactivate the hCMV-MIE promoter in permanent CHO cell lines. Nucleic Acids Res. 19, 319–325.

Fan, Z., Beresford, P.J., Zhang, D., Lieberman, J., 2002. HMG2 interacts with the nucleosome assembly protein SET and is a target of the cytotoxic T-lymphocyte protease granzyme A. Mol. Cell. Biol. 22, 2810–2820.

Fan, Z., Beresford, P.J., Oh, D.Y., Zhang, D., Lieberman, J., 2003a. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. Cell 112, 659–672.

Fan, Z., Beresford, P.J., Zhang, D., Xu, Z., Novina, C.D., Yoshida, A., Pommier, Y., Lieberman, J., 2003b. Cleaving the oxidative repair protein Ape1 enhances cell death mediated by granzyme A. Nat. Immunol. 4, 145–153.

Flint, S.J., 1986. Regulation of adenovirus mRNA formation. Adv. Virus Res. 31, 169–228.

Gorman, C.M., Gies, D., McCray, G., Huang, M., 1989. The human cytomegalovirus major immediate early promoter can be *trans*-activated by adenovirus early proteins. Virology 171, 377–385.

Greber, U.F., Webster, P., Weber, J., Helenius, A., 1996. The role of the adenovirus protease on virus entry into cells. EMBO J. 15, 1766–1777.

Halbert, D.N., Cutt, J.R., Shenk, T., 1985. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. J. Virol. 56, 250–257.

Haley, K.P., Overhauser, J., Babiss, L.E., Ginsberg, H.S., Jones, N.C., 1984. Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products. Proc. Natl. Acad. Sci. U. S. A. 81, 5734–5738.

Haruki, H., Gyurcsik, B., Okuwaki, M., Nagata, K., 2003. Ternary complex formation between DNA-adenovirus core protein VII and TAF-Ibeta/SET, an acidic molecular chaperone. FEBS Lett. 555, 521–527.

Haruki, H., Okuwaki, M., Miyagishi, M., Taira, K., Nagata, K., 2006. Involvement of template-activating factor I/SET in transcription of adenovirus early genes as a positive-acting factor. J. Virol. 80, 794–801.

Hosokawa, K., Sung, M.T., 1976. Isolation and characterization of an extremely basic protein from adenovirus type 5. J. Virol. 17, 924–934.

Iftode, C., Flint, S.J., 2004. Viral DNA synthesis-dependent titration of a cellular repressor activates transcription of the human adenovirus type 2 IVa2 gene. Proc. Natl. Acad. Sci. U. S. A. 101, 17831–17836.

Johnson, J.S., Osheim, Y.N., Xue, Y., Emanuel, M.R., Lewis, P.W., Bankovich, A., Beyer, A.L., Engel, D.A., 2004. Adenovirus protein VII condenses DNA, represses transcription, and associates with transcriptional activator E1A. J. Virol. 78, 6459–6468.

Jones, N., Shenk, T., 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell 17, 683–689.

Kawase, H., Okuwaki, M., Miyaji, M., Ohba, R., Handa, H., Ishimi, Y., Fujii-Nakata, T., Kikuchi, A., Nagata, K., 1996. NAP-I is a functional homologue of TAF-I that is required for replication and transcription of the adenovirus genome in a chromatin-like structure. Genes Cells 1, 1045–1056.

Kreppel, F., Biermann, V., Kochanek, S., Schiedner, G., 2002. A DNA-based method to assay total and infectious particle contents and helper virus contamination in high-capacity adenoviral vector preparations. Hum. Gene Ther. 13, 1151–1156.

Lee, C.K., Shibata, Y., Rao, B., Strahl, B.D., Lieb, J.D., 2004. Evidence for nucleosome depletion at active regulatory regions genome-wide. Nat. Genet. 36, 900–905.

Maizel Jr., J.V., White, D.O., Scharff, M.D., 1968. The polypeptides of adenovirus. II. Soluble proteins, cores, top components and the structure of the virion. Virology 36, 126–136.

Meier, J.L., Stinski, M.F., 1996. Regulation of human cytomegalovirus immediate-early gene expression. Intervirology 39, 331–342.

Montell, C., Fisher, E.F., Caruthers, M.H., Berk, A.J., 1982. Resolving the functions of overlapping viral genes by site-specific mutagenesis at a mRNA splice site. Nature 295, 380–384.

Okuwaki, M., Nagata, K., 1998. Template activating factor-I remodels the chromatin structure and stimulates transcription from the chromatin template. J. Biol. Chem. 273, 34511–34518.

Olive, D.M., al-Mulla, W., Simsek, M., Zarban, S., al-Nakib, W., 1990. The human cytomegalovirus immediate early enhancer-promoter is responsive to activation by the adenovirus-5 13S E1A gene. Arch. Virol. 112, 67–80.

Ornelles, D.A., Shenk, T., 1991. Localization of the adenovirus early region 1B 55-kilodalton protein during lytic infection: association with nuclear viral inclusions requires the early region 4 34-kilodalton protein. J. Virol. 65, 424–429.

Parks, R.J., Chen, L., Anton, M., Sankar, U., Rudnicki, M.A., Graham, F.L., 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. Proc. Natl. Acad. Sci. U. S. A. 93, 13565–13570.

Pilder, S., Logan, J., Shenk, T., 1984. Deletion of the gene encoding the adenovirus 5 early region 1b 21,000-molecular-weight polypeptide leads to degradation of viral and host cell DNA. J. Virol. 52, 664–671.

Pilder, S., Moore, M., Logan, J., Shenk, T., 1986. The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. Mol. Cell. Biol. 6, 470–476.

Prage, L., Pettersson, U., 1971. Structural proteins of adenoviruses. VII. Purification and properties of an arginine-rich core protein from adenovirus type 2 and type 3. Virology 45, 364–373.

Rekosh, D.M., Russell, W.C., Bellet, A.J., Robinson, A.J., 1977. Identification of a protein linked to the ends of adenovirus DNA. Cell 11, 283–295.

Russell, W.C., Laver, W.G., Sanderson, P.J., 1968. Internal components of adenovirus. Nature 219, 1127–1130.

Sandig, V., Youil, R., Bett, A.J., Franlin, L.L., Oshima, M., Maione, D., Wang, F., Metzker, M.L., Savino, R., Caskey, C.T., 2000. Optimization of the helper-dependent adenovirus system for production and potency in vivo. Proc. Natl. Acad. Sci. U. S. A. 97, 1002–1007.

Schaack, J., Bennett, M.L., Colbert, J.D., Torres, A.V., Clayton, G.H., Ornelles, D., Moorhead, J., 2004. E1A and E1B proteins inhibit inflammation induced by adenovirus. Proc. Natl. Acad. Sci. U. S. A. 101, 3124–3129.

Seo, S.B., McNamara, P., Heo, S., Turner, A., Lane, W.S., Chakravarti, D., 2001. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. Cell 104, 119–130.

Seo, S.B., Macfarlan, T., McNamara, P., Hong, R., Mukai, Y., Heo, S., Chakravarti, D., 2002. Regulation of histone acetylation and transcription by nuclear protein pp32, a subunit of the INHAT complex. J. Biol. Chem. 277, 14005–14010.

Shenk, T.E., 2001. Adenoviridae: the viruses and their replication, In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, Third ed. Lippincott William and Wilkins, Philidelphia.

Spector, D.J., 2007. Default assembly of early adenovirus chromatin. Virology 359, 116–125.

Straus, S.E., Ginsberg, H.S., Rose, J.A., 1975. DNA-minus temperature-sensitive mutants of adenovirus type 5 help adenovirus-associated virus replication. J. Virol. 17, 140–148.

Sullivan, C.S., Grundhoff, A.T., Tevethia, S., Pipas, J.M., Ganem, D., 2005. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. Nature 435, 682–686.

Thomas, G.P., Mathews, M.B., 1980. DNA replication and the early to late transition in adenovirus infection. Cell 22, 523–533.

Weber, J.M., Khittoo, G., Bhatti, A.R., 1983. Adenovirus core proteins. Can. J. Microbiol. 29, 235–241.

Witting, S.R., Brown, M., Saxena, R., Nabinger, S., Morral, N., submitted for publication. Helper-dependent adenovirus-mediated shRNA expression in the liver activates the interferon response.

Workman, J.L., 2006. Nucleosome displacement in transcription. Genes Dev. 20, 2009–2017.

Xue, Y., Johnson, J.S., Ornelles, D.A., Lieberman, J., Engel, D.A., 2005. Adenovirus protein VII functions throughout early phase and interacts with cellular proteins SET and pp32. J. Virol. 79, 2474–2483.