

ADENOVIRUS CHROMATIN:
The Dynamic Nucleoprotein Complex Throughout Infection

by
Andrea Giberson

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Abstract

Adenovirus (Ad) is a widely studied DNA virus, but the nucleoprotein structure of the viral genome in the cell is poorly characterized. Our objective is to study Ad DNA-protein associations and how these affect the viral life cycle. Most of the viral DNA condensing protein, protein VII, is lost within a few hours of infection and this loss is independent of transcription. Cellular histones associate with the viral DNA after removal of protein VII, with a preferential deposition of H3.3. Micrococcal nuclease accessibility assays at 6 hpi showed laddering of the viral DNA, suggesting the genome is wrapped in physiologically spaced nucleosomes. Although viral DNA continues to associate with H3.3 at late times of infection, the overall level of association with histones is greatly reduced. Knockdown of the H3.3 chaperone HIRA had no effect on the viral life cycle suggesting that other H3.3 chaperones are involved. Our studies have begun to elucidate the nucleoprotein structure of Ad DNA in the infected cell nucleus.

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Abbreviations

Ad	-	Adenovirus
bp	-	Base pair
CAR	-	Coxsackie-Adenovirus receptor
ChIP	-	Chromatin immunoprecititation
DIG	-	Digoxigenin
dsDNA	-	double stranded DNA
dox	-	Doxycyclin
DRB	-	5,6-dichlorobenzimidazole 1- β -D-ribofuranoside
fig.	-	Figure
hdAd	-	Helper-dependant Adenovirus
hr	-	Hour
hpi	-	Hours post infection
IP	-	Immunoprecititation
ITR	-	Inverted terminal repeats
MEM	-	Minimal essential media
MLP	-	Major late promoter
MOI	-	Multiplicity of infection
MNase	-	Micrococcal nuclease
NI	-	Non infected
NPC	-	Nuclear pore complex
PBS	-	Phosphate-buffered saline
PCR	-	Polymerase chain reaction
RFP	-	Red fluorescent protein
RNase A	-	Ribonuclease A
sec	-	Seconds
ssDBP	-	single-stranded DNA binding protein
TAF	-	Template-activating factor
TP	-	Terminal protein
wtAd	-	Wild type Adenoviruses
YFP	-	Yellow fluorescent protein

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Chapter 1 - Introduction

Adenoviruses (Ad) are a well characterized family of dsDNA viruses, of which there are more than 100 species and serotypes (Berk, 2007). They are known to infect a wide range of host species, from reptiles and amphibians to birds and mammals. The first specimen was reported by Rowe et al. in 1953, and named after the adenoid tissue from which it was isolated (Rowe et al., 1953). This novel virus was soon associated with human respiratory infections as well as tumour formation in rodents (Hilleman and Werner, 1954; Yabe et al., 1962). Research on Ads exploded from that point forward, resulting in a wealth of information on both the virus itself and many fundamental processes of the cell.

These days, Ad is better known as a viral vector in gene therapy as it can be easily manipulated (relatively large exogenous sequences can be inserted into the genome) and it is relatively safe. However, Ad is still a common child related respiratory infectious agent, as many (Hilleman and Werner, 1954; Yabe et al., 1962) as 26% of hospitalized children present with Ad (Hilleman and Werner, 1954; Yabe et al., 1962). Understanding the basic biology of Ad infection, especially chromatin state of the viral DNA in the cell, can perhaps provide a target for antiviral treatment. It could also lead to advances in gene therapy, by making vectors more efficient. The importance of chromatin in the regulation of gene expression in the cell has been demonstrated time and again (Luger, 2006).

1.1 - Adenovirus biology

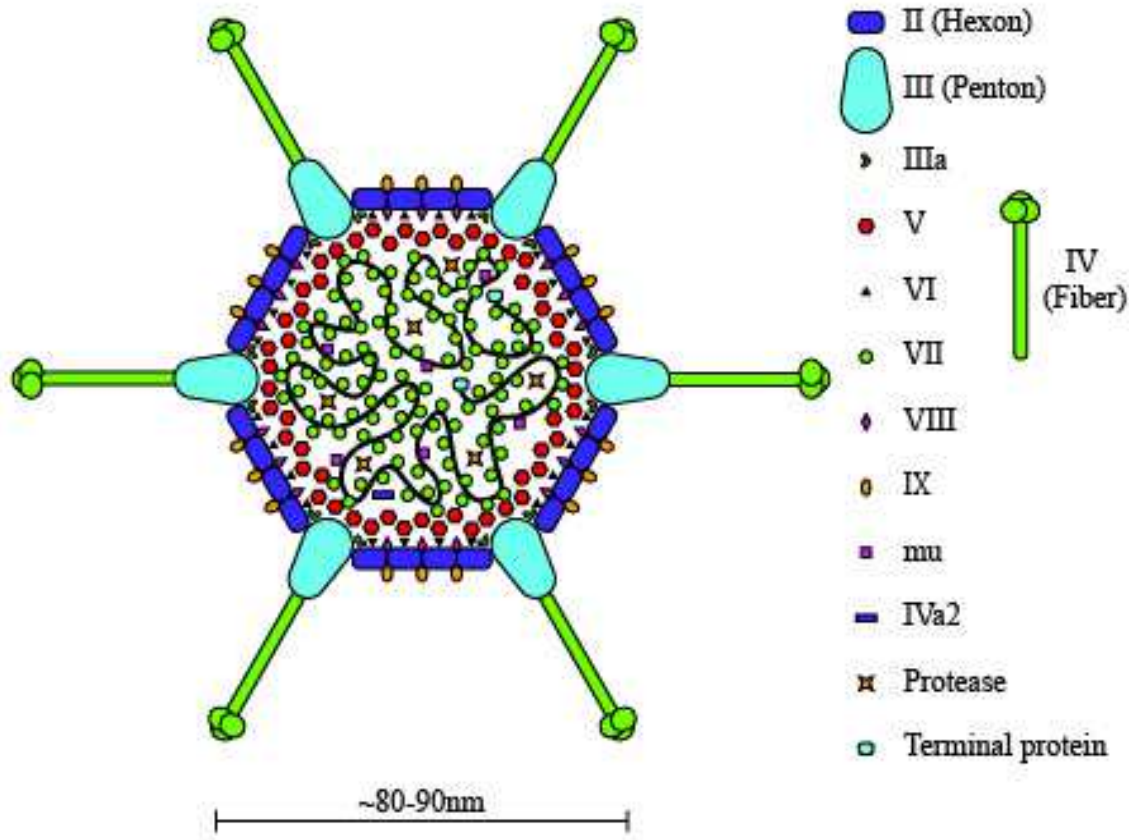
Structure

All adenoviruses have a conserved general structure, which is a non-enveloped icosahedral capsid, with a diameter of approximately 90nm (Fig. 1 A) (Berk, 2007) . The capsid is composed of three major (II, III and IV) and five minor (IIIa, IVa2, VI, VIII and IX) proteins (Christensen et al., 2008; Liu et al., 2010; Reddy et al., 2010; Russell, 2009). The hexons, which are a primary component of the capsid facets, are composed of a trimer of protein II, while the pentons at the vertices are made of five molecules of protein III. Fibre (trimer of IV) are the “arms” that are anchored at the pentons and give Ads their distinctive structure.

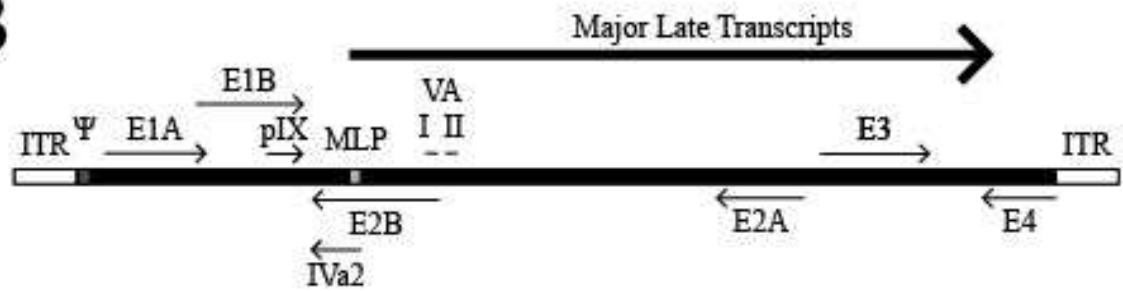
The nucleoprotein complex in the capsid has 3 protein components, core proteins VII, V and Mu (μ) (Chatterjee et al., 1986b; Maizel et al., 1968; Russell et al., 1968). Protein VII is highly basic and associates closely with the viral DNA, wrapping and condensing it (Mirza and Weber, 1982). The protein VII-DNA complex has a higher order structure, it has a dense core and twelve adenosomes, projections that are anchored at each vertex (Brown et al., 1975; Newcomb et al., 1984). Core protein V is the link between the nucleoprotein core and the capsid, by coating the protein VII-DNA core and interacting with the capsid components. Protein V has been shown to contact the base of the penton directly and hexons indirectly through proteins IIIa and VI (Chatterjee et al., 1985; Everitt et al., 1975; Liu et al., 2010; Matthews and Russell, 1998; Reddy et al., 2010; Stewart et al., 1993). Mu is a very small 19 amino acid protein produced when the viral protease cleaves the precursor protein, pre-Mu, during viral maturation (Anderson et al., 1989). Pre-Mu is thought to interact with the

Figure 1 - Adenovirus schematic. Adapted from (Giberson et al., 2012), with permission from publisher (See Appendix). (A) Model of the Ad5 virion. (B) Simplified Ad genome with main transcription units (E1-E4 and the major late transcripts). The transcripts from the major late promoter are extensively alternatively spliced to produce L1-L5 transcripts (not shown). Other transcripts include pIX, IVa2 and VA RNA I and II. The viral packaging element (Ψ) as well as the inverted terminal repeats (ITR) and the major late promoter (MLP) are shown. Of note, these features are not drawn to scale.

A



B



genome and participate with VII to condense the DNA. Cleavage of pre-Mu may release and relax the condensed DNA prior to viral entry (Pérez Berná et al., 2009). While the viral DNA does not interact directly with the capsid, it still has an important role to play in capsid integrity (Fabry et al., 2005; Silvestry et al., 2009). Virions packaged with DNA of sub-genomic length (<90%) are less stable than wtAd (Kennedy and Parks, 2009; Smith et al., 2009).

Genome and transcriptional organization

Ad genome organization is mostly conserved. As human Ad2 and Ad5, both of subclass C, are the most heavily studied Ads, their genome organization is best understood. Ad5 has a 36 kbp dsDNA genome which encodes 39 genes (Fig. 1 B). In accordance with viral nomenclature, genes expressed before DNA replication are called early and those expressed after are termed late (Davison et al., 2003). Ad has 4 early transcription units (E1a, E1b, E3 and E4) that encode proteins which play a role in coercing the cell, by altering the cellular micro-environment and subjugating the immune response. E2 proteins encode the viral DNA replication machinery. The majority of the late proteins are produced from extensive alternative splicing of a single transcript expressed from the major late promoter (MLP), leading to the transcription units L1-L5. Recently, L4-22K and L4-33K proteins, which play a role in regulating the MLP, have been found to be expressed from a novel promoter (Morris et al., 2010). Late-phase protein products generally encode the virion structural components. Four other late transcripts are expressed, pIX (minor capsid protein), IVa2 (involved in assembly and encapsidating the viral DNA into the immature virion) and VA RNA I and II (non-coding RNAs that block the interferon response).

Ad genomes have inverted terminal repeats (ITR) of 100 bp on both ends and a 5' covalently bonded terminal protein (TP), which are both essential for DNA replication. The ITR contain the origin of DNA replication and the TP acts as the primer for the new strand. The packaging sequence, of 150 bp, is adjacent to the left ITR.

Life cycle

The first step of the viral life cycle is attachment followed by entry into the cell. Ad binds the Coxsackie-Adenovirus receptor (CAR) with its fibre. CAR is the primary receptor for both Ad5 and the Coxsackie B virus, hence the name (Bergelson et al., 1997; Tomko et al., 1997). Secondary interactions between Ad penton and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins follow (Wickham et al., 1993). Alternative receptors have been observed; Ad can enter cells by interacting with heparin sulfate proteoglycans through the fibre shaft (Smith et al., 2003) or by interacting with blood factor IX or complement C4-binding protein (Kalyuzhniy et al., 2008; Shayakhmetov et al., 2005). Entry into the cell is accomplished by receptor-mediated endocytosis, and Ad escapes from the early endosome (Leopold et al., 1998). As the virion is trafficked to the nucleus along the microtubule network, it is slowly disassembled (Greber et al., 1993; Leopold et al., 1998). The protein VII-DNA complex enters the nucleus through the nuclear pore complex (NPC), while the remainder of the capsid remains at the nuclear membrane and is degraded (Chatterjee et al., 1986b; Greber et al., 1993; Leopold et al., 1998; Strunze et al., 2011). Viral DNA replication as well as virion assembly occurs within the nucleus. The entire viral life cycle, from entry to release of progeny by lysis takes 24 to 36 hours. A single infectious particle can produce as much as 10^4 daughter viruses per cell.

1.2 - Chromatin

Chromatin structure and organization

In eukaryotic cells, DNA is highly compacted and structured, with many levels of organization. At the base of this organization is the nucleosome, which is composed of 147 bp of DNA wrapped around a histone octamer. The histone octamer is composed of two sets of H2A–H2B dimers and one H3–H4 tetramer. Gone is the thinking that nucleosomes are “beads on a string”. It is now understood that nucleosomes play a critical role in gene regulation (Luger, 2006). Through post-transcriptional modifications of the histone tails to specific variants, histones serve as docking and recognition sites for regulatory proteins and provide information to guide gene expression (Jenuwein and Allis, 2001; Kouzarides, 2007).

Histone variants and their chaperones

Histone deposition is a regulated process that can happen by different mechanisms. The main instance of histone deposition is in S phase during DNA replication. Another is the deposition of histones after transcription of genes in G1 and G2 phases. There are specific histone variants and chaperones associated with each mechanism (Tagami et al., 2004). The canonical H3 variant is H3.1. It is expressed exclusively during S phase and is deposited by a replication-coupled mechanism (Smith and Stillman, 1989). The chromatin assembly factor I (CAF1) complex is specific to the H3.1 variant. The H3.2 variant differs from H3.1 by only one amino acid (Hake and Allis, 2006).

H3.3 is a histone variant that differs from H3.1 by only five amino acids, and is expressed ubiquitously. This variant is deposited in a replication-independent mechanism

((Tagami et al., 2004). H3.3 is associated with actively transcribed genes, transcription start sites, and enhancers, as well as telomeres and pericentric DNA-repeats. This association is conferred by the chaperones that recognize the H3.3 variant specifically. Chaperones HIRA and DAXX both have been shown to bind H3.3 preferentially (Drané et al., 2010; Goldberg et al., 2010; Lewis et al., 2010)(Drané et al., 2010; Goldberg et al., 2010; Lewis et al., 2010). HIRA has been demonstrated to shuttle H3.3 to actively transcribed regions, whereas DAXX tends to be associated with H3.3 at the telomeres and pericentric regions.

1.3 - Adenovirus and chromatin

Adenovirus and protein VII in early infection

The protein VII-DNA complex enters the nucleus through the NPC, leaving the remainder of the capsid at the nuclear membrane to degrade (Chatterjee et al., 1986b). While histone H1 has been shown to escort this DNA-protein complex through the NPC, it does not appear to have any structural role with Ad DNA (Trotman et al., 2001). At this stage in infection, protein VII protects the foreign DNA from activating the cell's DNA damage response (Karen and Hearing, 2011).

There is some debate as to the fate of protein VII after entry in the nucleus. Certain groups support the idea that protein VII remains associated with the viral DNA throughout early infection (Chatterjee et al., 1986b; Xue et al., 2005) while others maintain that VII is removed gradually from the DNA during this phase of the life cycle (Haruki et al., 2006; Komatsu et al., 2011). Yet another group has demonstrated that protein VII levels drop rapidly within the first few hours of infection, both overall and DNA-associated (Ross et al.,

2011). Another topic under debate is the requirement of active transcription to remove protein VII from the viral DNA (Chen et al., 2007; Ross et al., 2011; Xue et al., 2005).

The nature and properties of Ad core have been studied in cell-free systems designed to study viral replication. VII-wrapped DNA was found to be refractive to both transcription and replication (Matsumoto et al., 1993; Okuwaki and Nagata, 1998). These observations led to a search for proteins that could remodel and relax the viral core. Three such cellular proteins were identified: template activating factor Ib (TAF-Ib) [also known as SET (Matsumoto et al., 1993)], TAF-II [NAP-1 (Kawase et al., 1996)] and TAF-III [B23/nucleophosmin (Okuwaki et al., 2001)]. All three TAF's were able to stimulate replication in the cell-free system, while only TAF-Ib and TAF-II were able to stimulate transcription.

TAF-Ib forms a tertiary complex with the protein VII-wrapped DNA (Gyurcsik et al., 2006; Haruki et al., 2003; Xue et al., 2005). This complex increased the accessibility of the viral DNA to restriction enzymes and nucleases (Okuwaki and Nagata, 1998). It remains to be determined whether the increased accessibility is due to removal or shifting of protein VII. When TAF-Ib was knocked-down in infected cells there was a modest delay in viral gene expression, replication and viral yield (Haruki et al., 2006). At 4 hours post infection (hpi) there was no difference in the levels of protein VII association by chromatin immunoprecipitation (ChIP) in the TAF-Ib knockdown cells (Komatsu et al., 2011). It is likely that other cellular factors contribute to the removal or remodelling of the viral core when it enters the nucleus.

The evidence supporting the continued association of protein VII with the viral DNA

throughout the early phase of infection is as follows. Protein VII can be cross-linked to the viral DNA at nearly any stage of infection (Chatterjee et al., 1986a). Protein VII foci, which coincide with viral DNA, can be observed by immunofluorescence up to 10 hpi (Chen et al., 2007; Komatsu et al., 2011; Xue et al., 2005). Finally, VII was shown to be bound to the viral DNA up to 10 hpi by ChIP (Haruki et al., 2003; Johnson et al., 2004; Komatsu et al., 2011; Ross et al., 2011; Xue et al., 2005).

However, whether the levels of protein VII-association fluctuate or remain constant over the early phase of the viral infection is also contested. Some studies support an unchanging level of protein VII (Chatterjee et al., 1986a; Chen et al., 2007; Xue et al., 2005) while others support a model in which protein VII-association declines during this time period (Haruki et al., 2003; Karen and Hearing, 2011; Komatsu et al., 2011; Ross et al., 2011). ChIP experiments conducted by Komatsu et al., 2011, have shown that protein VII association with the viral DNA can vary depending of the loci of the genome examined. Between 1 and 10 hpi, association with the hexon loci, a late gene product, remains constant whereas association with the MLP declines over the same time period (Komatsu et al., 2011). This observation may be the key unifying these seemingly contradictory findings.

In a plasmid-based experiment, adding a small amount of protein VII was able to enhance transcription of the DNA when compared to naked DNA (Komatsu et al., 2011). This suggests that protein VII may play a role in preventing repressive structures from forming on promoter or regulatory regions. These results put forth a model in which protein VII is dynamically regulated throughout early infection, enough protein VII must be removed or remodelled in order to allow the transcription machinery to access to the viral

DNA, while some protein VII must remain to stimulate transcription.

The role transcription plays in removing or remodelling protein VII from the viral DNA is still in question. In some studies, inhibition of transcription led to retention of protein VII on the Ad DNA (Chen et al., 2007; Karen and Hearing, 2011), while other groups did not observe an effect (Komatsu et al., 2011; Ross et al., 2011).

The first expressed viral gene product, E1A, has been suggested to interact with protein VII and play a role in removing it from the viral genome (Chen et al., 2007; Johnson et al., 2004). However, this function is not essential, as removal of protein VII still occurs in the absence of E1A or active transcription (Komatsu et al., 2011; Ross et al., 2011).

Adenovirus and histones

Studies from the 1980s were separated into two views, one that suggested Ad viral DNA associates with cellular histones and one that it did not (Beyer et al., 1981; Daniell et al., 1981; Déry et al., 1985; Sergeant et al., 1979; Wong and Hsu, 1988). However, with advances in more sensitive biochemical techniques, this debated question has come under investigation once again. ChIP has been used to investigate the association of histones with Ad and Ad vectors (E1-deleted, replication defective, or helper-dependant (hdAd)) and has found that histones do associate with this foreign DNA within a few hours of infection, and as early as 1hpi (Komatsu et al., 2011; Ross et al., 2011; Ross et al., 2009). Using ChIP/re-ChIP experiments, it was shown that both histones and protein VII can be detected on the same piece of DNA (Komatsu et al., 2011). As the histones interact directly with the viral DNA, it is clear that some protein VII must be removed to allow the histone binding to occur.

As mentioned above, the exact mechanism by which protein VII is removed has yet to be elucidated.

Ad can infect both quiescent and replicating cells. Ad infection induces cell cycle progression, however this occurs as a result of viral gene expression. This suggests that any mechanism that chromatinizes Ad, is likely independent of the cell cycle. By ChIP, it was demonstrated that hdAd, E1-deleted Ad (Ross et al., 2011) and wt Ad (Komatsu and Nagata, 2012) preferentially associates with the histone three variant H3.3, supporting the hypothesis that chromatinization occurs in a replication-independent process. A similar observation was made with Herpes Simplex Virus 1 (HSV1), which was found to associate with H3.3 at early times in lytic infection (Placek et al., 2009). Knockdown of HIRA, an H3.3 specific chaperone, reduced the total association of H3 on the Ad and HSV1 DNA and reduced expression of viral genes. This indicates that efficient chromatinization of the HSV1 genome was required for optimal gene expression. This evidence supports the role of HIRA in the H3.3 deposition over DAXX, which is actively degraded during Ad infection by the viral E1B-55K in a proteasome-dependant manner (Schreiner et al., 2010). This may suggest a common mechanism for the chromatinization of dsDNA viruses, and also has parallels with the chromatinization of the male pro-nucleus after fertilization. The pro-nucleus is highly condensed by protamines, a small and highly basic protein, which must be removed to allow chromatinization. H3.3 is then deposited on the pro-nucleus DNA in a replication independent mechanism involving HIRA (Bonney et al., 2007). It would be intriguing if the chromatinization of dsDNA viruses co-opted the cell's natural pathways to deposit histones in the absence of DNA replication.

All four histones, H2A, H2B, H3 and H4, have been found associated with Ad DNA (Komatsu et al., 2011). This observation supports the fact that Ad DNA in the cell nucleus has been shown to be wrapped in physiologically spaced nucleosomes (Beyer et al., 1981; Bonnefoy et al., 2007; Daniell et al., 1981; Déry et al., 1985; Ross et al., 2011; Sergeant et al., 1979). The chaperone responsible for the deposition of H2A-H2B on Ad has not yet been identified. An estimation of the nucleosome coverage of the viral DNA is as much as 40% at 3hpi, with all regions of the genome represented in the fractions protected from micrococcal nuclease digestion (Sergeant et al., 1979).

As protein VII and histones have both been shown to associate with the same molecule of DNA at the same time, the viral chromatin is most likely unique and does not completely resemble cellular chromatin (Komatsu et al., 2011). HSV1 has been known to be chromatinized in its latent phase, in stable and regularly spaced nucleosomes. However in the HSV1 lytic cycle, the associated nucleosomes are “unstable” and generate heterologous sized pieces of DNA upon MNase digestion (Lacasse and Schang, 2010). It is unknown whether Ad associates into stable or unstable nucleosomes. Using electron microscopy, at late times in infection (16-18 hpi), nucleosomes were shown to be irregularly spaced, at approximately one tenth the density of cellular chromatin (3 versus 26 nucleosomes per μm of DNA) (Beyer et al., 1981). Whether this is representative of unstable nucleosomes, or the lack of available histones to deposit on the DNA because of the limited histone pool is unclear.

During HSV1 viral DNA replication, there is a switch in the preferential association of H3 variants on the genome, from H3.3 in the early phase to H3.1 during and after DNA

replication (Placek et al., 2009). It is unknown if a similar switch occurs with Ad. As Ad infection results in a shut-off of host cell protein synthesis, including histone gene expression (Hodge and Scharff, 1969; Tallman et al., 1977), Ad chromatin may proceed from H3.3 nucleosome bound to pre-protein VII bound in preparation for packaging into the capsid without a H3.1-bearing intermediate.

Adenovirus and chromatin late in infection

To complete the viral life cycle, the DNA must be condensed and packaged within the newly synthesized capsid. There are no cellular proteins within the virion, meaning that histones must be removed from the viral DNA and replaced with pre-protein VII, the immature precursor of protein VII. Little is known about the switch from nucleosome-bound to pre-protein VII-wrapped DNA. Expression of histones in the cell is tightly regulated by the cell cycle and coincides with cellular DNA replication. Although Ad infection induced cell replication, there is a dramatic decline in histone synthesis (Hodge and Scharff, 1969; Tallman et al., 1977). It is possible that the switch between histones and pre-protein VII is a passive one, with the pool of available histones drops while pre-protein VII amounts increase (Brown and Weber, 1980; Daniell et al., 1981; Déry et al., 1985).

The deposition of pre-protein VII on the viral DNA is likely mediated by a cellular chaperone, as cell free systems found that mixing purified pre-protein VII with DNA led to an insoluble complex (Burg et al., 1983). In co-IP experiments, TAF-III/nucleophosmin, was found to have a greater affinity for pre-protein VII than for the mature protein VII (Samad et al., 2007). In the same cell free system, if TAF-III was added to the system, pre-protein VII was deposited on the DNA, suggesting that TAF-III may be the cellular chaperone

responsible for depositing pre-VII on the Ad DNA. Further studies are required to confirm this hypothesis.

Once the pre-protein VII-wrapped DNA is packaged within the immature capsid, the final step of the viral life cycle occurs, maturation. Along with a number of other virion protein, pre-protein VII is cleaved by the viral protease (Berk, 2007). In this manner, the mature Ad is formed and is ready to subsequent infections to perpetuate the virus life cycle.

1.4 - Hypothesis

We hypothesize that effective chromatinization of the replication-competent Ad genome is a fundamental step of the viral life cycle in a productive infection, and that this is a dynamic process in which the cellular proteins involved will change during the different phase of the Ad life cycle.

1.5 - Objectives

The objectives of this project are three-fold. First to determine the dynamics of protein VII removal and deposition throughout the viral life cycle. Second, to examine the chromatinization of Ad DNA, such as the deposition of chromatin protein (total H3 and H3.1 vs H3.3) in the early phase (before DNA replication) and late phase of infection. Third, to investigate cellular chaperones that are necessary for the above mentioned processes

1.6 - Approach

Protein VII dynamics in the presence and absence of transcription will be investigated using a transcription inhibitor (DRB) and monitored by Western Blot and by chromatin

immunoprecipitation (ChIP). The DNA-chromatin structure will be investigated using a classic micrococcal nuclease digestion assay followed by southern blot. Cells lines expressing H3.1-YFP or H3.3-YFP will be used to investigate the association of the H3 and its variants with the viral DNA at various time points after infection using ChIP followed by qPCR. The role of active transcription on the deposition of histones will also be investigated, repeating the ChIP experiments in the presence of a transcription inhibitor (DRB). Cell lines that inducibly knockdown HIRA, the H3.3 specific cellular chaperone, were established using a lentiviral system. When HIRA was knocked down, the effect on viral transcription and life cycle was be investigated by Western Blot, and viral titer.

Chapter 2 - Methods

2.1 - Cell culture

Cell culture and growth

A549 (ATCC) and 293 cells (obtained from Frank Graham, McMaster University) (Graham et al., 1977) were maintained in culture medium (Minimal essential media (MEM), 10% foetal bovine serum, 2 mM Glutamax, and 1X antibiotic-antimycotic (Invitrogen)). Cell growth was monitored by microscopy as well as by a crystal violet staining protocol, described previously in (Hubberstey et al., 2002).

Transfections

Cells were grown to the desired confluency (50 to 90% depending on length of the experiment) in 6 or 12 well plates and transfected using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol.

Cell line creation

The plasmid, pCMV-H3.3-YFP expressing yellow fluorescent protein (YFP) – tagged H3.3 was obtained from Addgene, and the construction of pCMV-H3.1-YFP was described previously (Ross et al., 2011). The H3.1-YFP and H3.3 YFP genes were subcloned into expression plasmids containing an internal ribosome entry site – hygromycin resistance cassette, and designated pJR131 and pJR132, respectively. These plasmids were stably transfected into A549 cells and individual colonies were selected with 200µg/mL of hygromycin (Invitrogen), then maintained in culture medium supplemented with 50µg/mL of

hygromycin, creating A549 H3.1-YFP and A549 H3.3-YFP cells.

A549 – Scrambled and A549 – HIRA cells were created by infecting A549 cells with lentivirus (produced by using the Thermo Scientific Trans-Lentiviral packaging kit, and following the manufacturer's protocols, and pTripZ plasmids (clones RHS4743 and V3THS_407101, Open Biosystems). The cells were selected with 1.5µg/mL of puromycin (Sigma) and split into single cell/colony per well in 96 well dishes. The shRNA – RFP construct in these cells was induced with 1µg/mL of doxycyclin (Sigma) and monitored by the expression of red fluorescent protein (RFP).

2.2 - Adenoviruses

Adenovirus vectors

Both Ad VII-FLAG (Ad CC100) and Ad Δ E1 VII-FLAG (Ad JR46) (E1-deleted virus) contain a FLAG epitope tag on the C terminus of protein VII as previously described (Ross et al., 2011; Smith et al., 2009). Viruses were propagated as described previously (Ross and Parks, 2009). All vectors were purified by cesium chloride buoyant density centrifugation, using standard procedures (Palmer and Ng, 2003; Ross and Parks, 2009).

Infections

Viral titers required to perform infections were calculated based on multiplicity of infection (MOI), cell confluency, plate size and stock viral titer. Infection were carried out by rinsing the cells with PBS then adding infectious virus (stock virus diluted in MEM) in 1/10 of the usual maintenance volume of culture media. Infection was incubated for 1hr in a 37°C incubator, with periodic tilting of the plates to ensure cells remained hydrated and

spread the limited volume of infectious solution. At 1 hpi plates were filled to normal maintenance volumes with culture media, and incubated in the 37°C incubator.

Synchronized infections

To synchronize infection, cells were chilled on ice with virus for 30 min, allowing the virus to attach, but not penetrate into the cell. Infection was started by adding warm media and placing cells in a 37°C incubator.

2.3 - Immunoblots

Immunoblotting was performed using standard techniques. Polyvinylidene fluoride (PVDF) membranes were probed with the following primary antibodies: anti-FLAG M2 (1:10 000 dilution; Rockland), anti- α -tubulin (1:10 000; Calbiochem), anti-green fluorescent protein (GFP) (1:2 000; Invitrogen) (which cross-reacts with YFP), anti-Ad fiber (clone 4D2, 1:10,000; Neo-Markers), anti-Ad 5 E1A (M58) (1:10 000; Neo-Markers), anti-HIRA (clone WC119, 1:1000; Millipore) and anti-CAF1 p150 (1:1000; Santa Cruz). Anti-H3 (catalogue no. 2650; 1:10 000; Cell Signalling) was used in the majority of experiments (unless otherwise indicated), recognizes the C-terminus of the protein and does not cross react with the YFP-fusion constructs. Anti-H3 specific to the N-terminus (PA1-46377, Thermo Scientific) was used to determine the relative amounts of H3-YFP to endogenous H3. Secondary antibodies were either Biorad HRP coupled goat anti-rabbit IgG or anti mouse IgG (catalogue no. 170-6515 and 170-6516, respectively). Signal was developed using the Pierce ECL Western Blotting Substrate (product no.32209) and captured using X-ray film (Catalogue no. 34091, Thermo Scientific).

2.4 - RNA and transcription

RNA isolation and reverse transcription PCR

RNA was isolated using TRIzol reagent (Invitrogen) by following the manufacturer's instructions. First strand synthesis was conducted using random hexamers and Moloney Murine Leukemia Virus Reverse Transcriptase reverse transcriptase (Invitrogen) then treated with RNase H. Polymerase chain reaction (PCR) was conducted using E1A primers (Table A) and Taq DNA polymerase (Invitrogen) for 30 cycles of melt at 95°C for 30 seconds (sec), annealing at 60°C for 30 sec and extend at 72°C for 30 sec.

Table A - Primer sequences. Primers used for PCR.

E1A Forward	5-GTC CGG TTT CTA TGC CAA ACC TTG
E1A Reverse	5-CAA ACT CCT CAC CCT CTT CAT CCT
Hexon Forward	5-CTT ACC CCC AAC GAG TTT GA
Hexon Reverse	5-GGA GTA CAT GCG GTC CTT GT
Human β -actin Forward	5-CTG AAC CCT AAG GCC AAC CGT
Human β -actin Reverse	5-CCG TCA GGC AGC TCA TAG CT
Human RPL30 Exon 3	#7014, Cell Signalling

Pharmacological inhibition of transcription

Cells were treated for 1 hr before infection with culture medium containing 100 μ M DRB (5,6-dichlorobenzimidazole1- β -D-ribofuranoside). Viral infections proceeded as described above. DRB culture media was saved and used to fill plates once the 1hr infection

incubation period was complete.

2.5 - Micrococcal nuclease accessibility assay and Southern blots

A549 cells ($\sim 2.0 \times 10^7$) in 15-cm dishes were infected with Ad VII-FLAG at an MOI of 100 (for 6 hpi) or an MOI of 10 (for 18 hpi). At the indicated time points after infection, nuclei were isolated as described previously (Mymryk et al., 1997). Isolated nuclei in aliquots of 0.2 mL ($\sim 4.0 \times 10^6$) were treated with 200U of micrococcal nuclease (MNase; NEB) at 37°C and stopped at 0, 2, 3, 4 and 5 mins. Digests were terminated by addition of 50 μ L 5X stop solution (20 mM Tris-HCl at pH 8.0, 50 mM EDTA, 2.5% SDS) then digested with 4 μ g of proteinase K and 1 μ g of RNase overnight at 42°C. DNA was purified by phenol-chloroform extraction and ethanol precipitated. Purified DNA (6 μ g) was separated on 10-cm 1.25% agarose gels, transferred to a nylon membrane, and subjected to Southern blotting (Southern 1975) with a digoxigenin-labeled fragment from pCC100 plasmid (corresponding to the Ad VII-FLAG virus) or cellular SMN locus. DNA labelling and probe detection were performed using the digoxigenin (DIG) High Prime DNA labelling and detection starter kit II (Roche), according to the manufacturer's instructions.

2.6 - Chromatin immunoprecipitation and quantitative PCR

Confluent A549, A549 H3.1-YFP or A549 H3.3-YFP cells in 5 x 15-cm dishes ($\sim 1.25 \times 10^8$ cells) were infected with virus Ad VII-FLAG at a MOI of 10. At the indicated time points, cells were fixed by adding formaldehyde to a concentration of 1% for 10 min. Fixation was quenched with 0.125 M glycine (final concentration) and after 5 min of incubation, the monolayers were rinsed twice with cold phosphate-buffered saline (PBS) and

scraped into PBS supplemented with protease inhibitor tablet (Complete mini ETA-free; Roche). All subsequent buffers contained protease inhibitor. The cells were pelleted (1500 x g, 10 min, 20°C) and stored at -80°C. To isolate nuclei, the cells were re-suspended in 2mL cell swelling buffer (25 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.1% NP-40, 1mM DTT) and incubated on ice for 15 min followed by dounce homogenization (10-15 strokes using pestle B). The nuclei were pelleted by centrifugation (2 000 x g, 5 min, 4°C) and lysed in 1 mL sonication buffer + 1% SDS (50 mM HEPES pH 7.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium-deoxycholate). Chromatin was sheared to an average size of 300 bp by sonication in a Diagenode Bioruptor for 40 cycles (30 sec ON, 1 min OFF) at 4°C. Sheared chromatin was diluted 8-fold with ChIP dilution buffer (sonication buffer without SDS). Diluted chromatin was pre-cleared with 20 µL of a 50% slurry of protein G magnetic beads (Invitrogen) (which had been equilibrated with ChIP buffer + 1 µg sheared herring sperm DNA + 1 µg of BSA) for at least 2 hr at 4°C with constant rotation. Antibody (2 µg) was incubated with 40 µL of a 50% slurry of protein G magnetic beads for each sample for at least 2 hr. One mL of pre-cleared chromatin was incubated overnight with antibody-bead complex at 4°C with constant rotation. Antibodies used for ChIP analysis were rabbit IgG anti-GFP (A-11122; Invitrogen), anti-FLAG M2 (Rockland), anti-H3 (catalogue no. 2650; Cell Signalling), or control IgG. After immunoprecipitation (IP), beads were rinsed twice with low-salt immune complex wash buffer (50 mM HEPES pH 7.0, 140 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% Triton X-100, 0.1% sodium-deoxycholate), high-salt ICWB (50 mM HEPES pH 7.0, 500 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% Triton X-100, 0.1% sodium-deoxycholate), and lithium chloride ICWB (20 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% NP40 and 0.5% sodium-deoxycholate) and twice with Tris-

EDTA (TE). The beads were incubated twice for 10 min with 0.2 mL elution buffer (50 mM Tris-HCl pH 8.0, 1mM EDTA and 1% SDS). Eluates were combined, NaCl and RNase A were added to 200 mM and 10 μ g/mL, respectively, and the samples were incubated for 4 to 6 h at 65°C. After cross-link reversal, 4 μ L of 0.5 mM EDTA and 10 μ g of proteinase K was added to the samples and incubated for 2 h at 42°C. DNA was purified by phenol-chloroform extraction and precipitated with 2 X volume of 100% ethanol, 0.1 M sodium acetate and 100 μ g of glycogen. CHIP DNA was suspended in 100 μ L H₂O, and input DNA sample represents 0.02% of sample in the IP.

Quantitative PCR (qPCR) was performed using SYBR green JumpStart *Taq* ReadyMix (Sigma), according to the manufacturer's instructions, with the exception that the reaction was performed in a total volume of 20 μ L. The following primer sets for qPCR were used: E1A, Hexon, and Human RPL30 Exon 3 (Table A). The PCR was performed in duplicate using a MX3000P thermocycler (Stratagene), with 40 amplification cycles of melt at 95°C for 30 sec, annealing at 60°C for 30 sec, and extend at 72°C for 30 sec. Data was analyzed and presented as a percentage of input.

Chapter 3 - Results

3.1 - Protein VII is evicted shortly after infection

Ad DNA is highly condensed within the nucleocapsid by protein VII (Brown et al., 1975) and this condensed structure is refractory to viral gene expression (Matsumoto et al., 1995). This suggests that the DNA-protein VII complex must be remodelled to allow for efficient expression of virus-encoded genes. Using an otherwise wtAd which encodes a FLAG tagged protein VII (Ad VII-FLAG), we examined the fate of protein VII in A549 cells at various times post infection. A synchronized infection was performed at a high MOI (1000 infectious particles/cell) in order to detect protein VII from the infecting virions. The pool of protein VII in the infected cells declined gradually after 1-1.5 hpi continuing until 6 hpi, determined by immunoblot analysis of whole-cell lysates (Fig. 2 A). As the virion takes approximately 1 hr to reach the nucleus after entering the cells (Leopold and Crystal, 2007), this loss commences when the viral DNA-protein VII complex enters the nucleus (Leopold and Crystal, 2007). However, some protein VII is still detectable at 6 hpi, suggesting that a small proportion may remain associated with the viral DNA at this point. We observed an identical decline in protein VII levels when cells were infected with an E1 deleted Ad (Ad Δ E1 VII-FLAG) (Fig. 2 B). This pattern for loss of protein VII is consistent with protein VII dynamics observed in hdAd (Ross et al., 2011).

The association of protein VII with the viral DNA by chromatin immunoprecipitation (ChIP) was also assessed in A549 cells. At 6 hpi, protein VII is still associated with both the E1A and Hexon regions of the viral genomes (Fig. 3 A and B), consistent with the observation that some protein VII persists (Komatsu et al., 2011). However, the

Figure 2 - Ad protein VII, is rapidly lost over time. A549 cells were synchronously infected at a MOI of 1000 with a protein VII- FLAG (Ad VII-FLAG) tagged virus which is either wild type (A) or E1 deleted (Ad Δ E1 VII-FLAG) (B). Whole cell lysates were harvested at various time points post infection and immunoblotted to detect protein VII-FLAG. NI = non-infected. (Representative of two independent experiments)

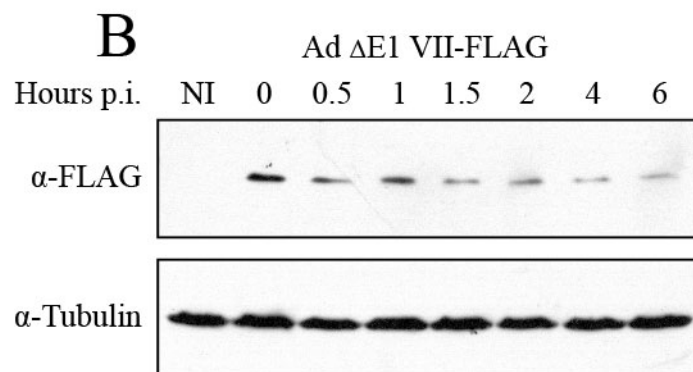
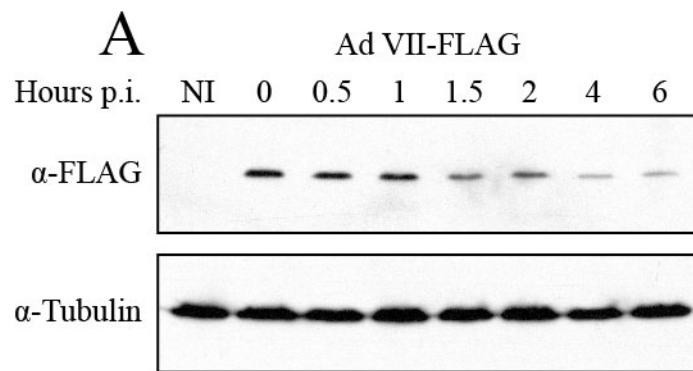
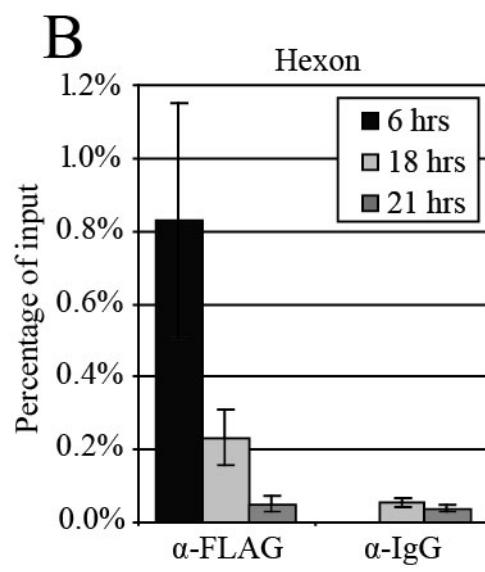
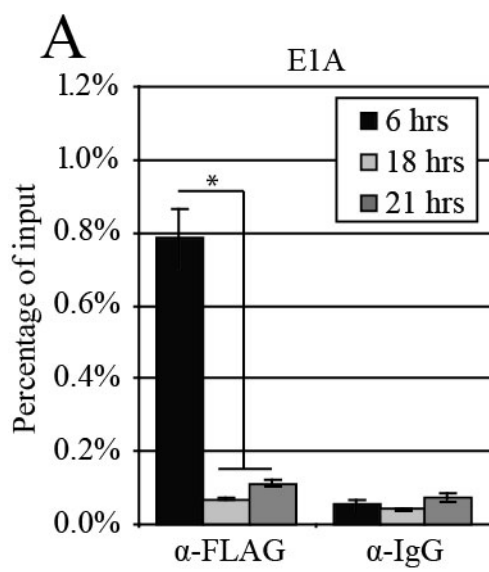


Figure 3 - Protein VII association with Ad DNA throughout the life cycle. A549 cells were infected with Ad coreVII-FLAG at an MOI of 10. Cells were processed for ChIP at 6, 18, and 21 hpi with the indicated antibodies. (A and B) The resulting DNA samples were analyzed by qPCR for two viral regions, E1A (A) and Hexon (B). Error bars represent standard error. * $P < 0.001$ (n=2, analyzed in duplicate)



association between protein VII and the viral DNA at late time in infection is significantly lower at the E1A region (Fig. 3 A).

3.2 - Transcription does not affect the loss of protein VII

There is some debate regarding the requirement for active transcription for the removal of protein VII from the Ad DNA (Giberson et al., 2012). Thus, we investigated whether transcription elongation was required for the loss of protein VII from wtAd. To do so, a preliminary experiment was conducted to determine the required concentration of the pharmacological inhibitor of transcription elongation, DRB. Cells were pretreated for 1 hr with various concentrations of the drug, then infected with Ad at an MOI of 1000. Protein was harvested 8h.p.i. and immunoblotted for the viral protein E1A (Fig 4). Viral protein was clearly detectable at a concentration of 25 μ M. Thus a concentration of 100 μ M was chosen to proceed in further experiments in order to assure that transcription elongation was inhibited.

We examined the kinetics for the loss of VII from the cell in the presence or absence of DRB. Cells were pretreated for 1 hr with 100 μ M DRB, and then infected with Ad VII-FLAG at an MOI of 1000. Viral transcription and protein expression, as indicated by E1A, was effectively blocked at 6 hpi by this treatment (Fig. 5 A and B). Treatment of cells with DRB had no effect on the timing for the loss of protein VII from the cell, suggesting that transcription elongation is not essential for removal of the bulk of protein VII from the viral DNA.

We also looked at the impact of blocking transcription elongation on the association of protein VII with the viral DNA by ChIP. In line with our expectations and our previous

Figure 4 - Concentration of DRB required to inhibit viral transcription. Cells were treated with a transcription elongation inhibitor (DRB) at various concentrations then infected at an MOI of 1000. At 8 hpi whole cell lysates were harvested and subjected to immunoblotting. (Representative of two independent experiments)

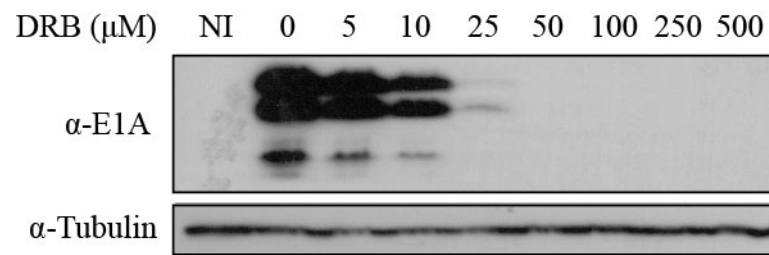
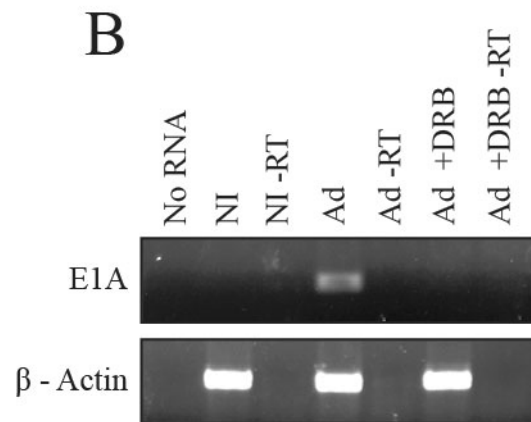
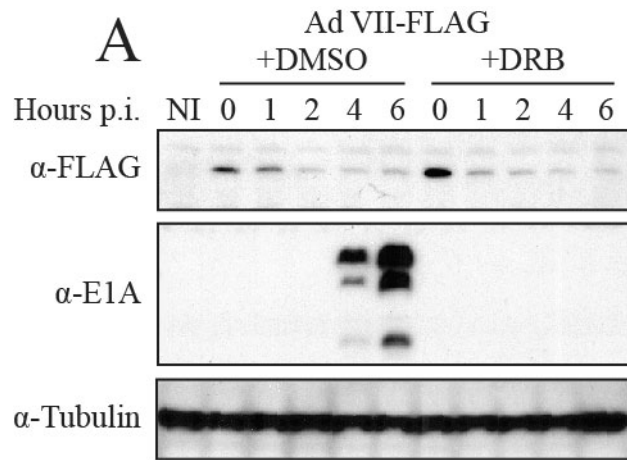


Figure 5 - Protein VII levels in the cell is not affected by transcription. (A) A549 cells were treated with a transcription elongation inhibitor (DRB). Cells were synchronously infected at a MOI of 1000 with a proteinVII- FLAG (Ad VII-FLAG) tagged virus which is either wild type. Whole cell lysates were harvested at various time points post infection and immunoblotted to detect protein VII-FLAG. (B) A549 cells were treated with DRB or vehicle (DMSO) and synchronously infected at an MOI of 1000. RNA was harvested at 6 hpi and then subjected to RT-PCR for viral (E1A) and control (β -actin) sequences. (Representative of two independent experiments)



biochemical data (Fig. 2 A), the levels of protein VII associated with the Ad genome was not altered when transcription was inhibited for both viral sequences (Fig. 6).

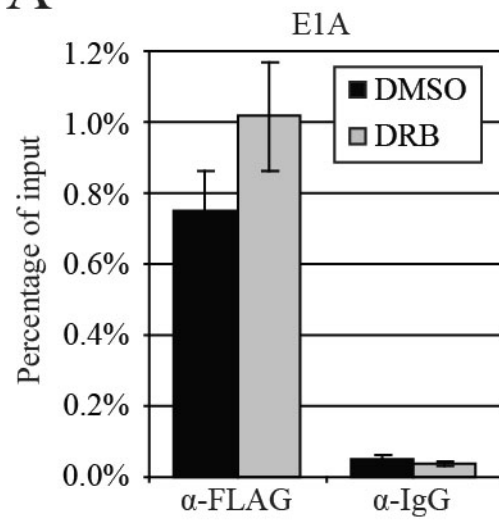
These results indicate that the majority of protein VII is removed from the viral DNA within the first few hours of infection, and this process is independent of transcription elongation.

3.3 - Adenovirus DNA associates into physiologically spaced nucleosomes

Whether Ad DNA was wrapped in a repeating nucleosome-like structure at early and late times of infection was investigated using a classical micrococcal nuclease (MCN) digestion assay. A549 cells were infected with Ad VII-FLAG at an MOI of 100 (for the early, 6hr time point) or 10 (for the late, 18hr time point). The DNA was isolated and visualized by Southern blot, probing for viral or cellular sequences. At both early and late times of infection, the cellular DNA was found to produce a clear laddering pattern (Fig. 7). On the other hand, while the viral DNA appears to be wrapped in physiologically spaced nucleosomes at early times, forming a laddering pattern like the cellular DNA (Fig. 7 A), there was no such evidence of laddering at 18hrs, although DNA fragments corresponding to mononucleosomes can be seen (Fig. 7 B). The absence of even di- and tri-nucleosomes suggests that at late times, the viral DNA is much less chromatinized by cellular histones. At both early and late times, a very heavy band is seen at top of the blots. These are inaccessible genomes, either intact virions at early times or de novo packaged genomes at late times. The existence of inaccessible packaged genomes at late times was confirmed by the use of a temperature sensitive Ad, in which packaging is blocked at the restrictive temperature. When packaging was blocked, the heavy protected band disappeared when

Figure 6 - Protein VII association with Ad DNA is not affected by transcription. A549 cells were treated with DRB or vehicle (DMSO) and infected with Ad coreVII-FLAG at an MOI of 10. Cells were processed for ChIP at 6 hpi with the indicated antibodies. The resulting DNA samples were analyzed by qPCR for two viral regions, E1A (A) and Hexon (B). Error bars represent standard error. (n=3, analyzed in duplicate)

A



B

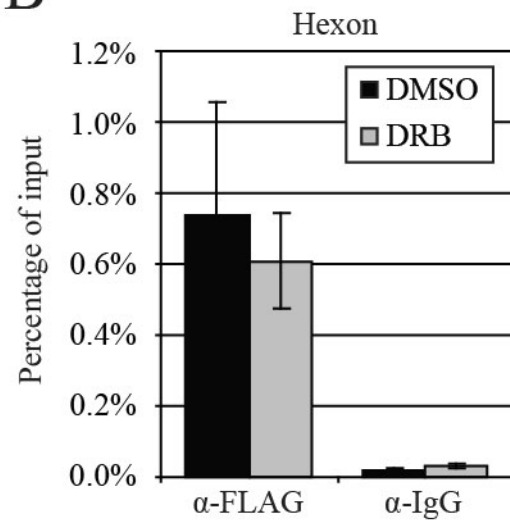
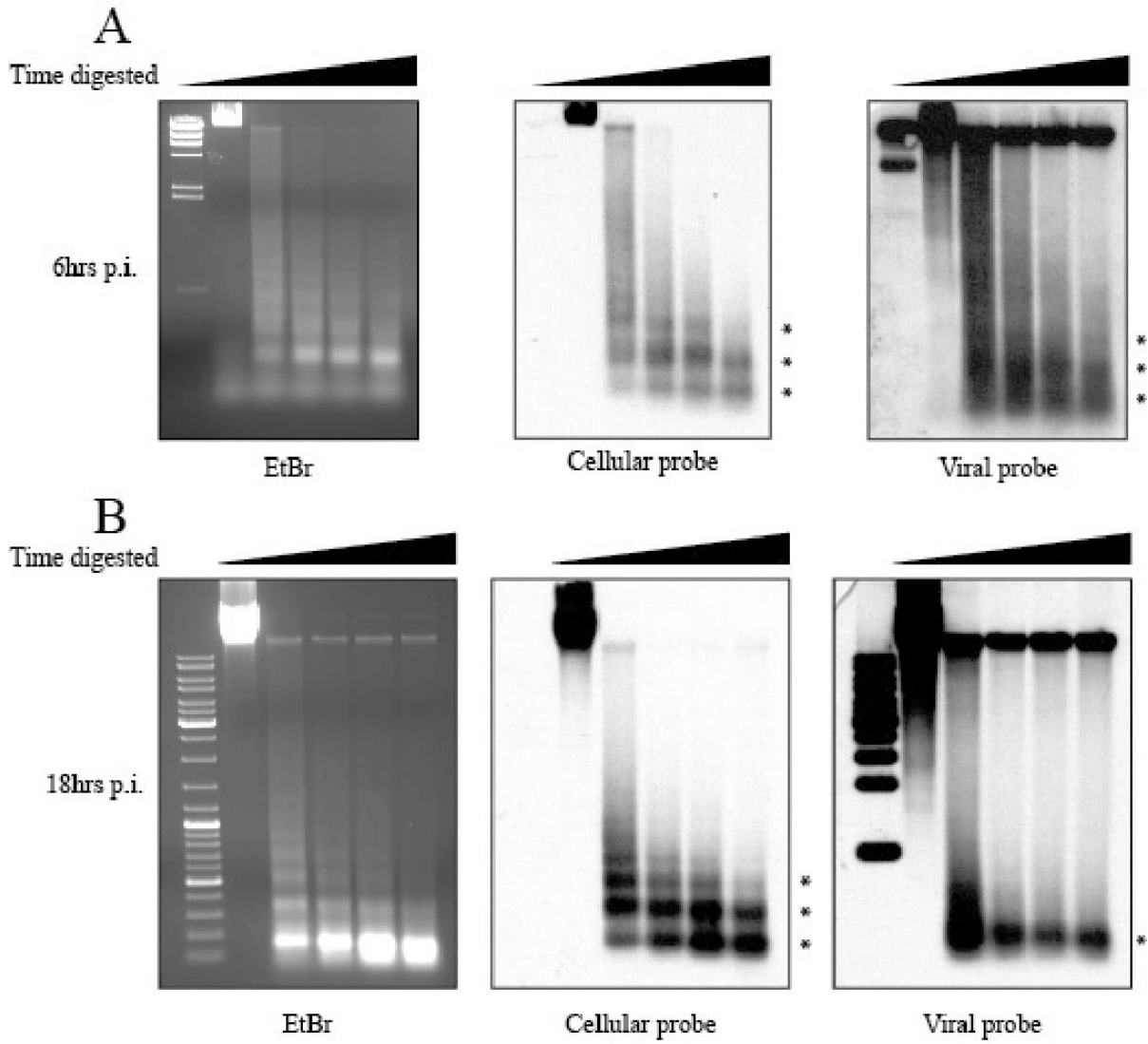


Figure 7 - Ad DNA assembles into nucleosomes. A549 cells were infected with Ad coreVII-FLAG at an MOI of 100 (A) or 10 (B). The samples were processed for micrococcal nuclease (MNase) digestion assay at the indicated times. The ethidium bromide (EtBr)-stained agarose gel (showing bulk cellular chromatin) and southern blots using cellular and viral probes are displayed. Bands representing DNA that was protected from MNase cleavage are indicated by asterisks. (Representative of two independent experiments)



treated with MCN (Brown and Weber, 1980; Daniell et al., 1981).

3.4 - Histone association with Adenovirus DNA varies during the life cycle

To determine if wtAd associated with H3, A549 cells were infected with Ad VII-FLAG at an MOI of 10 and the association of histones with viral DNA was investigated using ChIP at both an early (6hr) and a late (18hr) time point post infection (Fig. 8). We examined histone association with different regions of the Ad genome, E1A which is transcribed early, and hexon, which is not expressed until after DNA replication. H3 is associated with the viral DNA at a much higher level before DNA replication with the E1A locus than the hexon locus (compare Fig. 8 A and B). The overall level of H3 association drops significantly after DNA replication (Fig. 8 A and B). This observation is consistent with the lack of nucleosomes seen in the MCN digestion assay at late times (Fig. 7 B). As in internal control, association of H3 with the cellular locus RPL30 was not impacted by viral infection and was consistent in all samples (Fig. 8 C).

3.5 - Transcription does not affect the deposition of histones at early times

To investigate the effect of transcription on histone deposition on Ad DNA, A549 cells were treated with a transcription elongation inhibitor (DRB) and ChIP for H3 was performed at 6 hpi (Fig. 9). We observed no difference in the association of H3 with the viral DNA when transcription was inhibited (Fig. 9 A and B). The H3 association with the cellular RPL30 locus was also unaffected by the treatment with DRB (Fig. 9 C).

Figure 8 - Ad DNA chromatin associates with histone H3. A549 cells were infected with Ad coreVII-FLAG at an MOI of 10. Cells were processed for ChIP at 6, 18 and 21 hours p.i. with the indicated antibodies. The resulting DNA samples were analyzed by qPCR for two viral regions, E1A (A) and Hexon (B) as well as a cellular locus, RPL30 (C). Error bars represent standard error. * $P < 0.001$ (n = 2, analyzed in duplicate)

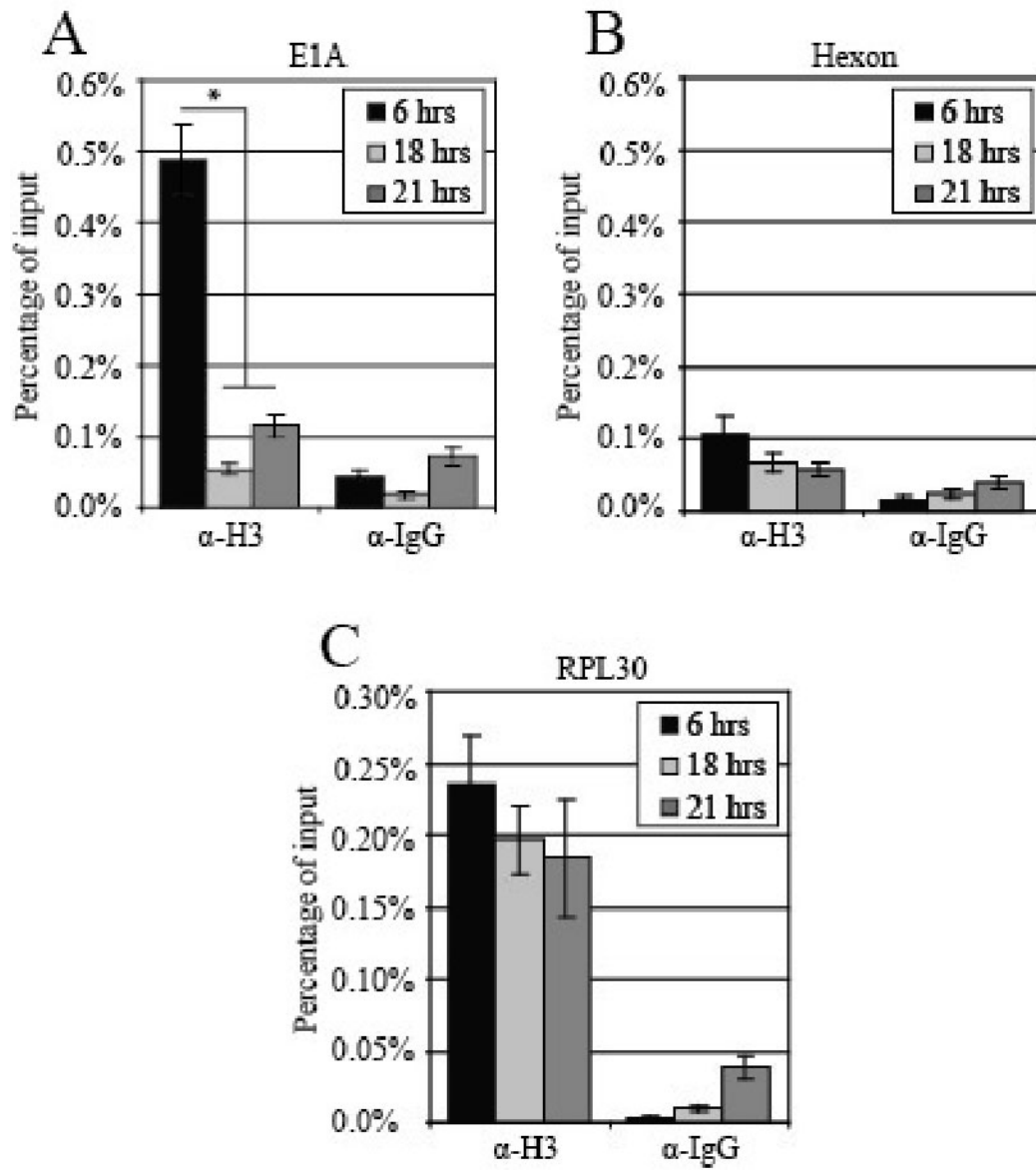
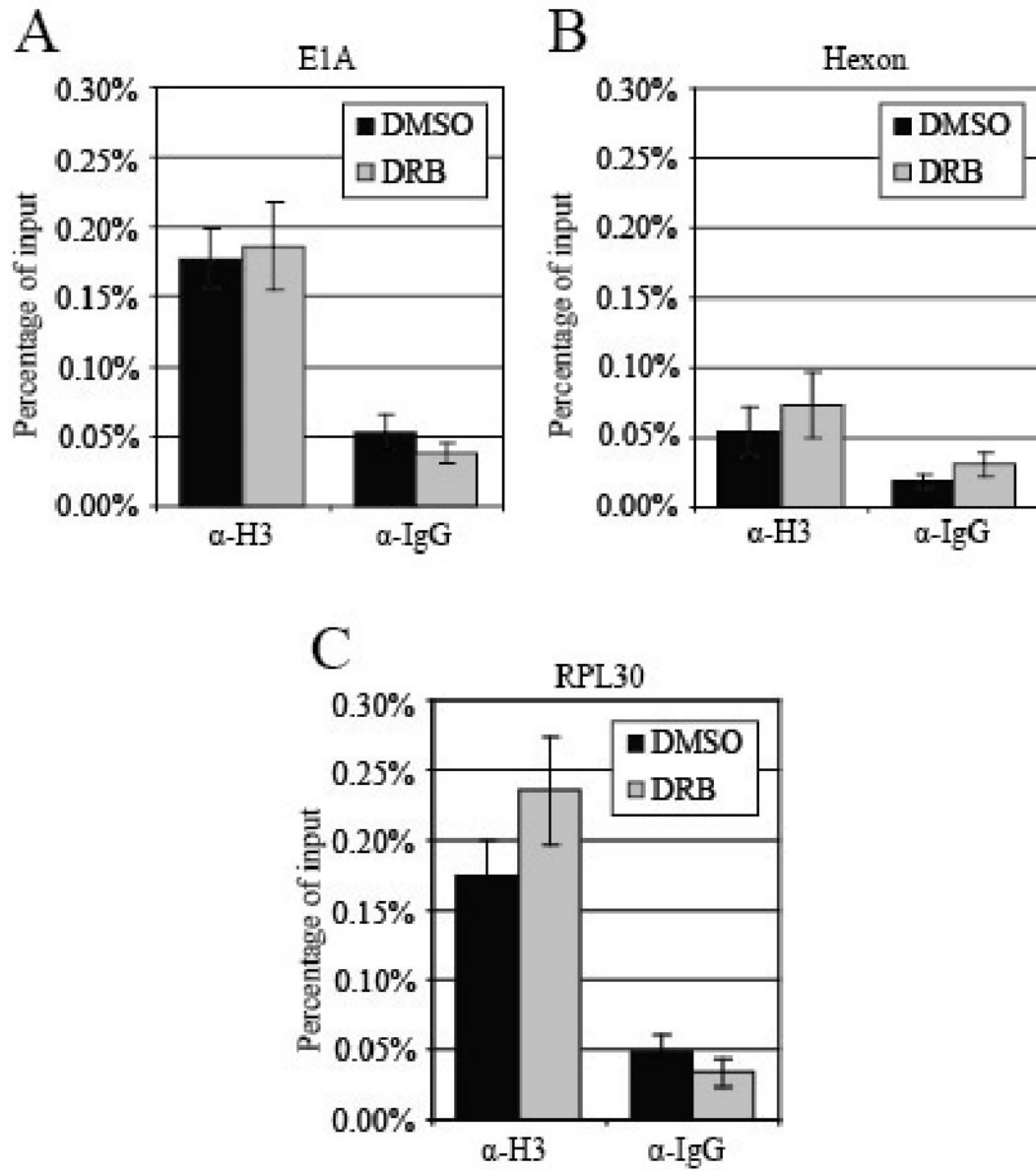


Figure 9 - H3 association with Ad DNA is not dependant on transcription. A549 cells were treated with DRB or vehicle (DMSO) and were infected with Ad coreVII-FLAG at an MOI of 10. Cells were processed for ChIP at 6 hpi with the indicated antibodies. The resulting DNA samples were analyzed by qPCR for two viral regions, E1A (A) and Hexon (B) as well as a cellular locus, RPL30 (C). Error bars represent standard error. (n = 3, analyzed in duplicate)



3.6 - Preferential association of Adenovirus DNA with histone variant H3.3

Characterization of H3-YFP cell lines

A recent study has shown that naked DNA is poorly tolerated in the nucleus, and deposition of the H3 variant H3.3 is the default mechanism by which the cell chromatinizes that DNA (Schneiderman Ji Fau - Orsi et al., 2012). As H3.3 was also preferentially associated with hdAd DNA, we decided to investigate the association of wtAd DNA with the two main H3 variants, H3.1 and H3.3. To investigate whether Ad DNA associates with histone variant H3.1 or H3.3, two cell lines stably expressing H3-YFP fusion proteins were created: A549 H3.1-YFP and A549 H3.3-YFP. The expression of the H3-YFP constructs was verified by immunoblot by probing whole cell lysates with anti-GFP or anti-H3 antibodies with similar levels of expression between the two cell lines (Fig. 10 A). To look at relative expression of the H3-YFP constructs versus the endogenous H3, we used a different H3 antibody, recognizing the N-terminus, and serial dilution of the lysates (Fig. 10 B and C, indicated by black boxes). In both A549 H3.1-YFP and A549 H3.3-YFP, the H3-YFP variant is expressed at approximately 10% of the level of endogenous H3, therefore neither H3-YFP variants are substantially over expressed compared to cellular H3.

We next examined the localization of the YFP tagged histones and gross cell morphology by fluorescence microscopy. As expected, the tagged histones localize correctly to the nucleus and are excluded from the nucleoli (Fig. 11). Gross cell morphology was normal. The growth of the two cell lines versus the parental A549's, to determine if expression of tagged H3 had an adverse affect on cell growth. Briefly, the

Figure 10 - Stable expression of H3-YFP tagged variants. (A) Immunoblot of whole cell lysates showing exogenous H3-YFP tagged variants (α -GFP) and endogenous H3 (α -H3). (B and C) Immunoblots of serial dilutions of A549 H3.1-YFP (B) or A549 H3.3-YFP (C) probed with an H3 Ab (α -H3 (N)) that recognizes the N-terminus and therefore both endogenous and YFP-tagged forms of H3 to estimate the relative levels of expression, indicated by the boxed bands. Of note, the α -GFP antibody cross-reacts with YFP. (Representative of three independent experiments)

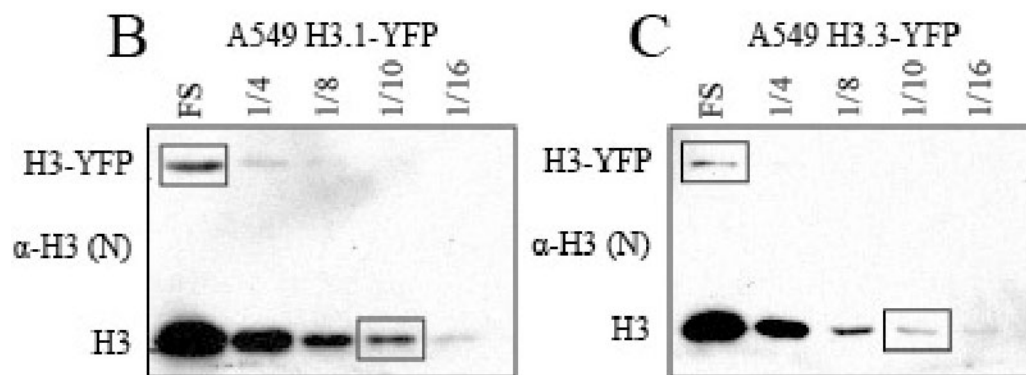
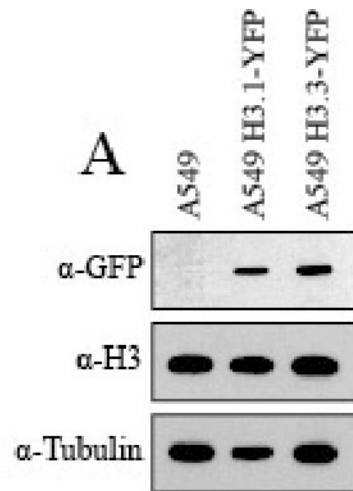
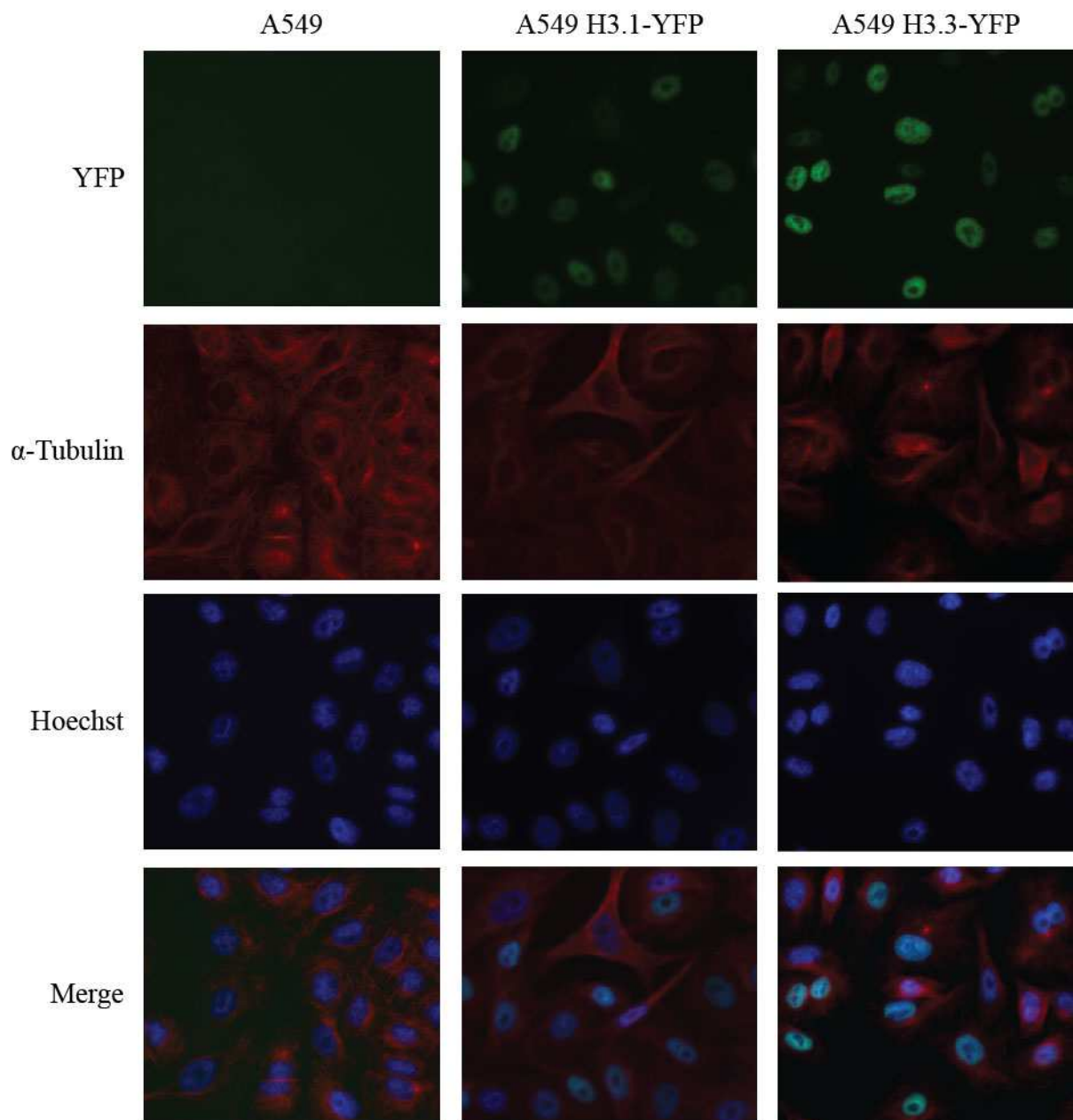


Figure 11 - H3 variants localize correctly to the nucleus. Immunofluorescence analysis of cell lines stably expressing YFP-tagged histone 3 variants (A549, A549 H3.1-YFP and A549 H3.3-YFP cells) stained with α -tubulin antibody (red) and Hoechst. (Representative of two independent experiments)



parental and both H3-YFP cell lines were seeded at low density, then fixed and stained with 0.1% crystal violet at varying time points. At the end of the assay, the crystal violet was solubilized in 10% acetic acid and the intensity of crystal violet staining correlates to the number of cells in the dish (Fig. 12). Expression of the H3-YFP variants does not affect cell growth as all three growth curves are not significantly different.

We also examined whether expression of the YFP-tagged variants affected viral growth. Each cell line (parental and H3-YFP variants) was infected with Ad at an MOI of 1 and the virus was harvested 24 hpi. The collected virus was then titered on 293 cells (Fig. 13). As the viral titer obtained after passage in either A549 H3.1-YFP or A549 H3.3-YFP cells was similar to that grown in the parental cell line, the expression of H3-YFP variants does not negatively impact the viral life cycle. Moreover, a time courses of Ad infection in all 3 cell lines investigating the timing of early and late gene products were all comparable (Fig. 13). Taken together, these data show that the cell lines stably expressing H3.1-YFP or H3.3-YFP do not exhibit any differences in cell or virus growth characteristics.

Adenovirus DNA and H3.3

Our previous work with hdAd showed that the incoming viral DNA-protein VII complex associated preferentially with the H3 variant H3.3. We were interested to see if wtAd DNA also preferentially associates with H3 variant H3.3. To determine this association with wtAd DNA, A549, A549 H3.1-YFP and A549 H3.3-YFP cells were infected with Ad VII-FLAG at an MOI of 10 and the association of histones with viral DNA was investigated using ChIP at both an early (6hr) and a late (18hr) time point p.i. (Fig. 14). We examined histone association with two different regions of the Ad genome, E1A which is

Figure 12 - Expression of H3-YFP variants does not affect cell growth. Growth curve of cell lines stably expressing H3-YFP variants as compared to parent cell line. Cells were seeded at low density, then fixed and stained with 0.1% crystal violet at various time points. The crystal violet was resuspended in 10% acetic acid and absorbance was measured at 595nm. Error bars represent standard error. (n=3)

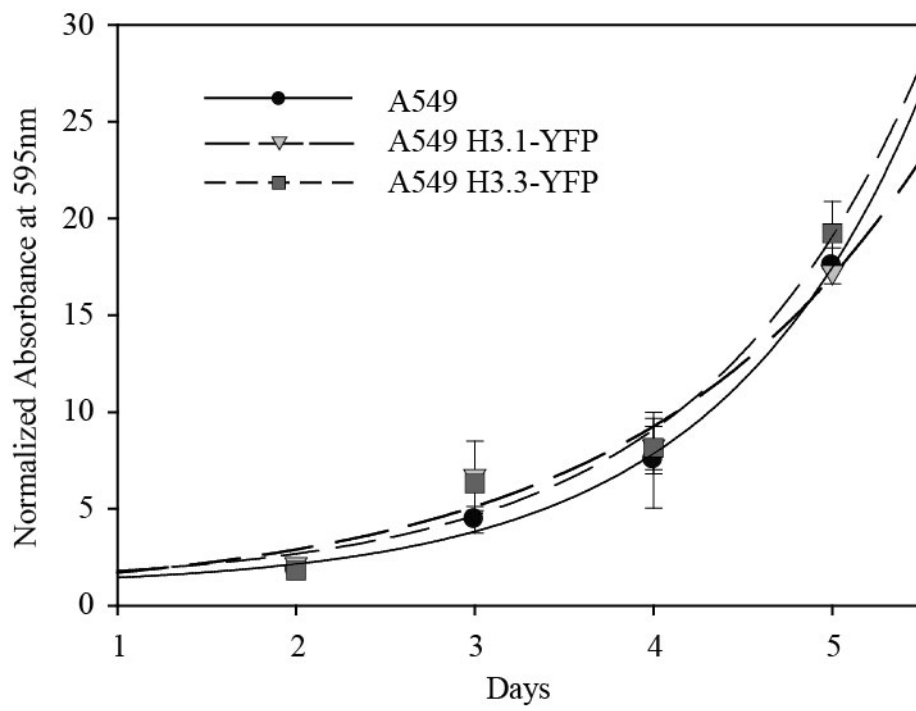


Figure 13 - Expression of H3-YFP variants does not affect viral growth. Cells were infected with wtAd at an MOI of 10. (A) Immunoblot of whole cell lysate harvested at various times showing the Ad fiber protein. (B) Virus was harvested at 24 hpi. The titer was obtained by plaque assay in 293 cells. NI = Non infected. Error bars represent standard error. (n=3)

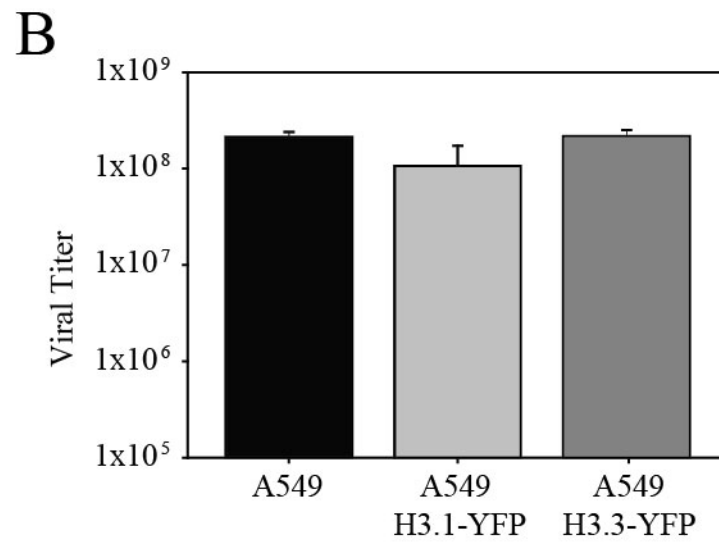
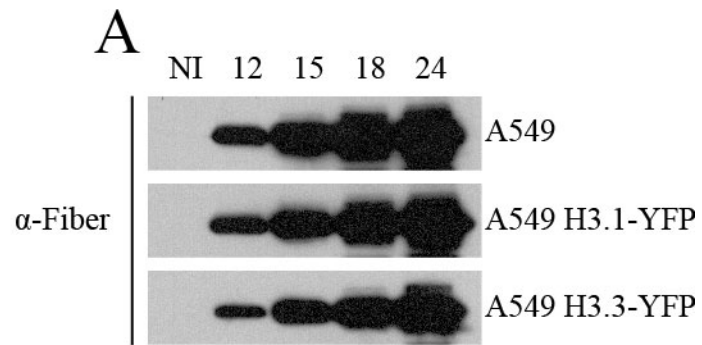
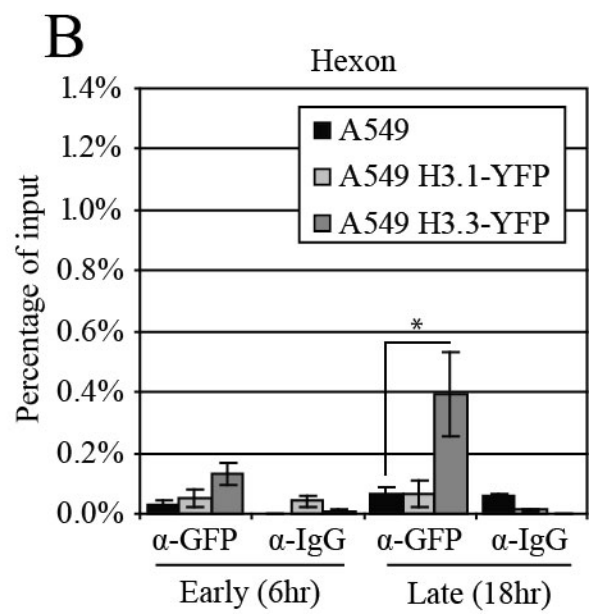
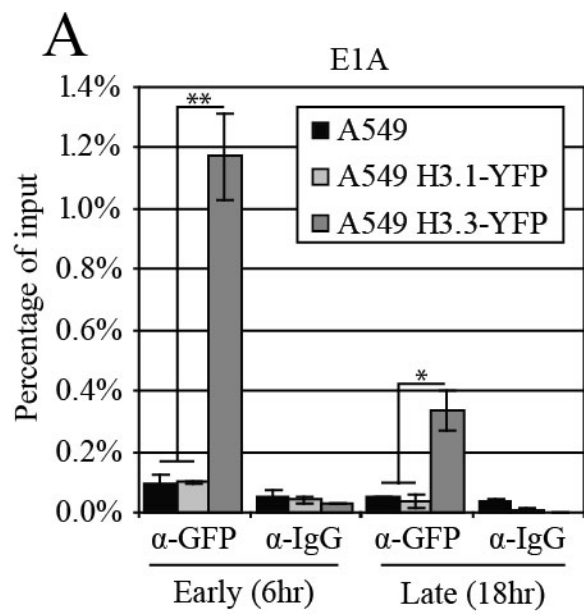


Figure 14 - Ad DNA chromatin preferentially associates with histone variant H3.3. Cells (A549, A549 H3.1-YFP or A549 H3.3-YFP) were infected with Ad coreVII-FLAG at an MOI of 10. Cells were processed for ChIP at 6 and 18 hours p.i. with the indicated antibodies. The resulting DNA samples were analyzed by qPCR for two viral regions, E1A (A) and Hexon (B). Of note, the GFP antibody cross-reacts with the YFP tag on H3.1 and H3.3. Error bars represent standard error. *P<0.05 **P<0.001 (n = 2, analyzed in duplicates)



transcribed early and hexon, which is not expressed until after DNA replication. There is a marked preference for the histone variant H3.3 over the replication-dependant H3.1 variant at the 6 hour time point (compare Fig. 14 A, α -GFP, samples A549 H3.1-YFP (light grey) and A549 H3.3-YFP (dark grey)). The preferential association of H3.3 with the Hexon locus becomes apparent at the late time point (Fig. 14 B, Late (18hr), α -GFP, A549 H3.1-YFP (light grey) and A549 H3.3-YFP (dark grey)). Interestingly, we did not observe a switch from preferential association with H3.3 to H3.1 after DNA replication, as has been observed in HSV1 (Placek et al., 2009).

3.7 - HIRA is not responsible for the deposition of H3 on viral DNA

Recent data have shown that the histone variant H3.3 specific chaperone HIRA plays a role in the deposition of H3.3 on hdAd and HSV1 at early times of infection (Placek, Huang et al. 2009; Ross, Kennedy et al. 2011). Thus, we examined the impact of HIRA on the viral life cycle. A549 derived cell lines containing an inducible shRNA construct, which was either scrambled or HIRA specific, were created. When the constructs are induced by doxycyclin, they concurrently express RFP as a marker, which is used to monitor expression of the shRNA. No RFP is detectable in the cells without doxycyclin, indicating tight regulation of the promoter (Fig. 15). The presence of the exogenous construct did not impact cell growth, whether or not the construct was expressed (Fig. 16).

With the addition of 1 μ g/mL of doxycyclin for 3 days, expression of the shRNA was induced and a knockdown of HIRA of 85-90% was achieved (Fig 17, HIRA - vs.+ dox). Cells were infected with wtAd at an MOI of 10. Whole cell lysates were harvested at various times p.i. and immunoblotted for both early and late gene products (Fig. 17 A and B). No

Figure 15 - Induction of the shRNA construct by dox. A549, A549-Scrambled and A549-HIRA cells were treated with doxycyclin or vehicle to induce expression of the shRNA construct which can be monitored by the expression of RFP by fluorescence microscopy. Shown is an overlay of the fluorescence image and a phase-contrast image. (Representative of four independent experiments).

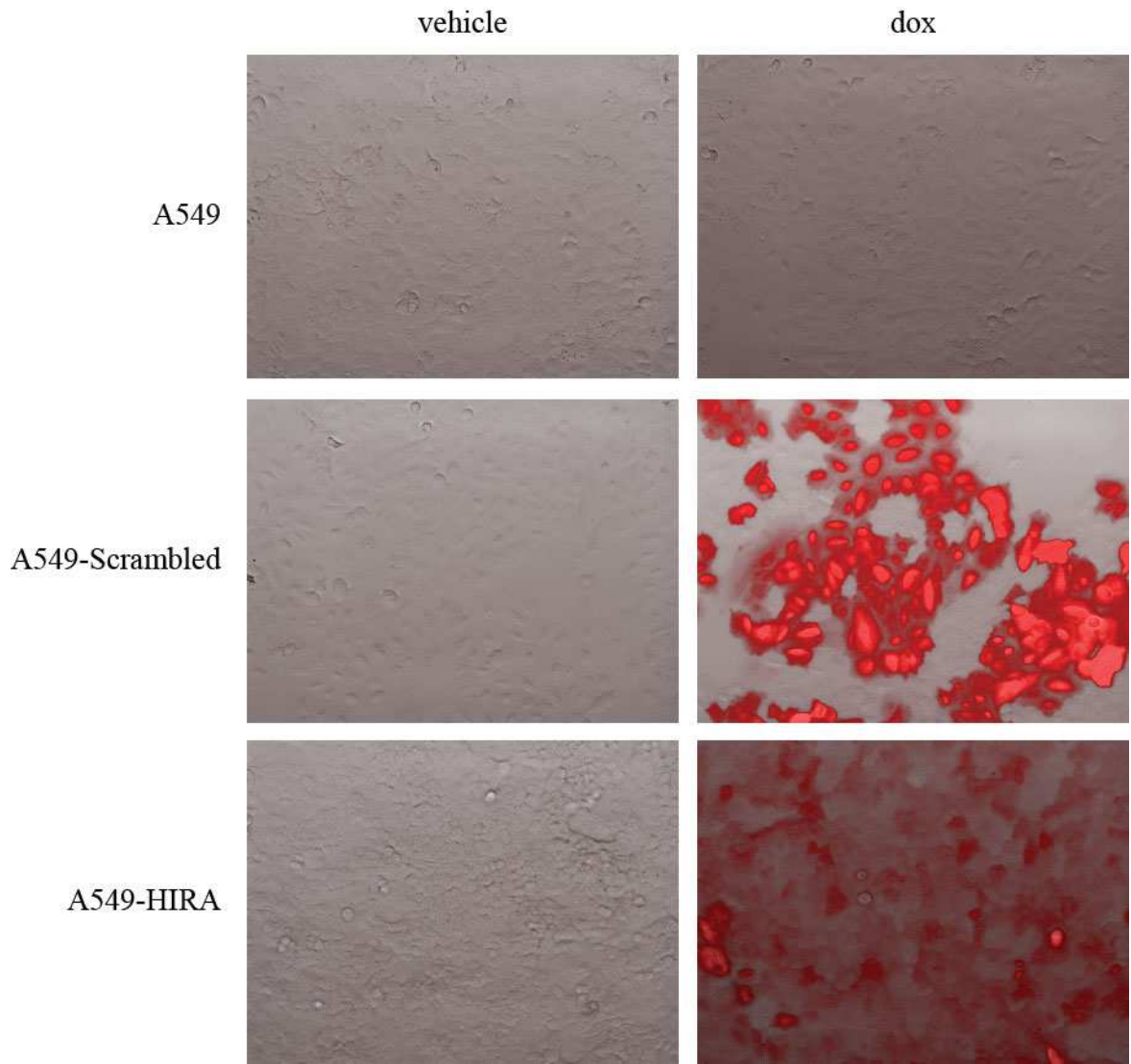


Figure 16 - Exogenous shRNA constructs do not affect cell growth. Growth curve of cell lines containing shRNA constructs compared to parent cell line, with and without induction of the shRNA. Cells were seeded at low density, then fixed and stained with 0.1% crystal violet at various time points. The crystal violet was resuspended in 10% acetic acid and absorbance was measured at 595nm. Error bars represent standard error. (n=3)

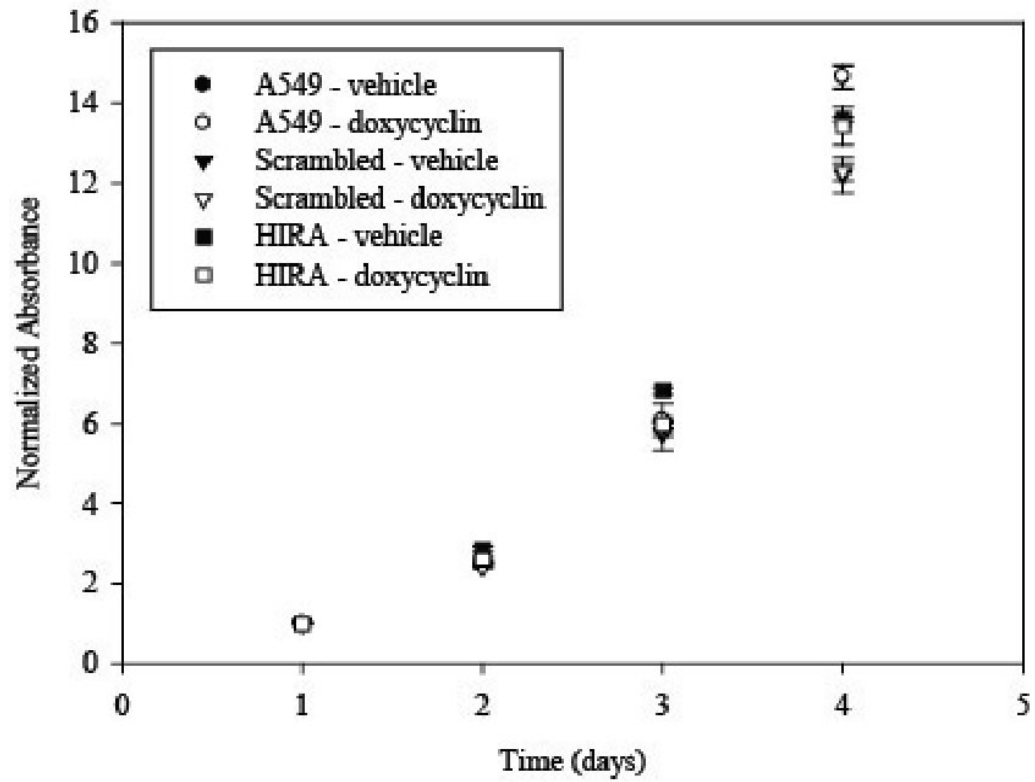
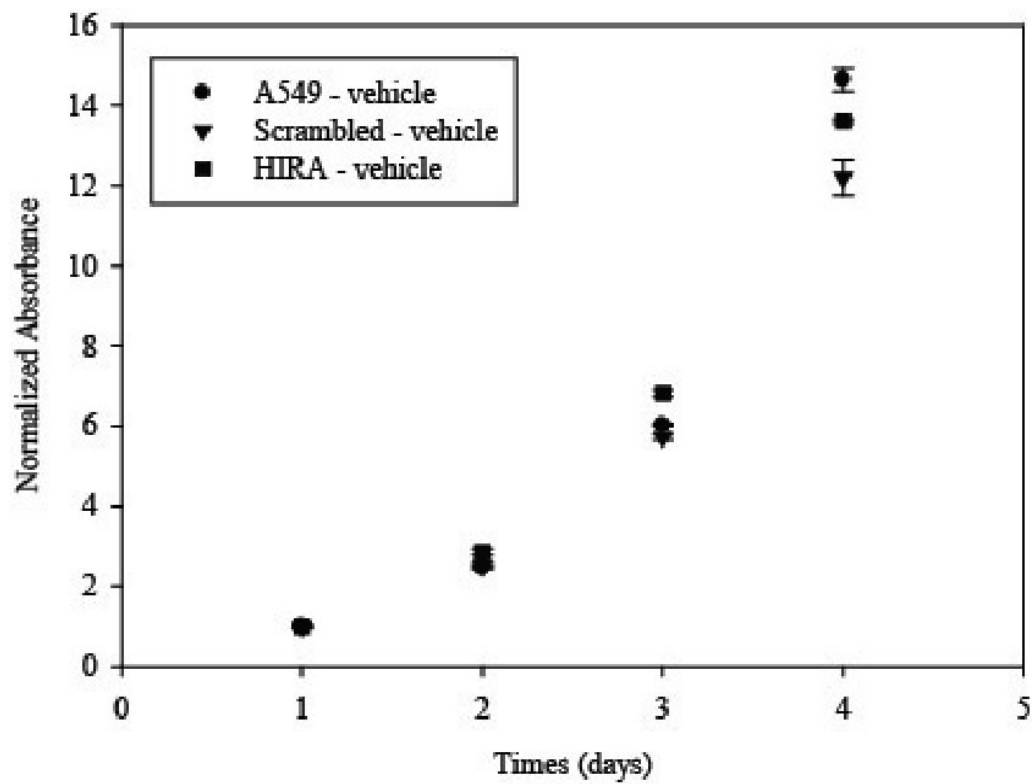
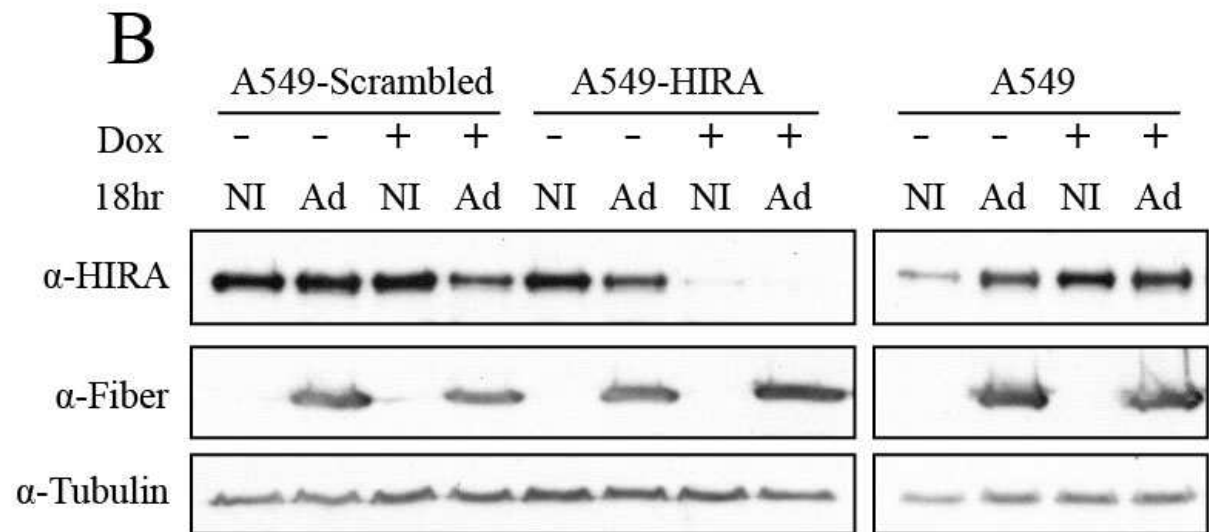
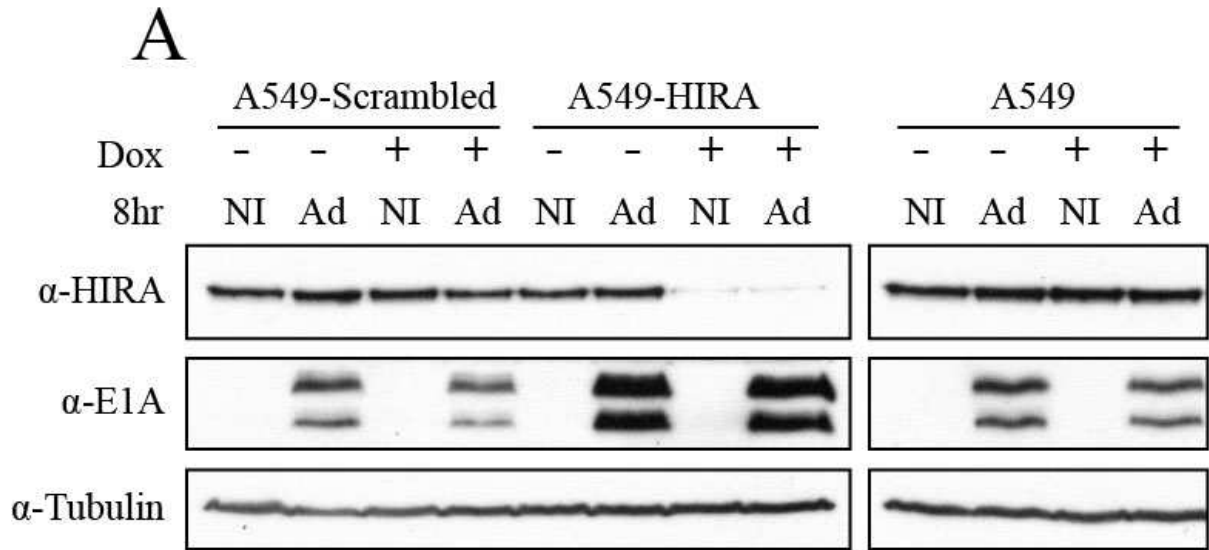


Figure 17 - Effect of HIRA on the viral life cycle. A549, A549-Scrambled and A549-HIRA cells were treated with doxycyclin or vehicle to induce expression of the shRNA construct. Cells were then infected with Ad coreVII-FLAG at an MOI of 10 and whole cell lysate was harvested at 8 (A) or 18 (B) hpi and subjected to immunoblotting. (Representative of two independent experiments).



change in either early or late gene products was detected.

HIRA did not have a significant impact on the viral growth. Each cell line (parental and shRNA) induced with doxycyclin for 3 days then infected with Ad at an MOI of 1. The virus was harvested 24 hpi and the titered on 293 cells (Fig. 18). As the viral titer obtained after a passage in all cells under all conditions were similar to that grown in the parental cell line, the depletion of HIRA does not negatively impact the viral life cycle.

Histone association with the viral DNA was investigated by ChIP in the context of a cell depleted of HIRA. After induction with doxycyclin for 3 days, A549-Scrambled or A549-HIRA cells were infected with wtAd and processed for ChIP at 6 hpi. No difference in the association of H3 was observed at any loci in cells with or depleted of HIRA (Fig. 19).

Taken together, these data indicate that HIRA is not responsible for the deposition of H3.3 on the wild type Ad DNA in A549 cells.

Figure 18 - Impact of knocking down HIRA on the viral life cycle. A549, A549-Scrambled and A549-HIRA cells were treated with doxycyclin or vehicle to induce expression of the shRNA construct. Cells were then infected with Ad coreVII-FLAG at an MOI of 1 and virus was harvested at various time points post infection. The titer was obtained by plaque assay in 293 cells. NI = Non infected Error bars represent standard error. (n=2)

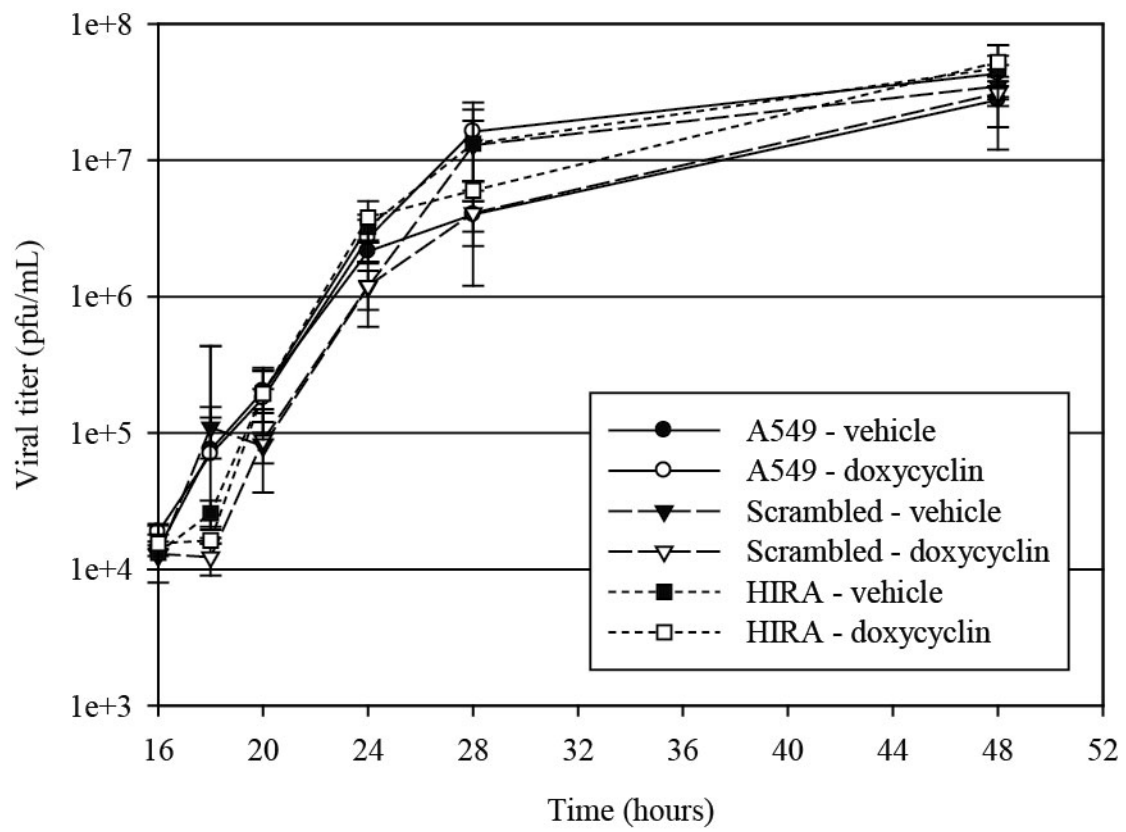
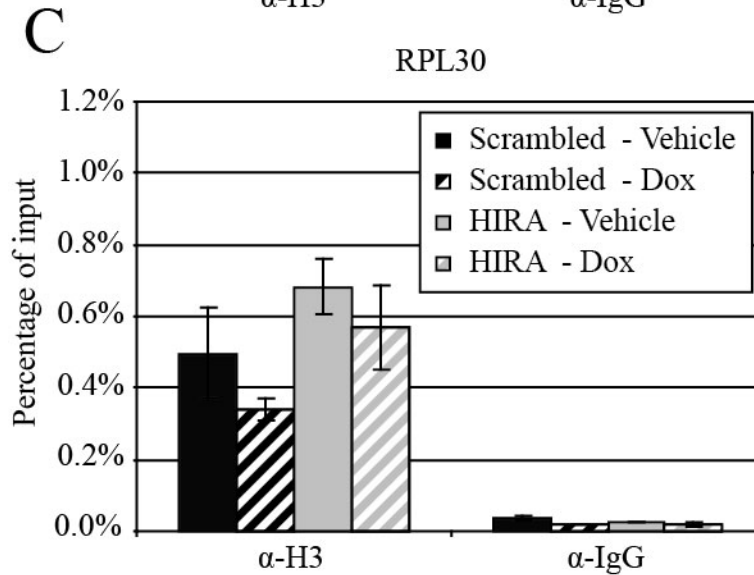
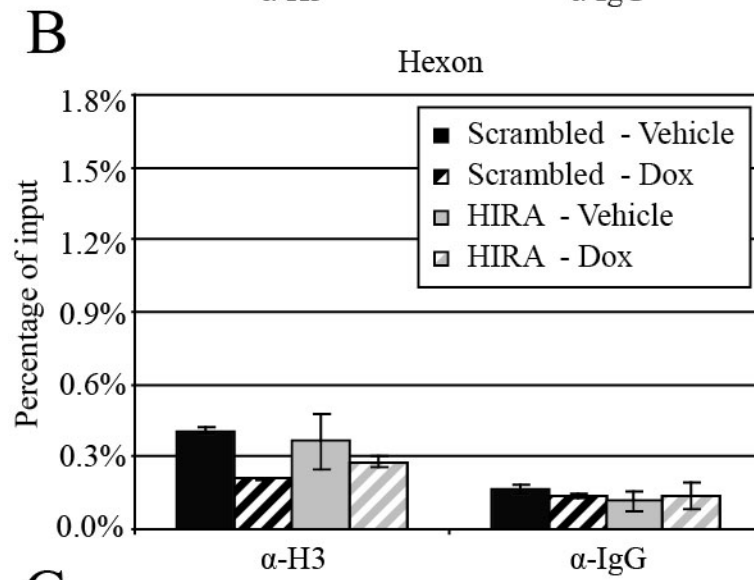
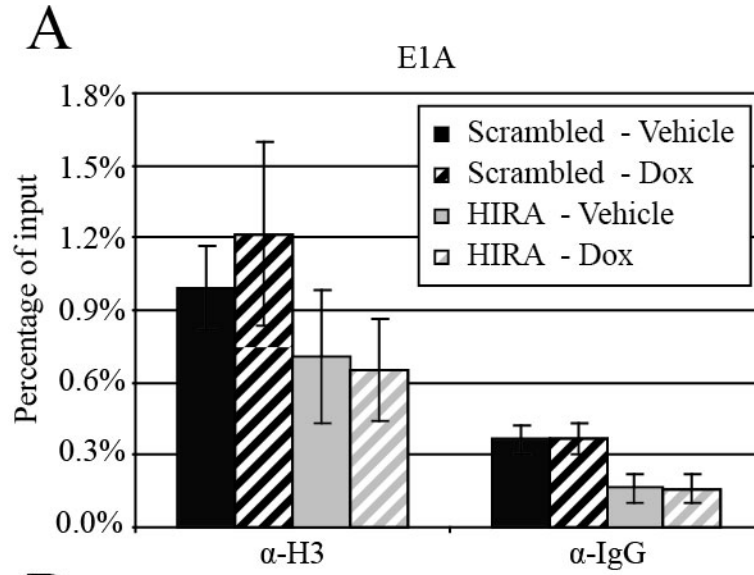


Figure 19 - Effect of the HIRA on the association of H3 with the viral DNA. A549-Scrambled and A549-HIRA cells were treated with 1 μ g/mL of dox or vehicle for 3 days then infected with Ad VII-FLAG at an MOI of 10. Cells were processed for ChIP at 6 hpi with the indicated antibodies. The resulting DNA samples were analyzed by qPCR for two viral regions, E1A (A) and Hexon (B) as well as a cellular locus, RPL30 (C). Error bars represent standard error. (n = 2, analyzed in duplicate)



Chapter 4 - Discussion

4.1 - Adenoviral association into chromatin varies throughout the life cycle

The goal of this study was to investigate the structure and chromatin state of wtAd DNA within the infected cell. Protein VII is a small, highly basic viral protein responsible for wrapping and condensing the viral DNA in the capsid (Mirza and Weber, 1981; Sung et al., 1983). It has been well established that it is the protein VII-DNA complex which enters the cell nucleus (Chatterjee et al., 1986a). Previous work with hdAd showed that protein VII was rapidly removed from the incoming DNA within a few hours of infection, although a small proportion persisted in the nucleus (Ross et al., 2011). We showed that similarly, for wtAd, protein VII levels drop dramatically starting at 1.5 hpi but can still be detected at 6hrs (Fig. 2). By ChIP, it was found that the levels of protein VII association on the viral genome was equivalent on both early (expressed, E1A) and late (silent, Hexon) regions (Fig. 3). Using immunoblotting, inhibition of transcription elongation by DRB did not affect the kinetics of protein VII loss over time (Fig. 5). Nor was a difference in the association of protein VII with the viral genome detectable by ChIP when transcription was inhibited (Fig. 6). As ChIP is a snapshot of the association at a specific time, it does exclude an immediate loss of protein VII upon reaching the nucleus, most likely mediated by TAFI (Xue et al., 2005).

The association of pre-protein VII with viral DNA remains quite low at late times in infection (Fig. 3), whereas a dramatic increase is expected. It is likely that the deposition of pre-protein VII and the packaging of the virion are linked and preventing the antibody from

interacting with the pre-protein VII target (Ostapchuk and Hearing, 2005). The isolation of the viral DNA in the virions would also protect it from digestion by MCN. The full length genome bands seen in the Southern blots (Fig. 7) are genomes that have not been uncoated at the early time point or *de novo* packaged virions at late time in infection (Brown and Weber, 1980; Daniell et al., 1981).

Ad DNA was found to preferentially associate with replication independent H3 variant H3.3 at the two investigated loci (Fig. 14). This is consistent with the observations with hdAd vectors, wtAd and HSV1 (Ahmad and Henikoff, 2002; Komatsu and Nagata, 2012; Placek et al., 2009; Ross et al., 2011). Unexpectedly, wtAd maintains a preferential association with H3.3, unlike HSV1 which switches to the canonical H3.1 variant after viral DNA replication (Placek et al., 2009).

Also contrary to expectations, Ad DNA does not seem to associate significantly with histones late in infection. At 18hrs, no clear laddering pattern is visible when digested with MCN and there is a significantly lower association with H3 as seen by ChIP (Fig. 7 and Fig. 8). Many studies have looked at the affect of viral infection on the production of various cellular house keeping genes, including histones. While histone synthesis is usually tightly coupled with cellular DNA synthesis (Robbins and Borun, 1967), Ad replication does not induce histone expression. In fact it was found that histone production is abolished at late times after infection (Hodge and Scharff, 1969; Tallman et al., 1977). This would greatly reduce the pool of available free histones at late time in infection, perhaps explaining the dramatic decline in association. Unlike previous studies, such as by Ross *et al*, 2011 and Placek *et al.*, 2009, no effect on viral gene expression was detected when the histone

chaperone HIRA was depleted from cells (Fig. 17, 18 and 19). We postulate that the cellular context in which the experiments were performed plays an essential role in the effects observed, as previous observations were conducted in Hela cells, whereas this study was conducted in A549 cells (Placek et al., 2009; Ross et al., 2011).

4.2 - Future directions

Many questions remain to be answered. For example, the identification and validation of the cellular factors that remove protein VII from the incoming viral DNA has yet to be determined. The template activating factors I, II and III have been identified to interact with protein VII in a cell free extract (Kawase et al., 1996; Matsumoto et al., 1993; Okuwaki et al., 2001; Okuwaki and Nagata, 1998). However, none of these have been shown to conclusively to remove or remodel VII on the viral DNA. Identification of further candidates by co-IP followed by mass spectrometry identification and definitive characterization by a series of knockdowns and ChIP are in order.

Another area that remains to be elucidated is the chaperone responsible for depositing H3.3 preferentially. HIRA, and DAXX are the main candidates identified in the literature with specificity for that particular histone variant. However, while some groups have shown positive results with HIRA (Placek et al., 2009; Ross et al., 2011), this work did not. The studies of DAXX in this role have generally been overlooked as it is actively degraded during a the course of infection (Schreiner et al., 2010). However, DAXX should be investigated as HIRA was not found to be responsible for the chromatinization on two loci of Ad DNA. Another aspect to consider is compensation of the chaperones. It is possible that when HIRA is eliminated from the system, DAXX takes up its function, and vice versa.

Simultaneous knockdowns and ChIP and well as more traditional biochemical methods should be used to look into these possibilities.

A question that was raised in the course of this research is what proteins associate with the viral DNA at late stages of infection, as neither H3 or pre-protein VII associate in high amounts. An intriguing possibility is that the viral encoded single stranded DNA binding protein continues to associate with the Ad DNA in late times after DNA replication, as has been suggested by Komatsu et al, 2012. One approach would be to produce an Ad with a biochemically tagged ssDBP to monitor its effect and investigate its association with the viral DNA at late times in infection.

4.3 - Conclusions

I have shown that the viral protein VII is lost from the viral genome within a few hours of infection and this loss is independent of transcription. The viral DNA was found to associate into physiologically spaced nucleosomes at early times in infection. Of the possible H3 variants, the transcription-dependant variant H3.3 was found to be preferentially deposited on the viral DNA. Although the viral DNA continues to associate with H3.3 at late times of infection, the overall level of association with histones is greatly reduced, which is consistent with the lack of nucleosomes as seen by MCN accessibility assays at that time. Knocking down the chaperone HIRA had no discernible effect on the viral life cycle or the levels of H3 on the viral DNA. Our work suggests that wtAd undergoes decondensation (removal of protein VII) and associates with cellular histones within the first hours of infection, with a preferential deposition of H3.3, and that the viral DNA is wrapped in physiologically spaced nucleosomes, at least in early times of infection.

This work is important because understanding fundamental steps in the viral life cycle can lead to better management of the organism as a pathogen, and contribute to our study of viruses as a tool to discover fundamental truths of molecular biology.

Chapter 5 - References

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Contributions of Collaborators

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Appendix III

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SURVEY AND SUMMARY

Chromatin structure of adenovirus DNA throughout infection

Andrea N. Giberson^{1,2,3}, Adam R. Davidson^{1,2,3} and Robin J. Parks^{1,2,3,*}

¹Regenerative Medicine Program, Ottawa Hospital Research Institute, ²Department of Biochemistry, Microbiology and Immunology and ³Centre for Neuromuscular Disease, University of Ottawa, Ottawa, ON, Canada

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ABSTRACT

For more than half a century, researchers have studied the basic biology of Adenovirus (Ad), unraveling the subtle, yet profound, interactions between the virus and the host. These studies have uncovered previously unknown proteins and pathways crucial for normal cell function that the virus manipulates to achieve optimal virus replication and gene expression. In the infecting virion, the viral DNA is tightly condensed in a virally encoded protamine-like protein which must be remodeled within the first few hours of infection to allow for efficient expression of virus-encoded genes and subsequent viral DNA replication. This review discusses our current knowledge of Ad DNA-protein complex within the infected cell nucleus, the cellular proteins the virus utilizes to achieve chromatinization, and how this event contributes to efficient gene expression and progression of the virus life cycle.

INTRODUCTION

Human Adenovirus (Ad) was first isolated from adenoid tissue in the 1950s as novel viral agents associated with respiratory infections (1,2). Over 100 Ad family members have been identified and characterized in a wide range of host organisms, from a variety of mammals and birds, to reptiles and amphibians (3). In the early 1960s, researchers showed that some human Ads can cause tumours in rodents (4,5), which led to a surge in studies of the molecular biology, genetics and physiology of Ads which continues to this day. Since Ads must manipulate the host cell to promote a microenvironment conducive to virus replication, studies of basic Ad biology have contributed a great deal of novel insight into all fields of cellular

biology, including DNA replication, tumourigenesis and control of gene expression in the host cell.

While the pool of knowledge regarding the Ad lifecycle is immense, few studies have investigated the structure and protein association of Ad DNA within the infected cell nucleus. Considering the fundamental importance of chromatin in regulating gene expression in host cells, it is surprising that, until recently, it remained unclear whether Ad DNA interacted with cellular histones or assembled into chromatin. This review summarizes our current knowledge of the nucleoprotein structure of the Ad genome within the infected cell.

ADENOVIRUS BIOLOGY

All Ads have the same general structural characteristics. The virion is a non-enveloped icosahedral capsid with a diameter of ~80–90 nm, containing a linear double stranded DNA genome of ~30–40 kb (Figure 1) (3). Of the human Ads, serotype 2 (Ad2) and 5 (Ad5), both of subclass C, are the most extensively characterized. The Ad5 genome is ~36 kb in size and encodes ~39 genes, which are classified as either early or late depending on whether they are expressed before or after DNA replication (Figure 1A) (6). Four early transcription units (E1a, E1b, E3 and E4) encode proteins that are required for transactivating other viral regions, modifying the host cellular environment or altering the immune response. E2 encodes proteins directly involved in viral DNA replication. All major late proteins, organized in the transcription units L1 to L5, are expressed from a common major late promoter and are generated by alternative splicing of a single transcript. However, recent work has shown that the L4-22K and L4-33K proteins, which are themselves involved in regulation of the major late promoter, are initially expressed from a novel promoter (7). The late transcripts generally encode virion structural proteins. Four other small late transcripts

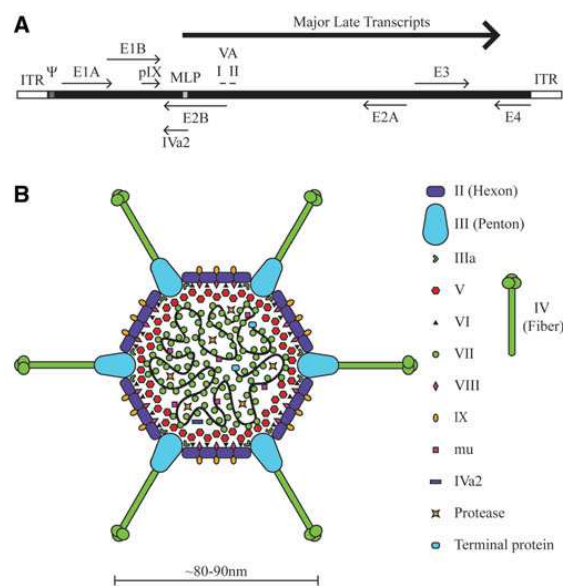


Figure 1. Schematic of the adenovirus genome and virion. (A) A simplified map of the Ad5 genome showing the early genes (E1–E4) and the region from which the major late transcript is produced (the extensively spliced L1–L5 transcripts produced from alternative splicing of the major late transcript are not shown). The relative position of pIX, VA RNA I and II and IVa2 are indicated. Also shown are the viral inverted terminal repeats (ITR) located at each end of the genome, the viral packaging element (Ψ) located adjacent to the left ITR, and the position of the major late promoter (MLP). Please note that these features are not drawn to scale. (B) Model of the Ad5 virion, adapted from (9), with modifications based on additional data provided by (8,10,11).

are also produced: protein IX (pIX, encoding a minor structural protein), IVa2 (encodes a protein involved in encapsidating the viral DNA into the immature virion) and VA RNA I and II (the RNA itself blocks activation of the interferon response). Inverted terminal repeats (ITR) of ~100 bp flank both ends of the viral DNA and contain the origins of replication. Directly adjacent to the left ITR is the viral packaging sequence (~150 bp). The genome organization is relatively conserved through all Ad species.

The Ad5 capsid is composed of three major (II, III and IV) and five minor (IIIa, IVa2, VI, VIII and IX) polypeptides (Figure 1B) (8–11). The facets are composed primarily of hexons (trimers of protein II) with pentons (five molecules of protein III) capping each vertex. The latter is the base from which extends fibre (trimer of protein IV), the distinctive projections at the Ad capsid vertices. Within the capsid, the viral DNA is associated with three highly basic proteins, core proteins VII, V and Mu (μ) (12–14). Protein VII is a protamine-like protein and is responsible for wrapping and condensing the viral DNA (15). The protein VII-DNA nucleoprotein complex is organized into a central dense core with 12 large spherical nucleoprotein projections, termed adenosomes, which extend into each vertex (16,17). A shell of protein V is

thought to coat the protein VII-DNA complex (16,18). Protein V is believed to make contact with the outer capsid in several different ways; protein V interacts directly with penton, and indirectly with peripentonal hexon and the remainder of hexon bridged through IIIa and protein VI, respectively (10,11,19–22). Mu is synthesized as a 79 amino acid precursor protein, pre-Mu, which is cleaved by the Ad-encoded proteinase to its final 19 amino acid, highly basic mature form (23). Pre-Mu is speculated to interact with and aid in tightly condensing the viral DNA within the capsid, and cleavage of pre-mu may serve to partially relax this structure prior to its entry into the nucleus (24). Although the viral DNA does not interact directly with the major capsid proteins (10,25,26), the DNA still appears to contribute to the physical stability of the virion; packaging of subgenomic sized DNA [$<90\%$ of the wild-type (wt) genome length] results in virions that are less stable than wtAd (27,28).

Many of the details of Ad5 infection of cells have been elucidated. Initially, the Ad fibre protein binds to the Coxsackie-Adenovirus receptor (CAR), which is the primary receptor for both Ad5 and Coxsackie B virus (29,30). This binding is followed by a secondary interaction between Ad penton and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins (31). Recent studies have shown that Ad5 can enter cells using heparin sulfate proteoglycans as an alternative receptor, either through direct binding to the Ad fibre shaft (32), or bridged through interaction of Ad with blood factors such as factor IX, factor X or complement component C4-binding protein (33–35). Ad is internalized by receptor-mediated endocytosis and evades degradation by escaping from the early endosome (36). The virion is transported through the cytoplasm to the nucleus along the microtubule network (36), and the capsid is slowly disassembled en route (37). Upon reaching the nuclear pore complex, the protein VII-wrapped Ad DNA enters the nucleus (14,37,38), while the rest of the capsid remains at the nuclear membrane and is subsequently degraded. Viral DNA replication and assembly of progeny virions occur entirely within the nucleus of infected cells. The life cycle takes 24–36 h, although the time for completion of the lifecycle is slightly extended in primary cells. A single cell infected with a single virus can produce $\sim 10^4$ daughter virions.

EARLY EVENTS WITHIN THE INFECTED CELL NUCLEUS

An overview of our current understanding of Ad DNA chromatin state in the infected cell is shown in Figure 2. Although a number of Ad capsid proteins reach the nucleus, it is only the protein VII-wrapped DNA that enters the nucleus (14). Histone H1 (H1) escorts the Ad DNA–protein complex through the nuclear pore (39); however, this function appears to be independent of any structural role for H1 on the viral DNA. During this phase of infection, protein VII protects the viral DNA from activating the DNA damage response (40). The ultimate fate of protein VII after entry into the nucleus is currently a topic of debate. Some studies suggest that

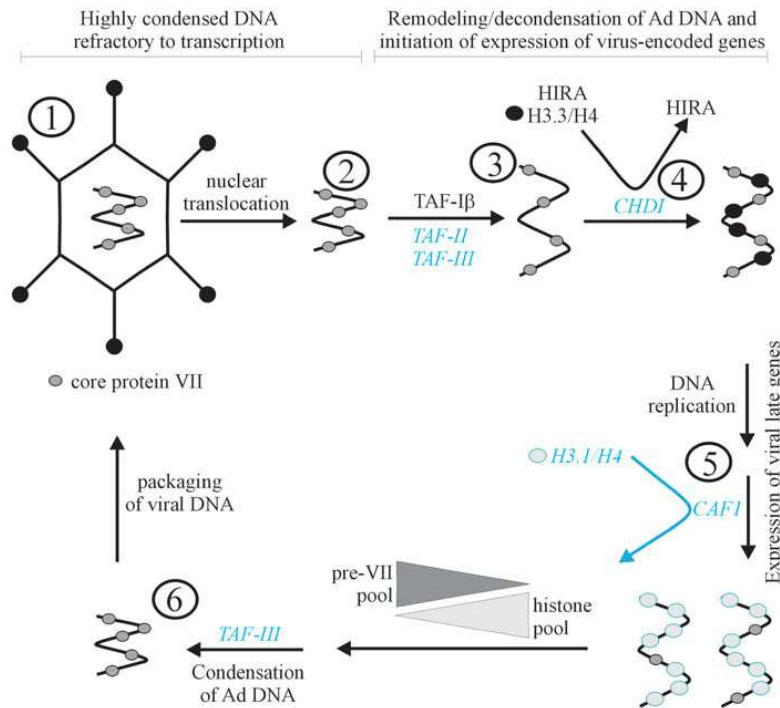


Figure 2. Adenovirus DNA chromatin structure throughout infection. In black—elements that we know. In blue and italics—speculation/areas of future research. DNA in the Ad capsid is highly condensed with core protein VII (1), along with protein V and μ (not shown). The protein VII-DNA complex transits to the nucleus (2), and undergoes remodeling to decondense the core before transcription of early genes can begin (3). Remodeling may involve loss of at least some VII. At the time when viral gene expression is first detected, histones can be found bound to the viral DNA along with VII. Histone variant H3.3 is preferentially deposited on the viral DNA, through the action of HIRA and CHD1 (4). Onset of viral DNA replication may be accompanied by a shift to deposition of H3.1 by the CAF1 complex on the DNA, as is observed for HSV1 (5). As the histone pool is depleted and the intracellular levels of newly synthesized pre-protein VII increase, there is a transition of Ad DNA association from nucleosomes to pre-protein VII, possibly mediated by TAF-III (6). The viral DNA condensed with pre-protein VII is packaged into the Ad capsid (1).

protein VII stably associates with Ad DNA throughout the early phase of infection (14,41), while other groups suggest that VII is removed gradually from at least certain regions of the genome during this same time period (42,43). Other reports have shown that the overall level of VII within the infected cell declines rapidly within the first few hours of infection with a concomitant decline in VII association with viral genomes (44). Whether the eviction of protein VII requires active transcription of the Ad genome is also in dispute (41,44,45).

Cell-free systems developed to study Ad DNA replication have shown that the compacted nature of the VII-wrapped viral DNA allows for only limited transcription and DNA replication (46,47). This observation suggests that the core/DNA structure must be remodeled to allow these processes to proceed with greater efficiency. Three cellular proteins have been identified that can remodel the Ad core in these cell-free systems: template activating factor I β (TAF-I β) [also known as SET (46)], TAF-II [NAP-1 (48)] and TAF-III [B23/nucleophosmin (49)]. Using the cell-free system, all three TAF's were shown to stimulate replication from the Ad core, while

TAF-I β and TAF-II were also shown to enhance transcription. TAF-I β , the best characterized TAF in the context of Ad core remodeling, forms a tertiary complex with the VII-wrapped DNA (41,50,51), which results in increased accessibility of the viral DNA to nucleases and restriction enzymes and, presumably, transcriptional activators (47). It is not clear if increased accessibility was due to actual removal or only shifting of VII on the DNA template. siRNA-mediated knockdown of TAF-I β in infected cells delayed virus gene expression, DNA replication and virus yield (42), although the effect was relatively modest. Knockdown of TAF-I β did not affect the binding level of protein VII on viral DNA as assessed by chromatin immunoprecipitation (ChIP), at least at 4 hpi (43). Thus, additional proteins are likely involved in preparing the Ad core for efficient gene expression and DNA replication within the infected cell nucleus.

Several lines of evidence suggest that VII remains associated with Ad DNA during the early stage of infection. First, VII can be cross-linked to the viral DNA at virtually all stages of infection (52). Second, based on immunofluorescence analysis, foci of VII can be observed in the nucleus of infected cells, which represent

the VII-wrapped viral DNA (40,41,45,53). Third, ChIP studies have shown directly that VII is bound to the viral DNA up to at least ~10 hpi (41,43,44,51,54). There is some disagreement in these studies regarding the level of VII association over time; some studies indicate that VII association is constant and does not change throughout the early stage of infection (41,45,52), while others suggest a gradual (or more rapid) decline in VII association with the viral DNA during this time period (40,43,44,51). Based on ChIP experiments by Komatsu *et al.* (43), it appears that the degree and timing of VII association with the viral genome during the early phase of infection can vary depending on the region of the genome that is analysed. For example, between 1 and 10 hpi, VII remains stably associated with the late-gene hexon coding region, but shows declining association over time with the major late promoter (43). This observation somewhat rationalizes the previous disparate studies. In plasmid-based *in vitro* assays, addition of small amounts of protein VII with the DNA actually enhanced transcription over naked DNA, suggesting that small quantities of protein VII may function in part to keep repressive histone/chromatin features from forming on certain promoter regulatory elements (43). Taken together, these results suggest that dynamic regulation of protein VII is necessary for optimal viral growth; sufficient protein VII must be removed or remodeled to decondense the viral DNA–nucleoprotein complex to allow access to the transcription machinery, but some protein VII must remain to stimulate transcription.

It is unclear whether transcription through the Ad DNA template is required for removal or remodeling of VII. Inhibition of transcription has been correlated with prolonged retention of VII on the Ad genome (40,45), although in other studies inhibition of transcription or transcription elongation did not affect loss of VII (43,44). Although it has been suggested that *de novo* expressed E1A (the first viral gene product expressed within the infected cell) associates with protein VII and is involved in stimulating transcription on the viral genome which subsequently strips VII from the viral DNA (45,54), this function is likely not completely necessary since VII is still removed in the absence of E1A or active transcription (43,44). It is possible that other proteins within the cell can perform this function in the absence of E1A; indeed, E1-deleted Ad can complete a full replication cycle in certain cell types, although the time required to complete the replication cycle is extended (55), suggesting that compensating proteins may exist.

AD DNA ASSOCIATES WITH CELLULAR HISTONES IN THE INFECTED CELL NUCLEUS

In eukaryotic cells, the basic unit of chromatin is the nucleosome, with 147 bp of DNA wrapped around a histone octamer, composed of two sets of H2A–H2B and H3–H4 dimers. The notion that nucleosomes are simply ‘beads on a string’ has been challenged by the realization that histones and nucleosomes play key roles in gene regulation (56). The post-translationally modified

N-terminal tails of histones serve as docking/recognition sites for other regulatory proteins (57), providing the epigenetic information governing gene expression, as dictated by the ‘histone code’ (58).

Conflicting data from the 1980s suggested that Ad DNA is or is not associated with cellular histones within the infected cell (59–63). With the development of more sensitive techniques, the subject of the Ad nucleoprotein structure within the infected cell has been revisited recently. Based on ChIP analysis, Ad and its derivative vectors (either E1-deleted, replication defective Ad or helper-dependent Ad [hdAd—devoid of all Ad protein coding sequences (64)]) do interact with cellular histones within a few hours of infection (43,44,65). Histones can be detected on the Ad DNA as early as 1-h post-infection, and ChIP/re-ChIP experiments show that both protein VII and histones can be found associated with the same DNA molecule in the cell (43). Since the histones almost certainly bind directly to the Ad DNA, at least some VII must be removed from the viral DNA at these time points to allow for binding of histones. The mechanism by which VII is removed, and the cellular protein(s) involved in this process, have yet to be determined.

Within the cell, deposition of cellular histones can occur either through a replication-coupled or replication-independent mechanism, and there are specific histone variants and chaperones associated with each mechanism (66). Histone variant H3.1 is expressed exclusively during S-phase and is deposited on *de novo* synthesized DNA by the Chromatin Assembly Factor I (CAF1) complex in what is considered a replication-coupled mechanism (67). In contrast, the replacement histone variant H3.3, which differs from H3.1 by only five amino acids, is expressed at all phases of the cell cycle, and is deposited through a replication-independent mechanism (66). H3.3 is deposited on actively transcribed genes by the histone chaperone HIRA, or on specific regions of the chromosome [such as pericentric DNA repeats and on telomeres (68–70)] by the H3.3 chaperone DAXX (68). The H3.3 variant is also deposited on incoming male pro-nuclear DNA shortly after fertilization utilizing the histone chaperone HIRA (71). Although TAF-1 β can act as a chaperone to transfer histones to DNA templates (47), it does not appear to perform this function during Ad infection (43).

As Ad can infect both dividing and non-dividing cells (and only induces cell cycle progression after viral gene expression has initiated), it suggests that Ad DNA is likely to be chromatinized by exploiting a mechanism independent of DNA replication. Chromatin immunoprecipitation (ChIP) experiments have recently demonstrated hdAd and E1-deleted Ad (44), and wtAd (our unpublished data) DNA preferentially associates with H3 variant H3.3 as early as 4 hpi, suggesting that chromatinization does indeed occur by a replication-independent mechanism. A preferential association with H3.3 was also found with Herpes Simplex Virus 1 (HSV1) DNA during the early phase of infection (72). siRNA-mediated knockdown of HIRA reduced the total association of H3 with the hdAd and HSV1 DNA, as well as reducing expression of virally encoded genes for both viruses, suggesting that

chromatinization was necessary for efficient expression. The involvement of the H3.3 chaperone HIRA, and not DAXX, is consistent with the observation that DAXX is actively degraded during normal Ad infection (73). As deposition of H3.3 on Ad (44) and on HSV1 (72) was dependent on HIRA, it suggests a common mechanism for deposition of histones on the genomes of invading dsDNA nuclear viruses. Moreover, the similarity between the chromatinization of sperm DNA and nuclear virus DNA suggests that both use a similar pathway to achieve chromatinization in the absence of cellular DNA replication. *In vitro* observations suggest that histone chaperones, such as HIRA, either do not assemble nucleosomes or assemble them at a greatly reduced rate in the absence of ATP-utilizing factors (74). In the male pronucleus, HIRA is necessary for delivery of H3.3 to the site of nucleosome formation (71,75), but it is the ATP-dependent chromatin remodeling complex CHD1 that is required for H3.3 deposition (74). In HeLa extracts, HIRA interacts directly with CHD1 (74). Thus, it is likely CHD1 that is directly involved in deposition of histones on the Ad DNA, although this has yet to be formally proven.

Work by Komatsu *et al.* (43) showed that wtAd can be found associated with all members of the nucleosome, H2A–H2B and H3–H4, as early as 1 hpi which, together with studies showing Ad DNA in the nucleus is wrapped in a repeating ~200 bp structure, suggests that the DNA may be wrapped in complete, physiologically spaced nucleosomes (44,59–62,76). The chaperone responsible for deposition of H2A/H2B is unknown. It has been estimated that up to 40% of infecting wtAd DNA is contained in nucleosomes at 3 hpi, and all regions of the genome are represented in micrococcal nuclease-protected fractions (59). The observation that both protein VII and histones can be found bound to the same viral DNA molecule at the same time suggests that the viral chromatin may not completely resemble that of the host cell (43). Interestingly, HSV1 genomic DNA associates with regularly spaced nucleosomes in a latent infection, but the spacing becomes ‘unstable’ during a lytic infection and generates heterogeneously sized fragments upon MNase digestion (77). Whether wtAd assembles into stable or unstable chromatin during a productive infection remains to be determined. Electron microscopy analysis of viral genomes isolated during late time points of infection (16–18 hpi) showed irregularly spaced nucleosome-like particles at approximately one-tenth the density of cellular chromatin in HeLa cells [3 versus 26 nucleosomes per μm of DNA, respectively (61)]. However, it is not clear whether this is due to ‘unstable’ chromatin or the limited quantities of histones that are available late in Ad infection. During the replicative phase of the HSV1 lifecycle, there is a switch from early association with H3.3 to deposition of H3.1 (72). Whether a similar phenomenon occurs with wtAd has yet to be determined. The observation that Ad-induced shut-off of host protein synthesis results in a reduction in histone gene expression (78,79) suggests that the virus may simply switch from association of nucleosomes containing H3.3 to re-deposition of pre-protein VII in preparation for DNA packaging into

the viral capsid at the final stage of the virus lifecycle (discussed below).

THE IMPORTANCE OF AD DNA CHROMATINIZATION

Since Ad DNA is chromatinized, this suggests that epigenetic regulation may be as important for expression of Ad-encoded genes as is it for expression of genes encoded by the host cell. ChIP analysis showed that there was an increase over time in the level of association of acetylated H3 at all Ad promoters tested (43). Since acetylated histones are commonly associated with actively transcribed genes, it suggests that as these promoters become active, they adopt an epigenetic status similar to cellular genes, which may aid in recruiting appropriate co-factors for optimal gene expression. Interestingly, the cell also uses an epigenetic approach in an attempt to down-regulate expression from some foreign, invading DNAs, including Ad. Indeed, DAXX can act as an anti-Ad defense factor and down-regulate gene expression during wtAd infection (73,80). Thus, Ad-induced degradation of DAXX at late times during infection may be a mechanism that the virus uses to evade down-regulation of its expressed genes (73). A similar phenomenon occurs for vectors based on Ad (65), and this can affect vector function *in vitro* and *in vivo* (81). In these latter studies, the vector chromatin was preferentially associated with deacetylated histones, which is a marker of transcriptionally inactive chromatin (65); thus the DNA was ‘marked’ to reduce expression of vector-encoded genes. These observations clearly illustrate the ongoing battle between host and pathogen, and the importance of epigenetic regulation of viral DNA at the chromatin level.

LATE STAGE PROTEIN VII REPLACEMENT

During the final stage of virus replication, the viral DNA must be condensed once again into the compact structure required for packaging within the viral capsid. The histones must therefore be displaced from the Ad DNA and replaced with pre-protein VII, the precursor of the mature protein VII [the N-terminus of pre-protein VII is cleaved by the viral-encoded protease during virion maturation (3)]. Little is known about how this switch occurs.

In eukaryotic cells, expression and synthesis of new histones is tightly regulated to coincide with cellular DNA replication (82). Interestingly, however, there is a dramatic decline in histone synthesis at late times during Ad infection (78,79). This puts forth the hypothesis that at the late stage of infection, the decline of available histones relative to the increased levels of pre-protein VII leads, by default, to the deposition of pre-VII on the newly synthesized viral DNA (60,62,83). Experiments in cell free systems have shown that simply mixing Ad DNA and purified pre-protein VII leads to the formation of an insoluble complex, suggesting that a specific cellular chaperone(s) mediates the placement of pre-protein VII

on Ad DNA (84). Based on co-immunoprecipitation studies using extracts from infected cells, TAF-III/nucleophosmin was shown to have a greater affinity for pre-protein VII than the mature protein VII, suggesting that TAF-III may be involved in placing pre-protein VII on the viral DNA (85). In a cell free system, TAF-III was able to transfer pre-VII onto DNA, suggesting that TAF-III is indeed a pre-VII chaperone. However, additional studies are required to further support the role for TAF-III as a chaperone during normal Ad infection of a cell.

CONCLUSION

Insight into the mechanism of Ad chromatinization has the potential to impact three specific areas of research. First, Ad is widely used as a gene delivery system for basic studies and gene therapy applications (64), and improved understanding of the parameters that aid in establishing gene expression within the host cell will improve vector efficacy and safety. Second, Ad is a significant and often overlooked human pathogen (86), and understanding early events in the cell that permit expression of viral genes may lead to the identification of new therapeutic targets to limit or prevent wtAd-induced morbidity and mortality. Finally, numerous studies of basic aspects of Ad biology have contributed significantly to our understanding of how the host cell works (3). Undoubtedly, delineation of the proteins and pathways involved in Ad DNA chromatinization will also improve our understanding of this process within the host cell.

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Curriculum Vitae

Andrea Giberson

EDUCATION

Masters of Science, Microbiology September 2010- April 2013
University of Ottawa, Ottawa, Ontario, Canada

Honours Bachelor of Science, Microbiology September 2006-April 2010
University of Guelph, Guelph, Ontario, Canada

PUBLICATIONS

Giberson, A.N., A.R. Davidson, and R.J. Parks. (2011) Chromatin structure of adenovirus DNA throughout infection. *Nucleic Acids Res.* **40 (6):** 2369-2376.

AWARDS

- Queen Elizabeth II Scholarship in Science and Technology (2011)
- Faculty of Medicine Award of Excellence in Graduate Studies (2011)
- Ontario Graduate Scholarship in Science and Technology (2010)
- University of Ottawa Excellence scholarship (2010-2011)
- Undergraduate Student Research Award funded by NSERC (2009)
- Dean's List for every semester (University of Guelph) (2006-2010)
- Robert Orr Lawson Microbiology Entrance Scholarship for highest entrance average in microbiology (University of Guelph) (2006-2010)
- University of Guelph Entrance scholarship (2006)

CONFERENCES AND PRESENTATIONS

Oral Presentation

- DNA tumour Virus Meeting, Montreal, 2012. "Structure of the Adenovirus DNA-Protein Complex in the Infected Cell"

Posters

- DNA tumour Virus Meeting, Montreal, 2012. "Adenovirus chromatin: nucleoprotein complex of the viral genome in the infected cell"
- 55th Annual Conference of the CSMB – Epigenetics and Genomic Stability, Whistler, 2012. "Adenovirus chromatin: nucleoprotein complex of the viral genome in the infected cell"
- Ottawa Hospital Research Institute Research Day, Ottawa, 2011. "Structure of the Adenovirus DNA-protein structure in the infected cell"
- Biochemistry, Microbiology and Immunology Poster Day, Ottawa, 2011. "Structure of the Adenovirus DNA-protein complex in the infected cell"

- Ottawa Hospital Research Institute Research Day, Ottawa, 2010. “Adenovirus DNA-protein structure in the infected cell”

Attended

- DNA tumour Virus Meeting, Montreal, 2012.
- 55th Annual Conference of the CSMB – Epigenetics and Genomic Stability, Whistler, 2012.
- International Congress on Human Genetics, Montreal, 2011.
- New Directions in Biology and Disease of Skeletal Muscle Conference, Ottawa, 2010.

LABORATORY SKILLS

- Adept with a large variety of laboratory protocols including genomic and plasmid DNA extraction, polymerase chain reaction, agarose gel electrophoresis, southern blots, western blots, chromatin immunoprecipitation, protein purification, and thin layer chromatography
- Working knowledge of laboratory instruments such as centrifuges, autoclaves, pH meters, phase and fluorescence microscopes, homogenizers and sonicators
- Very familiar and proficient with Prokaryotic cloning and protein expression including the making of competent cells, chemical transformation, electroporation, IPTG or arabinose induced protein expression and targeted genomic knockouts
- Completely comfortable with Eukaryotic cloning and protein expression including techniques and protocols such as total and mRNA extractions, reverse-transcriptase/PCR, cell line culturing in a biological safety cabinet and immunoprecipitation

EMPLOYMENT EXPERIENCE

Laboratory Researcher

May 2010- Current

Laboratory of Dr. Parks, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON

- Work conducted on the structure of the Adenovirus DNA-protein structure in the infected cell
- Supported by a Ontario Graduate Scholarship in Science and Technology (OGSST)

Laboratory Researcher

September 2009-April 2010

Laboratory of Dr. Wootton, Department of Pathobiology, University of Guelph, Guelph, ON

- 450+ hours of laboratory work over two semesters as part of a fourth year Research Project (unpaid)
- Work conducted on six ovine proteins to confirm their interaction with the Enzootic Nasal Tumor Virus envelope protein and possibly play a role in pathogenesis

Laboratory Researcher

May-August 2009

Laboratory of Dr. Whitfield, Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON

- Recipient of an Undergraduate Student Research Award partially funded by NSERC

- 640 hours of laboratory time during a 16 week period working on phospholipid retrograde transport and LPS transport in *Escherichia coli*
- Confidently presented my findings in a formal scientific poster presentation

Security Guard

June-August 2008

Commissionaire Ottawa, Ottawa, ON

- Diligently and attentively assured the security of people and property through access control, by restricting entry to those without proper clearance and patrolling the surroundings
- Efficiently and cheerfully dealt with guests by registering them promptly
- Quickly and accurately wrote, transcribed, sorted and filed reports and personal information sheets alphabetically

Front Line Receptionist

May -August 2007

Bossart Salon and Spa, Ottawa, ON

- Effectively and gladly provided a high standard of customer service by welcoming customers, answering any questions and answering the phones
- Responsibly and efficiently dealt with monetary matters, by cashing out customers promptly, and calculating the day's final totals

Swim Staff

June - August 2006

Camp Trillium, Children's Oncology Camp – Rainbow Lake, Waterford, ON

- Competently and effectively took part in high stress teamwork, while life guarding in teams of 2 to 5 guards in various environments (pool, lake, docks)
- Enthusiastically and diligently cared for disabled and ill children from ages 1 to 16, by ensuring their safety and prompt, timely medication, bringing them to meals, supervising them 24 hours a day, etc
- Creatively organized and led activities, games and swimming lessons, averaging one hour in length, for groups ranging from 2 to 200 people

COMMUNICATION SKILLS

- Completely bilingual (French and English) and basic Spanish
- Very strong knowledge of office software including Microsoft Office, Corel WordPerfect Suite and OpenOffice
- General computer skills such as email and browsing the internet
- Comfortable with public speaking in a formal or informal setting including scientific poster presentations, seminars and lab meetings

OTHER SKILLS

- Secret level Government security clearance
- Possessed Standard First Aid, CPR C and National Lifeguard Service (NLS) certifications
- Possessed Ontario Private Security and Investigative Services licence
- NAUI Advanced scuba diving certification