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Viral and Cellular Interactions During Adenovirus DNA Replication

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Abstract

Adenoviruses represent ubiquitous and clinically significant human pathogens, gene-delivery vectors, and oncolytic agents. The study of adenovirus-infected cells has long been used as an excellent model to investigate fundamental aspects of both DNA virus infection and cellular biology. While many key details supporting a well-established model of adenovirus replication have been elucidated over a period spanning several decades, more recent findings suggest that we have only started to appreciate the complex interplay between viral genome replication and cellular processes. Here we present a concise overview of adenovirus DNA replication, including the biochemical process of replication, the spatial organization of replication within the host cell nucleus, and insights into the complex plethora of virus-host interactions that influence viral genome replication. Finally, we identify emerging areas of research relating to the replication of HAdV genomes.

Keywords

Adenovirus; DNA; virus; viral replication compartment; liquid-liquid phase separation

Introduction:

Adenoviruses (family *Adenoviridae*) are medium-sized (90–100 nm), non-enveloped viruses with an icosahedral nucleocapsid containing a double-stranded DNA genome. The human adenoviruses (HAdV) comprise 7 species of the Mastadenovirus genus (A-G), including over 50 serotypes as defined by the absence of serological cross-neutralisation [1–3]. Serotypes of the Mastadenovirus C species, in particular serotypes 2 and 5 (Ad2 and Ad5), are the best characterized in regard to their molecular biology, and are often utilized experimentally to study adenovirus DNA replication and other aspects of adenovirus biology. HAdV are prolific pathogens that represent a significant human disease burden [4]. In addition to their

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clinical importance as pathogens, HAdV can also be utilized scientifically and clinically as gene expression vectors, oncolytic agents, and vaccine vectors [5–9]. Furthermore, the study of HAdV as a prototype DNA virus has provided fundamental insights into important cellular processes, including DNA replication, the cellular DNA damage response (DDR), and RNA processing [10–15]. HAdV therefore present excellent opportunities to investigate fundamental aspects of DNA virus and cellular biology.

Viruses are obligate intracellular pathogens, and must therefore infect a host cell and co-opt cellular processes in order to replicate viral genomes and ultimately produce infectious progeny. HAdV replicates in the host cell nucleus, utilizing components of the host nuclear machinery for viral processes, including expression of viral genes, and replication of viral DNA genomes. Following attachment and entry, viral particles undergo a complex process of disassembly to yield viral nucleocapsids, which are transported along microtubules to the nuclear pore complex, where viral genomes are imported into the nucleus [16–18]. Replication of the HAdV genome generates *de novo* genomes that are required for several essential viral processes, including further amplification of viral genome copy number and expression of viral late genes necessary for production of viral particles. In addition, viral genomes are also packaged into particles to produce infectious progeny virions (Figure 1). Therefore, replication of HAdV genomes is essential for the production of viral progeny. Thus, our understanding of viral genome replication fundamentally underpins our understanding of HAdV infection. Decades of work have contributed to a detailed model of biochemical processes of HAdV DNA replication, defining its minimal requirements *in vitro*, characterizing the replication machinery, and elucidating the fundamental mechanism [12,13,19]. However, viral genome replication within the host nuclear environment presents a number of challenges as well as opportunities. The virus must redirect cellular processes and co-opt cellular proteins, manipulate the nuclear architecture, and create its own nuclear sub-compartments to facilitate viral genome replication. Here we present an overview of adenovirus DNA replication, including the biochemical mechanism of replication, the spatial organization of genome replication within the host cell nucleus, and the recruitment of cellular proteins to replicating viral genomes. In doing so, we highlight the importance of understanding adenovirus replication in the context of the complex nuclear environment, and identify emerging areas of research relating to the replication of HAdV genomes.

Replication of the adenovirus genome:

Following entry into the nucleus, viral DNA genomes initiate a program of viral early gene expression that includes expression of genes encoding components of the viral DNA replication machinery. DNA replication machinery assembles on viral genomes and replication takes place. This enables productive infection, further genome amplification, and the full complement of viral gene expression. In this section we cover key viral components required for viral DNA replication and discuss how they function in concert to replicate viral genomes.

The adenovirus genome:

HAdV contain a linear, double-stranded DNA genome, typically 30-36 kb in length. By utilizing overlapping open reading frames and allowing transcription from both strands of the genome, as well as alternate splicing to maximize coding potential, this small viral genome encodes multiple genes (Figure 2). Viral early genes encode, amongst other proteins, the pre-terminal protein (pTP, encoded by E2B), DNA polymerase (Ad Pol, E2B), and DNA-binding protein (DBP, E2A), which together constitute the viral replication machinery. Late genes including the major late transcriptional unit (MLTU) are expressed following the onset of viral DNA replication, and encode the capsid and packaging proteins required for assembly of viral particles and encapsidation of the viral genome [20–22]. The MLTU comprises 5 transcriptional units (L1-L5), which are typically expressed from the major late promoter (MLP) utilizing alternate splicing and differential polyadenylation [23–27]. IX and IVa2, which are not encoded within the MLTU are often described as intermediate-late genes, since pIX and IVa2 facilitate the transcription of MLTU genes, in addition to their role in virion assembly [28–33]. The central coding region of the genome is flanked by inverted terminal repeats (ITR) of approximately 100 bp at each of the 5’ and 3’ ends. The terminal ends of each ITR possess the origin of replication. These origins span approximately 50 bp and contain a minimal core origin at the extreme terminal ends, as well as an auxiliary origin. The core origin sequence contains a binding site for pTP and Ad Pol, while the auxiliary origin contains binding sites for the cellular transcription factors Nuclear Factor 1 (NF1) and the POU domain containing protein Oct-1 [34,35]. These proteins function with DBP to make up the pre-initiation complex that associates with the origin and initiates replication [13,35]. In addition, one end of the genome also contains a packaging sequence (ψ) proximal to the ITR. This packaging sequence can be bound by viral packaging proteins, and is required for encapsulation (packaging) of the viral genome within the viral particle [36,37]. Viral genomes present within capsids also have two copies of a mature form of the viral terminal protein (TP) covalently attached to the 5’ ends, resulting from the addition of pTP during replication and its subsequent proteolytic cleavage during virion maturation [38].

The viral replication machinery:

Three viral proteins - pTP, Ad Pol, and DBP - are essential for replication of the viral genome. Reconstitution of HAdV DNA replication *in vitro* has demonstrated that these viral proteins represent the minimal components necessary for replication, whilst the host proteins NF1 and Oct-1 can enhance replication [39–43]. pTP is a 76.5 kDa protein that forms a heterodimer with Ad Pol, and along with Ad Pol and DBP forms part of the DNA replication pre-initiation complex where it functions in replication priming and is covalently coupled to the viral DNA as part of this process. Following encapsidation of the viral genome, pTP is cleaved by the adenovirus protease at two sites to yield the 37 kDa mature form of TP via a processing intermediate (iTP) [44]. Thus, replicating viral DNA is associated with pTP, while mature TP is present on viral genomes within mature virions and on incoming viral genomes [38,45].

The Ad Pol is a 120 kDa protein that belongs to a subset of family B polymerases that utilize a protein primer for the initiation of replication [46,47]. Sequence comparisons to polymerases

of known structure, in particular the polymerase of the φ29 bacteriophage, have provided great insight into the likely structure of Ad Pol [48–50]. In addition, many biochemical and mutagenesis studies have characterized its functional domains and demonstrated the requirement of these domains for its activity [12,51–55]. The C-terminal half of Ad Pol contains the palm, finger, and thumb subdomains that are common to DNA polymerases and function in their 5' to 3' polymerase activity [12,56]. In common with many other DNA polymerases, Ad Pol contains a 3' to 5' exonuclease domain within its N-terminal half that is necessary for its proofreading activity [12]. An interesting feature of Ad Pol is the presence of two sub-domains, termed Terminal Protein Region 1 and 2 (TPR1 and TPR2), that are specific to family B polymerases that initiate replication by a protein-primed mechanism, and are thought to facilitate protein-primed replication [47,57–59].

DBP is a 70 kDa protein that likely performs several functions during HAdV genome replication. DBP was first isolated based on its ability to bind ssDNA [60], but can also bind dsDNA [61–63], and even RNA [64–66]. A combination of biochemical assays, electron microscopy, and x-ray crystallography have demonstrated that DBP binds to ssDNA in a co-operative manner, and can oligomerize to form a protein chain associated with ssDNA [43,60,67–69]. This binding along ssDNA is sequence-independent, with one DBP monomer every 10-15 nucleotides [70]. Although DBP has been best characterized in relation to its function in the replication of viral DNA, it has been implicated in a number of different processes including transcription, mRNA stability, and even capsid assembly [29,71–76]. DBP is essential for viral DNA replication recapitulated *in vitro* [77–79]. Temperature-sensitive DBP mutant viruses fail to replicate in infected cells at non-permissive temperatures [73,77–80]. DBP may contribute to viral DNA replication in multiple ways. Firstly, DBP is part of the replication pre-initiation complex and enhances the rate of initiation of replication [63,81–83]. DBP also enhances Ad Pol processivity, and can facilitate template elongation and strand displacement by de-stabilizing the dsDNA helix [84–86]. This ability to destabilize dsDNA is dependent on the multimerization of DBP on the displaced ssDNA during replication [87,88]. In addition, DBP protects ssDNA intermediates generated during strand displacement from nuclease digestion, and regulates annealing of displaced ssDNA [89,90].

The mechanism of AdV genome replication:

HAdV replication occurs via a mechanism of strand-displacement similar to φ29-like dsDNA bacteriophages of the *Podoviridae* family [35,47,58,91,92] (Figure 3). Multiple protein-protein and protein-DNA interactions between the viral replication machinery, NF1, Oct-1 and the origin of replication result in the formation of the pre-initiation complex [35,93,94]. NF1 and Oct-1 facilitate replication initiation by altering the origin conformation [35,95,96]. The initiation of viral genome replication occurs by a protein priming mechanism in which pTP is covalently coupled to what will be the first nucleotide of the nascent chain. In the case of Ad5, the Ad Pol first covalently attaches a dCMP nucleotide to Ser580 of pTP to yield pTP-dCMP (pTP-C) [97,98]. The generation of pTP-C acts as the first stage in the formation of the pTP-linked trinucleotide intermediate pTP-CAT. Although initiation occurs at position 4 of the template strand, a "jumping back" mechanism results in this pTP-CAT intermediate base pairing with positions 1-3 [99]. This is made possible by the presence of a short repeat sequence at the beginning of the AdV origin of replication [35]. DBP facilitates

initiation by enhancing binding of Ad Pol and NF1 to the origin of replication and enhancing coupling of the first nucleotide to pTP. This occurs on dsDNA, suggesting that DBP does not enhance initiation through origin unwinding, but instead may facilitate this process by modulating origin conformation [63]. After replication priming, the Ad Pol dissociates from pTP, and synthesis of the nascent strand can proceed. The pre-initiation complex disassembles, with NF1 dissociating early during initiation and Oct-1 displaced following progression of the replication fork through the origin [94]. Ad Pol elongates the nascent strand, facilitated by the dsDNA unwinding ability of DBP as it progressively oligomerizes along displaced ssDNA as the replication fork proceeds. This generates a new DNA duplex while displacing the non-template strand. Displaced ssDNA can subsequently be replicated to generate a new dsDNA genome. Interestingly, ssDNA replication intermediates do not appear to be short-lived, as they accumulate during infection [100–103]. However, functional consequences of ssDNA accumulation are unclear. The ITRs of displaced single strands anneal together either through intramolecular or intermolecular interactions to form new dsDNA origins of replication [104,105]. Thus, newly generated dsDNA genomes as well as ssDNA replication intermediates can enter into subsequent rounds of replication resulting in amplification of genome copy number that drives productive infection.

Spatial organization of adenoviral genome replication and the role of genome replication in viral late processes:

The replication of HAdV genomes takes place within the host cell nucleus, which is a complex environment home to a vast repertoire of cellular processes. Accordingly, HAdV must co-opt or antagonize many cellular pathways, while reorganizing the existing nuclear environment. These architectural changes include the formation of virus-induced membrane-less nuclear compartments that harbor viral genome replication, termed viral replication compartments (VRCs). The formation of VRCs represents a strategy that is common to DNA viruses which replicate in the nucleus and is thought to provide the means to organize and concentrate viral and cellular factors beneficial to the virus, while excluding factors that are inhibitory [106,107]. Despite their obvious importance, there is much we still do not understand about how VRCs form, how these compartments change as infection progresses, and how virus-host interactions influence the organization of viral processes at these sites. In this section we address the fate of incoming viral genomes in relation to initiation of VRCs at dedicated sites within the nucleus, discuss details of VRC formation, and review compartmentalization and spatial organization of HAdV genome replication at these sites. In addition, we discuss how viral genome replication is required for viral late processes, and the changes in VRC morphology that occur as infection progresses. In doing so, we highlight emerging questions regarding spatial organization of HAdV genome replication.

The fate of incoming viral genomes:

Following successful viral entry and import of the viral genome into the nucleus, several challenges must be overcome to begin replicating. Viral genomes must avoid cellular intrinsic antiviral defenses and homeostatic regulatory pathways such as the DDR that respond to the presence of foreign DNA and act to suppress viral gene expression and DNA

replication [14,15,108–112](**See also the review by P. Hearing in this issue**). Incoming HAdV genomes that avoid restriction by cellular pathways undergo replication and initiate VRC formation in close proximity to nuclear bodies (NBs) marked by the promyelocytic leukemia (PML) protein [113,114]. PML NBs regulate a number of important cellular processes including DNA repair, transcription regulation, cell senescence, apoptosis, and the interferon response, and have been proposed as major sites of the post-translational modification of proteins by small ubiquitin-like modifier (SUMO) [108,115–118]. The initiation of VRCs at or in close proximity to PML NBs is common amongst DNA viruses that replicate in the nucleus [106,119]. However, PML and components of PML NBs are recruited to sites associated with incoming genomes of DNA viruses and are known to restrict gene expression and replication of DNA viruses as part of an intrinsic antiviral defense. Indeed, many DNA viruses utilize strategies to antagonize this intrinsic defense [108,117–119]. This has long raised the question whether the relationship between incoming viral genomes and PML NBs is the result of a viral strategy to utilize these nuclear domains, or a cellular strategy to resist infection. A study on HAdV demonstrated that incoming viral genomes do not localize to PML NBs at early times post-infection, suggesting instead that ongoing HAdV replication may be required to recruit PML NB proteins to VRCs [108]. Thus, at least in the case of HAdV, initiation of VRCs at sites of PML NBs does not appear to result from a cellular response to target incoming viral genomes. PML NBs undergo a dramatic reorganization during HAdV infection, largely mediated by viral early proteins. For example, E4orf3 is responsible for a drastic redistribution of PML from spherical PML NBs into PML ‘tracks’ [120,121], while E1B-55K facilitates the proteasome-dependent degradation of DAXX to relieve DAXX-ATRX mediated suppression of viral gene expression [122,123]. It has also been suggested that PML NB proteins may be utilized by HAdV, since both anti-viral and pro-viral roles for PML NB proteins have been reported [118–126]. Interestingly, some PML NB proteins re-localize to VRCs during infection, including NDP55, specific isoforms of Sp100, and SUMO2/3 [124–126]. In the case of Sp100, the isoforms B, C, and HMG are reportedly sequestered at VRCs to inhibit their anti-viral function, while Sp100A is maintained in PML tracks so that it can be utilized to promote viral gene expression [126]. Therefore, while some PML NB proteins are degraded or sequestered to limit their negative impact on virus infection, it is possible that others may be utilized by the virus to promote viral processes. It is likely that as our understanding of nuclear architecture and spatial and biophysical properties associated with sites of PML NBs advances, so too will our understanding of the role played by PML NBs during HAdV infection.

The formation of viral replication compartments:

Multiple VRCs can be observed in adenovirus infected nuclei, with the number of VRCs initiated dependent on MOI, up to a point of saturation where addition of more virus does not result in an increase in VRC number [114]. This suggests that the number of VRCs formed is dependent on the number of incoming viral genomes, with each VRC likely initiated by a single genome. It also implies that the cell is only capable of supporting a limited number of VRCs. Evidence in support of this model can also be extrapolated from studies of other DNA viruses [127–131]. The factors limiting the maximum number of VRCs initiated within each nucleus is unknown. However, given that VRCs form at PML NBs which reside within

interchromosomal spaces, it is possible that VRC numbers may be limited by the number of sites viable for their initiation and growth. Interestingly, imaging of HAdV VRCs has revealed that host chromatin is excluded from these virus-induced domains, as they stain weakly for cellular histones and the DNA intercalator DAPI when compared to the rest of the nucleus [132–134] (**see also the review by H. Wodrich in this issue**). This suggests that VRC growth may require the reorganization of cellular chromatin. Exactly how cellular chromatin is excluded from VRCs is unknown, but viral proteins as well as chromatin remodeling factors and components of the cellular DDR have been implicated [132,135] (**See also the review in this issue by D. Avgousti**). The VRCs of HAdV and other DNA viruses also demonstrate the liquid-like ability to coalesce. Thus, as VRCs grow they may merge with nearby VRCs resulting in fewer, larger compartments that may contain progeny derived from more than one founder genome [131,136,137].

A particularly interesting question regarding the initiation and formation of viral VRCs is how the viral genomes and viral and cellular proteins are concentrated within these membrane-less compartments. An exciting emerging area of cell biology addresses the formation of biomolecular liquid-liquid condensates (LLCs) by the process of liquid-liquid phase separation (LLPS). It is now believed that many cellular membrane-less compartments exist as LLCs, including nuclear domains such as nuclear speckles, Cajal bodies and PML NBs [138,139]. The formation of LLCs can be driven by frequent low-affinity interactions between biomolecules, and thus can be facilitated by interactions with proteins containing intrinsically disordered regions (IDRs) and the interaction of proteins with nucleic acids. Hallmarks of LLCs include liquid-like properties. For example, they typically exhibit spherical or rounded morphologies and the ability to coalesce with similar condensates. These features are also exhibited by viral VRCs [106,131]. Furthermore, VRCs contain high concentrations of viral nucleic acids and viral proteins, and the presence of IDRs is a common feature in many viral proteins [140,141]. Indeed, many HAdV proteins contain or are predicted to contain IDRs including E1A, Penton, E1B-55K, and EIB-93R [142–144]. This has led to the suggestion that VRCs may be LLCs resulting from LLPS [107,145]. However, a recent study found that although the VRCs of HSV-1 exhibit many of the microscopic hallmarks of LLCs, they were not dissolved by treatment with 1,6-hexanediol, a feature of other cellular LLCs. Furthermore, the authors report that the recruitment of RNA Pol II to VRCs and its movement within VRCs are best explained by a model other than LLPS [146]. However, it is also necessary to consider that the role of LLPS in biological processes may be more subtle and complex than current prototype LLCs suggest [147]. Nonetheless, it remains an interesting notion that LLPS may play a role in formation of VRCs, including those of HAdV. Alternatively, understanding how such seemingly liquid-like structure might come about independently of classical LLPS, and how biophysical properties of these compartments may influence viral processes is likely to be equally important. Thus, how the properties of resident biomolecules influence formation of VRCs, and how the biophysical properties of VRCs influence HAdV DNA replication, remains an exciting and interesting question.

Viral replication compartments as the sites of viral genome replication:

VRCs play a role in compartmentalizing the infected nucleus, concentrating factors beneficial to the virus and thus providing an environment conducive to viral processes. Consistent with VRCs as the site of adenovirus DNA genome replication, the three viral replication proteins (pTP, Ad Pol and DBP) and the co-opted cellular protein NF1 are known to localize to VRCs [101,103,113,148,149]. Viral DNA can also be visualized at VRCs using fluorescent in-situ hybridization (FISH), or techniques that utilize the labelling of DNA through the incorporation of the nucleoside analogues such as 5-bromo-2'-deoxyuridine (BrdU), 5-ethynyl-2´-deoxyuridine (EdU) or 5-ethynyl-2'-deoxycytidine (EdC) during DNA replication [101–103,150–152]. Since HAdV infection induces shutoff of host DNA replication, these nucleoside analogues are preferentially incorporated into viral DNA during infection [150,153]. By labelling replicating viral DNA using short pulses of EdU, it is possible to mark replicating (nascent) DNA [150,154]. Furthermore, DNA replication activity can be detected in VRCs isolated from HAdV-infected cells [155]. Thus, VRCs have been confirmed as the *bona fide* sites of viral genome replication.

Adenovirus VRCs are also believed to be the sites of viral genome transcription and RNA processing, and may even act as sites of viral particle assembly and packaging [106,151]. Investigations into the structure of VRCs and the localization of cellular and viral factors to these virus-induced domains suggest that VRCs may facilitate the spatial organization of viral DNA replication, as well as the aforementioned viral processes. Electron microscopy (EM) and immunofluorescence microscopy studies utilizing nucleoside-analogue labelling and FISH identified the accumulation of viral ssDNA replication intermediates within compact fibrillar structures termed single-stranded DNA accumulation sites (ssDAS) [101–103] (Figure 4A–B). Labelling and visualization of replicating DNA using tritiated thymidine identified ongoing viral genome replication within ssDAS in only a limited number of VRCs, suggesting that replication is intermittent within the ssDAS [101]. In contrast, the same study found that viral genome replication was consistently detected in the surrounding fibrillo-granular areas of the nucleoplasm, termed the peripheral replicative zone (PRZ) (Figure 4A–B). Consistent with viral genome replication at the PRZ, Ad Pol and pTP predominantly label these sites, as does FISH of viral dsDNA [102,148]. Similarly, detection of viral RNA by FISH and the labelling of nascent RNA using tritiated uridine suggest that the PRZ is the major site of viral transcription [156]. However, another report suggest that DNA replicated at the PRZ subsequently moves out to the surrounding nucleoplasm, where both viral RNA and cellular RNA processing factors localize, suggesting that transcription of viral genomes and viral RNA biogenesis occurs in proximity to the PRZ [103]. These somewhat conflicting interpretations may represent spatial differences in the sites of transcription *vs.* RNA processing. However, in light of more recent findings that HAdV VRCs undergo morphological changes during the late phase of infection [136,157,158], the possibility that reported differences may represent changes in spatial organization of viral processes at different stages in the viral replication cycle must also be considered. Indeed, the fate and function of viral genomes that are displaced from VRCs remains an interesting question, given that the viral processes of DNA replication, transcription, and perhaps even packaging are thought to be closely associated spatially with VRCs.

Viral genome replication and late gene expression:

Replication of HAdV genomes is a pre-requisite for expression of viral late genes. This dependency on genome replication for late gene expression has been demonstrated in multiple studies that prevented viral DNA replication using inhibitors of DNA replication, inhibitors of protein synthesis, or mutant viruses incapable of DNA replication upon shifting to a non-permissive temperature [80,159–161]. Continuous replication does not appear to be essential for late gene expression, as ongoing late gene expression can be detected following inhibition of DNA replication provided that some viral genome replication has taken place prior to inhibition [80]. Studies in which cells were superinfected with adenovirus demonstrated that progression of the initial infection into the late phase prior to superinfection was not sufficient to support late gene expression of superinfecting virus if replication of this virus was inhibited [159,162]. This suggests that expression of late genes from the MLP may require genome replication *in cis*. Interestingly, inhibition of viral genome replication also results in an increase in expression of viral early genes [28,72,73,163–167]. It is therefore likely that a modification of the viral genome or an interaction with a trans-acting factor that is dependent on replication *in cis* is required to switch from early to late transcriptional programs. It has been suggested that cellular chromatin organizing protein CTCF may play a role in this switch, since CTCF interacts with viral genomes in a replication-dependent manner, and knockdown of CTCF attenuates DNA replication and late gene expression, but not early gene expression [168]. The authors of this study proposed that CTCF could promote HAdV genome replication and late gene expression by regulating viral chromatin, since CTCF is known to regulate cellular chromatin architecture and chromatin conformation of Kaposi's sarcoma-associated Herpesvirus (KSHV) and Epstein-Barr virus (EBV) genomes [169–171]. Others have suggested that viral core proteins or cellular histones associated with viral genomes during early stages of infection may need to be removed in a replication-dependent manner before late gene expression can proceed [135]. This hypothesis is particularly attractive given recently reported findings that CTCF can displace nucleosomes from DNA [172]. Ultimately, more work is required to understand mechanisms underlying the replication-dependent switch from early to late viral gene expression, which represents an essential feature of the adenovirus replication cycle.

Changes in spatial organization of viral replication compartments during late stages of infection:

VRCs form with the onset of viral DNA replication, following the early phase of infection. Although decades of work have contributed to our understanding of spatial organization of adenovirus genome replication at VRCs, it has only recently become apparent that changes in VRC morphology and nuclear organization during a final phase of infection may represent biologically significant diversions from the traditional view of VRCs organization [136,157,158]. During the late phase of infection, DBP-marked VRCs transition from the more commonly described spherical and crescent shaped morphologies, into structure that first appear more diffuse and broken up before forming ring-like assemblies of smaller globular regions marked by DBP. This suggests that early VRCs transition from an early to late

morphology as infection progresses (Figure 4C). These morphological changes coincide with the formation of structures recently referred to as virus-induced post-replicative (ViPR) bodies. These structures co-stain with DAPI, suggesting that they are enriched for DNA. In addition, ViPR bodies stained positive for viral core protein VII and packaging protein IVa2, but not capsid proteins pVI and IX [136,157,158]. Visualization of viral genomes by EdU pulse-chase or an ANCHOR3/Par3 *in vivo* DNA-tagging system revealed that, although viral genome replication occurs at VRCs throughout the course of infection, viral genomes produced late in infection accumulate at ViPR bodies [136,157] (**see also the review by H. Wodrich in this issue**). This led to the suggestion that ViPR bodies may function as sites of DNA accumulation, playing a role in packaging of viral genomes into virions. The nucleolar proteins Mybbp1A, nucleophosmin (NPM1), UBTF, and nucleolin also localized to ViPR bodies [136,157,158]. Depletion of NPM1 delayed ViPR body formation, increased resistance of viral genomes to nuclease digestion, and reduced production of infectious virions. Thus, the authors suggest that ViPR bodies contain viral genomes that have been remodelled in preparation for packaging into capsids, and that this remodelling is required for ViPR body formation [158]. However, it is uncertain to what extent these phenotypes may also be influenced by the proposed role of nucleophosmin during viral genome replication [173]. It is also noteworthy that although prevailing dogma suggests HAdV packages its genomes into pre-formed capsids as described by a sequential model of packaging [37], there is also evidence to support an alternate packaging model. A recent study that included immunofluorescence and electron microscopy identified viral DNA, the 52-55K packaging protein, and formed particles, as well as putative assembly intermediates, within the PRZ, leading to the suggestion that the PRZ is not only the site of viral DNA replication, but also the site of assembly and packaging [151]. The model proposed by the authors suggests that packaging proteins interact with nascent viral genomes and facilitate concurrent assembly and packaging as the viral genome is replicated. Rationalizing these different findings will likely require future work to elucidate in detail the fate of viral genomes, and the spatial and temporal regulation of key interactions between viral genomes and cellular and viral proteins that are indicative of viral processes such as packaging.

Recruitment of cellular proteins to replicating viral genomes:

Although HAdV DNA replication can be recapitulated *in vitro* with minimal components, it is becoming increasingly clear that viral DNA replication within the infected host-cell nucleus is more complex. In addition to NF1 and Oct-1, which play a key role in replication initiation, many other cellular proteins can interact with replicating viral DNA. These include proteins that interact with viral DNA as part of cellular antiviral or homeostatic pathways, proteins that are recruited to viral genomes to facilitate replication, and proteins that are recruited to viral genomes to facilitate other viral processes. It is also evident that viral genomes exist within the nucleus not just as naked viral DNA bound by replication or transcription complexes, but as viral chromatin associated with viral core proteins and/or cellular histones. In this section we highlight recent advances in identification of cellular proteins associated with replicating HAdV DNA and review roles of recruited cellular proteins in viral processes.

Identification of factors recruited to or excluded from replicating viral genomes:

Recent advances allow for identification of proteins on nascent DNA (iPOND) by incorporating EdU into replicating DNA, covalently linking EdU to biotin in a click reaction, and purifying the labelled DNA in association with interacting proteins[174–177]. This approach has been used in conjunction with mass spectrometry by us and others to identify cellular proteins associated with replicating viral DNA during infection with DNA viruses [150,154]. iPOND of HAdV identified cellular proteins involved in DNA replication, DNA repair, and chromatin remodelling, as well as proteins involved in transcription, RNA processing and nucleolar proteins. Identified cellular proteins representative of these processes were confirmed to localize to VRCs, validating their recruitment to the sites of viral genome replication [150]. It is interesting to note that cellular proteins recruited to replicating HAdV DNA include not only proteins involved in DNA replication, but also those that interact with nascent genomes to influence subsequent genome functions. The identification of cellular proteins involved in transcription and RNA processing supports this notion and is consistent with the close spatial relationship between these processes (**see earlier section:** Viral replication compartments as the sites of viral genome replication). In addition, many viral proteins were found to be associated with replicating viral DNA. These included the viral replication machinery (pTP, Ad Pol and DBP), as well as viral proteins involved in RNA splicing, packaging, and even capsid proteins [150]. This suggests that replication and transcription of viral genomes are intimately linked both spatially and temporally.

In addition to identification of cellular proteins recruited to replicating viral genomes, analysis of cellular proteins that were under-represented on replicating HAdV DNA compared to replicating host DNA also provided insight into host factors that may be actively excluded from viral genomes during replication. Amongst under-represented proteins were the components of the MRN complex - MRE11, RAD50, and NBS1 - consistent with known antagonism of the MRN complex by viral early proteins [11,178–183]. Interestingly, Claspin was the most underrepresented protein on replicating HAdV DNA compared to host DNA and was excluded from VRCs [150]. During replication stress and DNA damage, Claspin associates with the Chk1 kinase to facilitate Chk1-mediated signalling, slowing or stalling DNA replication as a result [184–186]. It therefore seems likely that exclusion of Claspin from replicating viral genomes is the result of a viral strategy to antagonize the otherwise inhibitory effects of the DDR. Another such under-represented protein, TFII-I decreased in abundance during WT HAdV infection, and was re-localized to foci distinct from VRCs. In contrast, TFII-I localized to VRCs during infection with an E4-deleted virus lacking key antagonistic functions provided by E4orf3 and E4orf6, and was not reduced in abundance [150]. TFII-I functions as a transcriptional repressor in many cellular processes [187], raising the possibility that TFII-I is prevented from interacting with replicating viral genomes to antagonize repression of viral gene expression. Interestingly, although many DDR proteins were excluded from replicating HAdV DNA, the structure-specific endonuclease subunit SLX4 was enriched [150]. SLX4 promotes DNA repair as part of multiple DDR pathways, functioning as a SUMO E3 ligase and coordinating structure-

specific endonucleases [188–193]. Depletion of SLX4 demonstrated that SLX4 promotes viral genome accumulation and protein production, suggesting SLX4 is recruited to replicating viral genomes where it functions to promote viral processes [150]. Thus, DDR proteins are not only antagonized by HAdV, but also exploited. In summary, comparing proteomes associated with replicating DNA in HAdV infected cells to uninfected cells presents an excellent tool to identify cellular factors that facilitate viral processes, as well as factors that are excluded from VRCs to prevent their interaction with replicating viral genomes.

Viral chromatin and the recruitment of nucleolar proteins:

Within the HAdV capsid, the viral genome exists as viral chromatin. Specifically, viral DNA in association with highly basic core proteins V, VII and Mu (μ) [17,37,194,195]. During uncoating, μ and V dissociate from viral genomes, while VII and viral genomes are imported into the nucleus as a VII-DNA complex [16,17,135]. As infection progresses, VII is lost from viral genomes, which become increasingly associated with cellular histones, in particular histone H3.3 [135,196–198]. The extent to which VII is lost, and cellular histones are added during the early phase of infection is still a matter of debate, but it is likely that a balance of both VII and cellular histones may be required for efficient viral early gene expression [135,196]. However, the extent to which viral chromatin must be modified to promote replication during infection is less clear. *In vitro* replication assays in which viral genomes in association with viral core proteins are used as a template indicate that compacted core-associated genomes undergo only limited replication, suggesting that modification of viral chromatin may be required for efficient DNA replication. The cellular histone-chaperone proteins SET (TAF-Iβ) and nucleosome assembly protein 1 (Nap-1 aka TFA-II) have been shown to enhance replication *in vitro* when viral chromatin is used as template [173,199,200]. The ability of these template-activating factors to promote replication is thought to be due to remodelling of viral chromatin, as has been suggested for SET [201–203].

It is also interesting to note that V, VII, and μ have been implicated in disruption of the nucleolus during HAdV infection, and redistribution of nucleolar proteins including nucleolin, nucleophosmin, nucleolar and coiled-body phosphoprotein 1 (NOLC1), and upstream-binding factor 1 (UBTF) [134,204–208]. Similarly to SET and Nap-1, nucleophosmin has been shown to promote replication of viral chromatin *in vitro,* raising the possibility that nucleophosmin is also able to modify the structure of viral chromatin to facilitate replication [173]. Furthermore, UBTF also appears to promote viral genome replication [208]. It is therefore interesting that nucleolar proteins - including NOLC1, treacle protein (aka TCOF1), and components of the RNA polymerase I (POL I) complex and the small subunit (SSU) processome - were identified by iPOND to associate with replicating HAdV DNA, and localized to VRCs [150]. NOLC1 and TCOF1 regulate ribosomal RNA biogenesis and processing via recruitment of POL I and SSU processome components [209–212]. Depletion of TCOF1 resulted in failure of NOLC1, POL I, and SSU processome components to localize to VRCs in HAdV infected cells, and reduced viral protein production and genome copy number [150]. This suggests that TCOF1 and TCOF1-mediated recruitment of POL I and SSU processome components promotes viral processes. Together, these findings suggest that redistribution of nucleolar proteins and their recruitment to VRCs represents a viral strategy to harness key nucleolar processes. Given the link between nucleolar proteins, viral core

proteins, and genome replication, investigating how these nucleolar proteins function to promote viral processes may also shed more light on how the nature of viral chromatin influences replication of viral genomes.

While it is likely that the modification of viral chromatin influences viral DNA replication, how the presence of cellular histones on viral DNA may influence this process is unknown. Although cellular histones may be present on both incoming and *de novo* viral genomes [133,135,213], it is unclear whether these histones are removed by the process of replication, or indeed whether they must be removed or temporarily displaced for replication to take place. What is clear is that encapsidated viral genomes are devoid of cellular histones [37,135]. This suggests either that cellular histones must be removed from viral genomes for encapsidation to occur, or that a proportion of viral genomes must avoid the addition of cellular histones altogether. Factor responsible for removal of cellular histones from viral genomes have not been identified. Thus, how adenovirus deals with the addition of cellular histones on its DNA to transcribe, replicate, and package genomes remains a fascinating question.

Conclusions and future perspectives:

Although many decades of research have contributed to a detailed model of HAdV DNA replication and to the characterization of viral replication machinery, we have only just begun to understand the complexity of HAdV genome replication in the context of the host-cell nucleus. Important questions regarding the formation and biophysical properties of VRCs remain. Furthermore, recent advances suggest that genome replication, transcription, and possibly even packaging are closely linked, both spatially and temporally. Thus, it will be interesting to determine how the vast repertoire of host proteins that interact with replicating viral DNA may impact not only viral genome replication, but also subsequent genome functions. Identification of morphological changes in VRCs raises further questions as to how viral late processes are organized spatially as infection progresses, and we can only begin to speculate as to how these late viral processes are coordinated in space and time to maintain concurrent DNA replication, transcription and packaging. Ultimately, future work aiming to elucidate in greater detail HAdV DNA replication in the context of the nuclear environment will be highly pertinent to our understanding of DNA viruses and nuclear processes.

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Abbreviations

Ad Pol	adenovirus DNA polymerase
Ad2	adenovirus serotype 2
Ad5	adenovirus serotype 5

BrdU	5-bromo-2’-deoxyuridine
DBP	DNA-binding protein
DDR	DNA damage response
EBV	Epstein-Barr virus
EdC	5-ethynyl-2’-deoxycytidine
EdU	5-ethynyl-2´-deoxyuridine
EM	electron microscopy
FISH	fluorescent in-situ hybridization
HAdV	human adenovirus
IDR	intrinsically disordered region
iPOND	identification of proteins on nascent DNA
iTP	intermediate terminal protein
ITR	inverted terminal repeats
KSHV	Kaposi’s sarcoma-associated Herpesvirus
LLC	liquid-liquid condensate
LLPS	liquid-liquid phase separation
MLP	major late promoter
MLTU	major late transcriptional unit
NB	nuclear bodies
NF1	nuclear Factor 1
NOLC1	nucleolar and coiled-body phosphoprotein 1
PML	promyelocytic leukemia
POL I	RNA polymerase I
PRZ	peripheral replicative zone
pTP	pre-terminal protein
ssDAS	single-stranded DNA accumulation sites
SSU	small subunit
SUMO	small ubiquitin-like modifier
TP	terminal protein

TPR1	terminal protein region 1
TPR2	terminal protein region 1
UBTF	upstream-binding factor 1
ViPR	virus-induced post-replicative
VRC	viral replication compartment
WT	wild-type
ψ	packaging sequence

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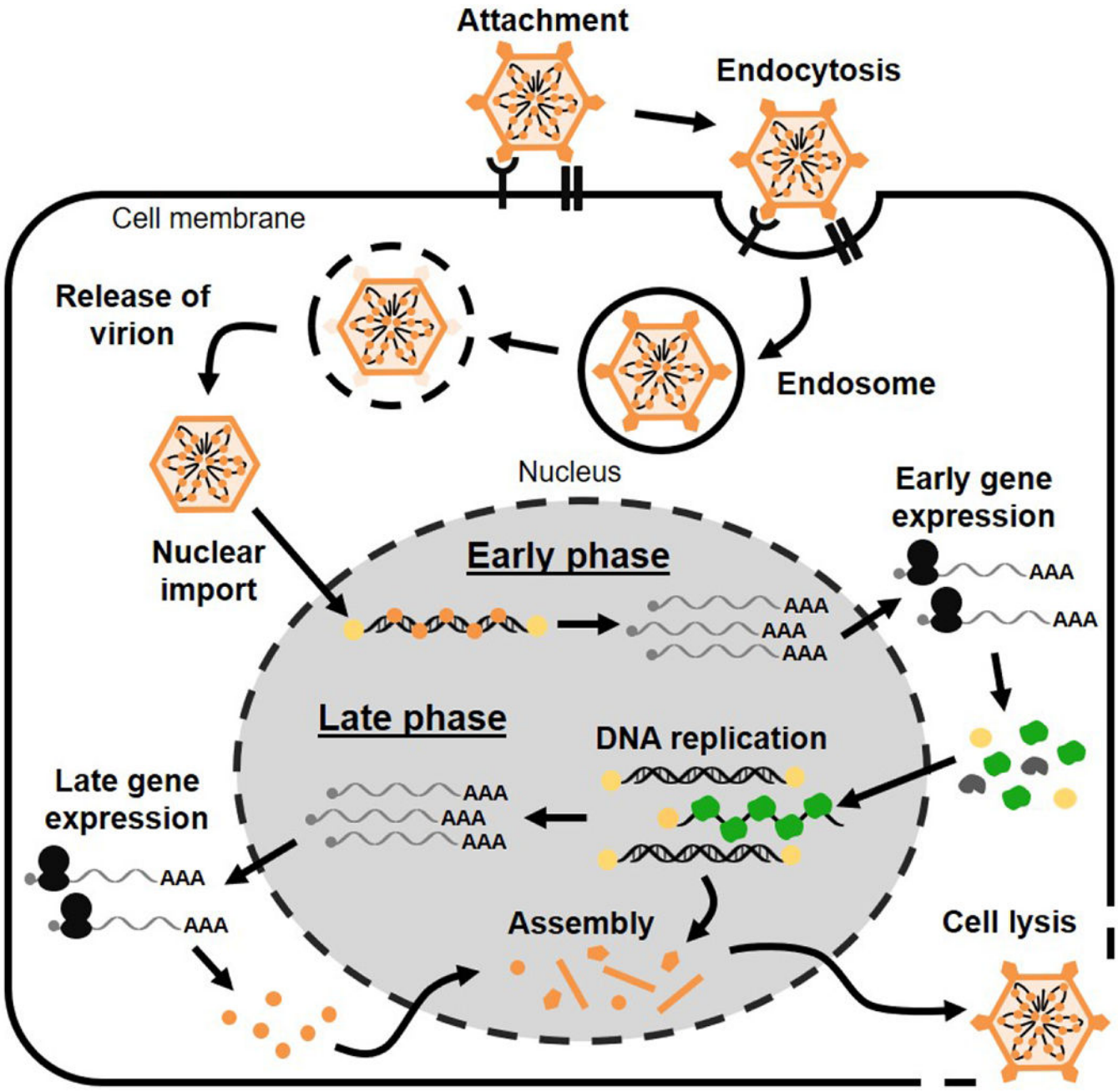

Figure 1. Overview of HAdV replication cycle.

Virus entry and import of viral genomes into the nucleus leads to a program of early gene expression that includes the viral replication machinery. The onset of viral DNA replication marks progression from the early to the late phase of infection, and is a pre-requisite for both late gene expression and virion assembly.

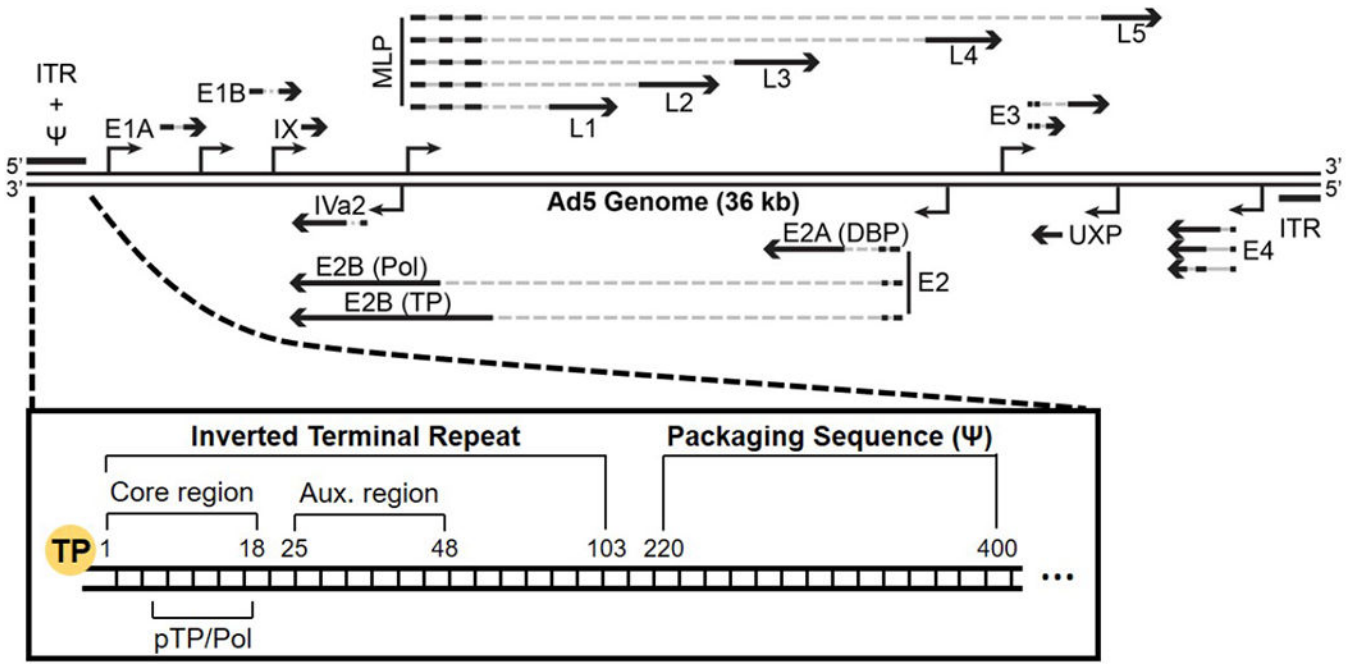

Figure 2. Schematic representation of the Ad5 genome.

The organization of genes within the central coding region is shown, as are inverted terminal repeats (ITR) and packaging domain (ψ). The zoom-in provides further details of the terminal end that contains the packaging domain, including the core region, auxiliary (Aux.) region, and binding site for pre-terminal protein and the viral polymerase (pTP/Pol).

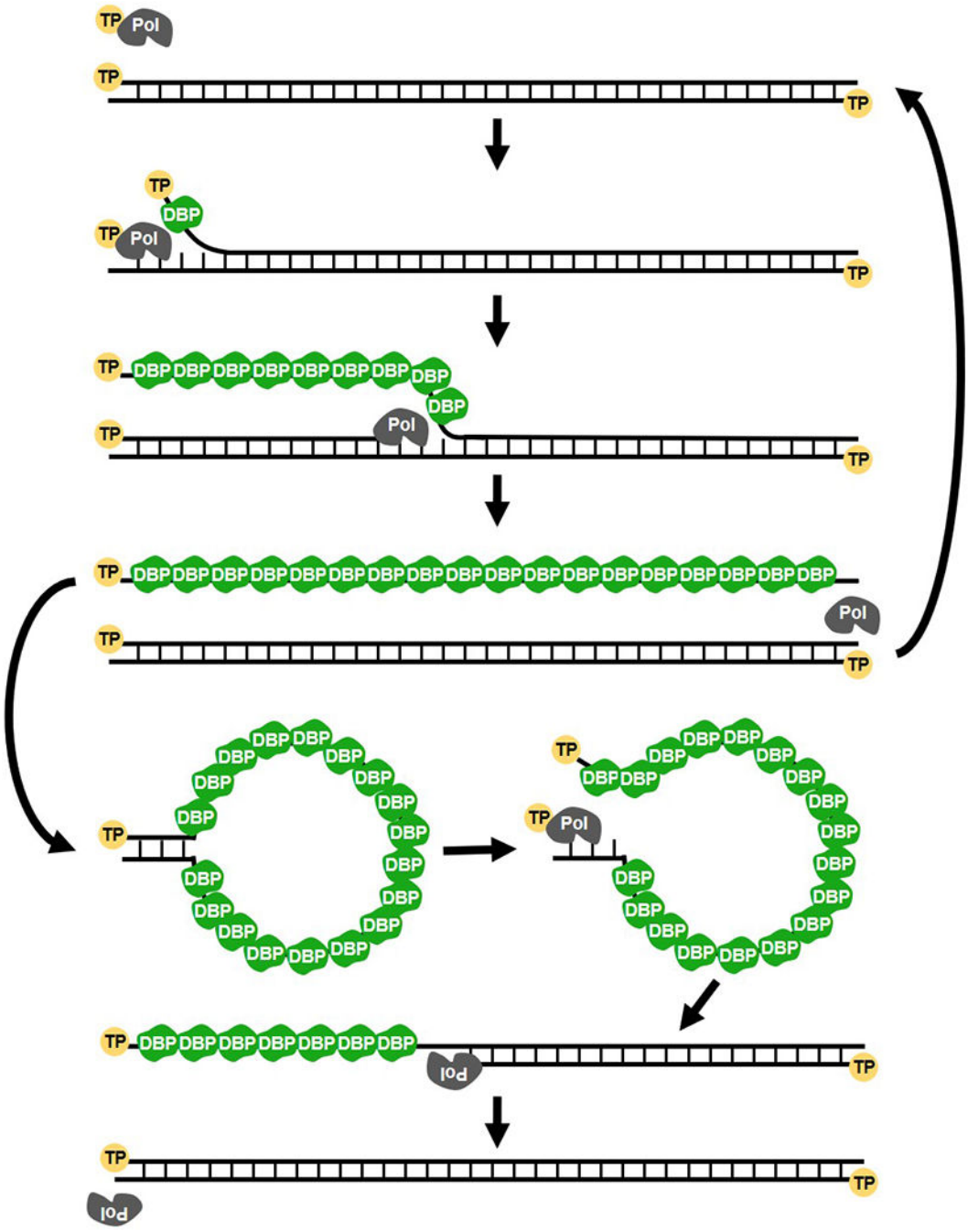

Figure 3. Replication of the HAdV genome by strand displacement.

Following replication initiation, the viral polymerase (Pol) elongates the nascent strand displacing the existing strand and generating a new dsDNA template. This process is aided by the viral DNA-binding protein (DBP), which oligomerizes along displaced ssDNA. Both the newly generated dsDNA genome and displaced ssDNA intermediate can be used as templates for further replication.

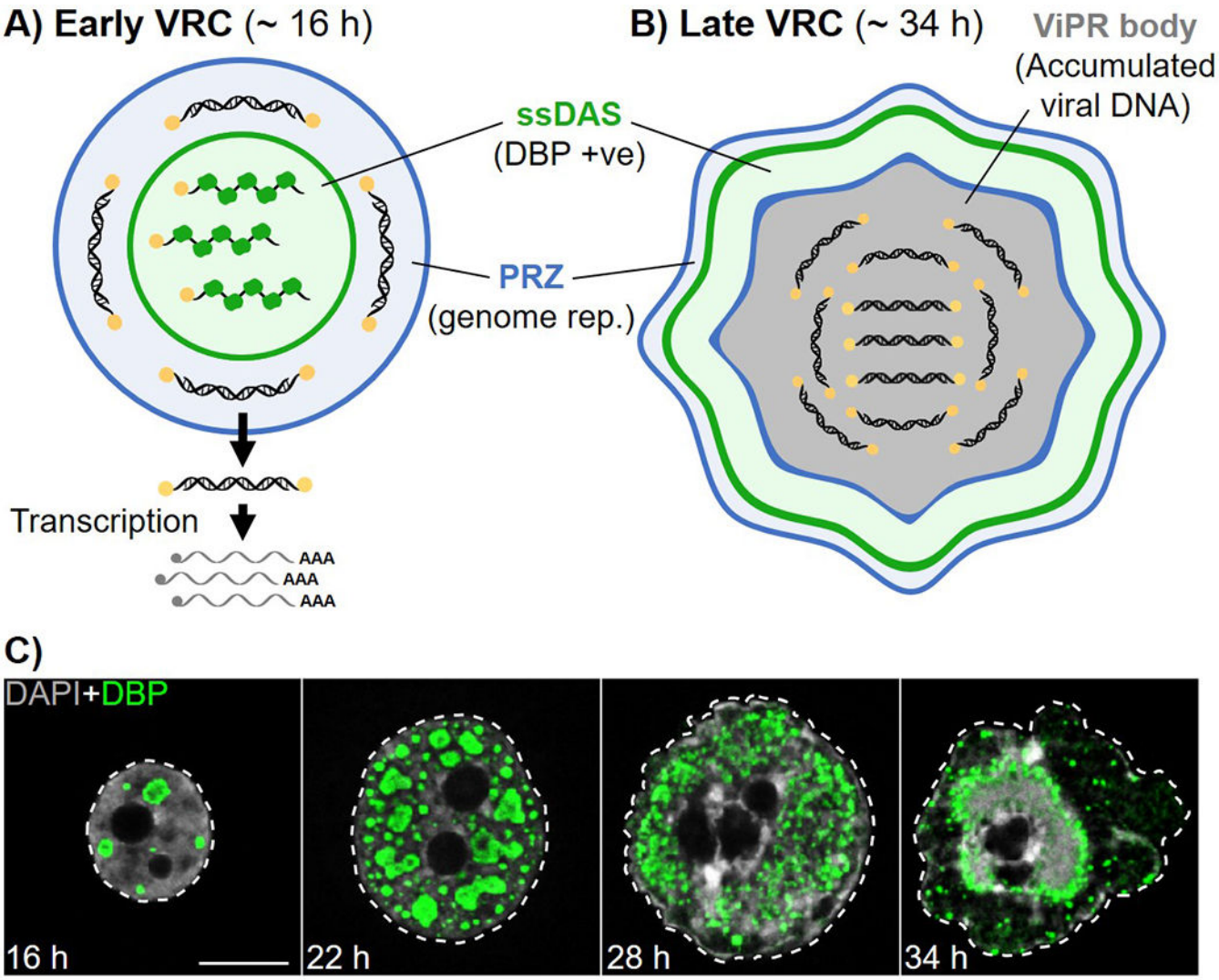

Figure 4. Viral replication compartments are reorganized during the late stage of infection.

A. Schematic representation of an early viral replication compartment (VRC), showing the single-stranded DNA accumulation site (ssDAS) in which ssDNA replication intermediates bound by the viral DNA-binding protein (DBP) are present (green), and the peripheral replicative zone (PRZ) where viral DNA replication takes place (blue). Viral dsDNA genomes move away from VRCs where transcription and RNA processing take place. **B.** Schematic representation of a late VRC including virus-induced post-replicative (ViPR) body. **C.** Human bronchial epithelial cells infected with Ad5 showing VRC morphology at 16, 22, 28 or 34 hours post-infection (h). Nuclei were visualized by confocal microscopy with DBP immuno-labelled (green), and DNA labelled with DAPI (grey). Dashed lines outline nuclei. Scale bar = 10 μm. Note, at 34 h VRCs can be observed as a ring surrounding a single large ViPR body.