Cellular response to adenovirus and adeno-associated virus coinfection

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FINAL APPROVAL OF DISSERTATION
Doctor of Philosophy in Biomedical Sciences

Cellular Response to Adenovirus and Adeno-Associated Virus Co-Infection

Submitted by:
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In partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences

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Cellular Response to Adenovirus and Adeno-Associated Virus Coinfection

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The University of Toledo

2009
Dedication

I would like to dedicate this work to my parents Jack and Jean Bevington, my brothers Jim and John, and my fiancé Roy Collaco, Ph.D. They have always been there for me and have supported me through this entire process.
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Introduction

Adeno-associated virus (AAV) is a single-stranded linear DNA virus in the genus Dependovirus of the Parvoviridae family (Tattersall, 2006). A helper virus is needed for AAV to efficiently replicate inside a host cell (Ward, 2006). The most studied helper-virus is adenovirus (Ad). In the absence of a helper-virus, AAV integrates its genome site-specifically into host chromosome 19 or persists as an episome in the nucleus (Owens, 2006). Adeno-associated virus is a non-pathogenic virus that can transduce both dividing and quiescent cells and has been studied for use as a gene therapy vector (Carter, 2006). Besides AAV being non-pathogenic, AAV also has anti-oncogenic and anti-proliferative effects (Kerr and Linden, 2006). These effects have been attributed to the AAV non-structural proteins (designated Rep proteins), the viral DNA, terminal repeats, and the intact capsid (Alam et al., 2006; Khleif et al., 1991; Raj et al., 2001).

The AAV genome is approximately 4.7 kb long and encodes three structural and four non-structural proteins (Cassinotti et al., 1988; Ruffing et al., 1994; Srivastava et al., 1983). The three structural proteins are designated VP1, VP2, and VP3 and make up the non-enveloped icosahedral capsid in a 1:1:10 ratio of VP1:VP2:VP3 (Xie et al., 2002). The four non-structural proteins are designated Rep78, Rep68, Rep52, and Rep40 based on their replicative function and apparent molecular weight (Hermonat et al., 1984; Mendelson et al., 1986; Tratschin et al., 1984a). This variety of Rep proteins is achieved using a common reading frame where mRNA transcript initiation is started at two different promoters creating differences between Rep78/68 and Rep52/40 and the use of a splicing signal creating the differences between Rep78/52 and Rep68/40 (Mendelson et
al., 1986). Portions of the Rep proteins are identical in sequence. Rep78 and Rep68 differ in sequence only at the carboxy-terminal end where the last 92 amino acids (a.a.) of Rep78 are exchanged for nine residues. Rep52 and Rep40 are amino-terminal truncated forms of Rep78 and Rep68, respectively, where the first 124 a.a. common in Rep78/68 are absent in Rep52/40 (Srivastava et al., 1983).

Rep78 and Rep68 have site-specific DNA binding activity, endonuclease activity, helicase activity, and ATPase activity (Im and Muzyczka, 1990; Im and Muzyczka, 1992). A ligase activity has also been associated with Rep78/68 although the underlying importance of this activity in replication is not known (Im and Muzyczka, 1992; Smith and Kotin, 2000). Rep52/40 have helicase and ATPase activities (Collaco et al., 2003; Smith and Kotin, 1998). Rep78/68 are essential for AAV replication; they are important for transcriptional regulation, viral genome replication, and viral genome integration into the host chromosome during latent infection (Dutheil and Linden, 2006; Nuesch, 2006). Rep52/40’s role in AAV replication mainly concerns packaging of the viral genome into the preformed viral capsid (Chejanovsky and Carter, 1989).

Previous studies looking at cellular proteins that interact and mediate the Rep proteins’ replicative functions have identified several cellular proteins that interact with Rep78/68 and some of these proteins are involved in transcriptional regulation. In the papers that describe Rep protein interaction with cellular proteins, the authors identified Rep-interacting proteins either by yeast-two-hybrid approaches or based on knowledge of Rep78/68’s transcriptional regulation and trying to identify Rep78/68’s role in the regulation of certain promoters. In our approach to identify other Rep78/68-interacting proteins, we utilized affinity chromatography to screen for potential Rep78/68-interacting
proteins. Initial experiments were performed to identify proteins that might interact with Rep78/68 and also determine the importance of the interaction. Patrick Needham, Ph.D., performed the initial screen using affinity chromatography with poly-histidine tagged Rep68 to extract potential Rep78/68 interacting partners from HeLa nuclear extracts (Bevington et al., 2007). The proteins identified by mass spectrometry (Venkatesh Basrur, Ph.D.) were nucleolin (C23), nucleophosmin (B23, NPM, or NPM1), high mobility group chromosomal protein 1 (HMG-1), template activating factor 1/set (SET/TAF-1), and acidic Leu-rich nuclear phosphoprotein B (Anp32B) (Bevington et al., 2007). Rep78 interaction with HMG-1 and Rep68 interaction with Anp32B and SET/TAF-1 has been previously reported (Costello et al., 1997; Pegoraro et al., 2006). Nucleolin interaction with intact viral capsids has also been previously reported (Qiu and Brown, 1999). Of the proteins identified by mass spectrometry, NPM was further investigated to confirm the interaction and also to determine a possible role in the interaction. Nucleophosmin interacts with other viral proteins like the Rev protein of the human immunodeficiency virus (HIV) (Fankhauser et al., 1991). Adeno-associated virus replicates in the nucleus and is no stranger to the nucleolus where NPM resides (Wistuba et al., 1997). The AAV VP proteins accumulate in the nucleolus and require the Rep proteins, which transiently exist in the nucleolus, to escape the nucleolus (Wistuba et al., 1997). Nucleophosmin is a nucleolar protein that is involved in a variety of cellular functions including: ribosome biogenesis, centrosome duplication, and nuclear-cytoplasmic shuttling of proteins (Okuda et al., 2000; Savkur and Olson, 1998; Szebeni et al., 1997; Szebeni and Olson, 1999; Yung et al., 1985). Nucleophosmin has several activities including chaperone characteristics, DNA and RNA binding, and ribonuclease
activity (Dumbar et al., 1989; Herrera et al., 1995; Savkur and Olson, 1998; Szebeni and Olson, 1999; Wang et al., 1994). Two isoforms of NPM/B23 exist, B23.1 and B23.2, where 36 amino acids at the C-terminus of B23.1 are replaced with 2 amino acids in B23.2 (Chang and Olson, 1989).

The Rep68/78-NPM interaction was further supported by crosslinking experiments performed by Roy Collaco, Ph.D. Crosslinked proteins from infected nuclear extracts immunoprecipitated by antibodies against either Rep proteins or NPM demonstrated that both Rep proteins and NPM were co-eluted (Bevington et al., 2007). Functional experiments were conducted to both confirm a functional interaction and to determine a possible role in the interaction. Electromobility shift assay (EMSA) experiments were performed and it was noted that there was increased binding of a bacterially-expressed, maltose binding protein tagged Rep78 (MBP-Rep78) to the probe in the presence of purified bacterially-expressed B23.1. This increased binding of MBP-Rep78 coincided with increased nicking activity of the Rep protein in the presence of B23.1 demonstrated in an endonuclease assay. This increased binding and nicking indicated that Rep78/68 might be a substrate for NPM’s chaperone activity.

Investigation by immunofluorescence of NPM and Rep protein localization demonstrated that NPM localized also with AAV VP proteins in the nucleolus and at small punctate spots within the nucleus. Wistuba et al. suggested based on immunofluorescence experiments that viral capsid assembly occurs in the nucleoli (Wistuba et al., 1997). The immunofluorescence experiments were followed up with immunoprecipitation experiments, performed by Roy Collaco, Ph.D., of infected nuclear extracts that were treated with a crosslinking agent (Bevington et al., 2007). The
immunoprecipitation experiments indicated that aside from the Rep-NPM interaction there was a possible interaction between NPM and VP proteins since VP proteins were co-eluted with NPM using antibodies against NPM. However, this interaction between VP proteins and NPM may be mediated through Rep proteins since it has been demonstrated that VP proteins interact with Rep proteins (Dubielzig et al., 1999). There is strong evidence based on the column purification, EMSA experiments, and endonuclease assay experiments that the larger Rep proteins interact with NPM. The interaction of NPM with Rep and VP proteins, or at least VP-containing Rep complexes, as well as the immunoflorescence experiments suggests a possible role of NPM at AAV assembly and/or encapsidation centers. The chaperone activity of NPM might explain the enhanced binding and nicking by bacterially expressed purified proteins in *in vitro* studies.

When studying host-pathogen interactions, the cellular proteins important for a productive infection as well as the effects of the viral infection and viral proteins have on the cell needs to be addressed. A few studies have been completed looking at the effects of an AAV infection on the cell cycle. In part, these studies tried to address the observation that AAV infection has anti-proliferative effects on infected cells. One of the effects noted upon infection of primary fibroblasts was a halt at the G0/G1 as well as the G2/M phases of the cell cycle (Bantel-Schaal and Stohr, 1992; Hermanns et al., 1997). Other studies have looked at the effects of Rep78/68 on the cell cycle. Studies using retroviral-expressed Rep proteins looked at the individual effects of Rep68 and Rep78 on cell cycle and found that both Rep68 and Rep78 induce G1 and G2 phase arrest, while Rep78 also induces S phase arrest (Saudan et al., 2000). Berthet et al. tried to discern
how Rep78 was able to induce the additional S phase arrest using Rep78 transfection experiments. They demonstrated that both Rep78-mediated inactivation of Cdc25A as well as activation of ATM and Chk2 was needed in the S phase arrest (Berthet et al., 2005).

While there have been studies on the individual effects of either Ad infection or AAV infection on the cell cycle, there have not been any extensive studies on the dual effect of both Ad and AAV coinfection. Batchu et al. reported using protein mobility studies with Western blots of a potential AAV-mediated protection of retinoblastoma (pRb) family member proteins from post-translational modifications seen in Ad infections (Batchu et al., 2002). In particular, Batchu et al. noted that AAV infection protected the transcription factor E2F-1, which is important for cell cycle progression, from activation by Ad proteins (Batchu et al., 2002). This seems to be a paradoxical action by AAV, since Ad protein disruption of the transcriptionally inactive pRb-E2F-1 complex allows the E2F-1 transcription factor to be released and activate transcription of cellular proteins important for DNA replication and for the progression of the cell cycle (Berk, 2007). AAV requires Ad to push cells into S phase so that AAV can use the cellular replication machinery (Weitzman, 2006). In another study by Batchu et al., Ad infection-induced p53 degradation was partially inhibited in Ad and AAV coinfected extracts; the implications of this are unknown since the effects on downstream effector proteins of p53, like p21 expression, was not reported (Batchu et al., 1999). There are no reports on Ad and AAV coinfection effects on cell cycle proteins and no other reports on Ad and AAV coinfection effects on cell cycle-related protein effects.
To better understand Ad and AAV coinfection effects on cell cycle proteins during a coinfection, experiments were undertaken to determine if there were any effects on cell cycle protein expression when comparing AAV and Ad coinfection versus Ad infection alone. There were no significant differences noted when we compared expression of cell cycle regulatory proteins or Cdk-cyclins in Ad and AAV coinfected cells to Ad infected cells. Hermanns et al. has shown that cyclin A was reduced in AAV infected cells (Hermanns et al., 1997); the experiments discussed herein comparing AAV and Ad coinfected cells to Ad infected cells showed a decrease in expression of cyclin A, however, it was not statistically significant. To determine if there is any effect on cyclin A expression, down-stream targets would need to be assessed. We looked at cell cycle-related proteins and found no statistically significant differences in protein expression of pRb, cell division cycle 25 homolog A (Cdc25A), or proliferating cell nuclear antigen (PCNA). However, differences were seen in the specificity protein 1 (Sp1) Western blotting pattern, where in Ad infected cells there were two bands and in Ad and AAV coinfected cells the faster migrating band disappeared. The slower migrating band appears to be modified by phosphorylation (Collaco, unpublished). Post-translational modifications of Sp1 impacts Sp1 transcriptional regulatory activity (Wierstra, 2008). Sp1 is involved in regulation of AAV transcripts at the $P_{19}$ and $P_{40}$ promoters (Pereira and Muzyczka, 1997a; Pereira and Muzyczka, 1997b). It should be noted that the experiments determining the differences in effects on the cell cycle-related protein expression between Ad and AAV coinfected cells and Ad infected cells are preliminary and needs to be verified in other cell lines, especially in primary cell lines that contain no other viral DNA used to transform the cell line.
Once AAV infects a cell with a helper-virus present, production of both viruses occurs; however, production of infectious Ad particles in coinfected cells is reduced up to 50-fold compared to Ad infection alone (Timpe et al., 2006). In a coinfected cell both viruses are competing for the cellular replication machinery. The exact mechanism by which AAV replication occurs at the expense of helper-virus replication is not known. In Rep transfection experiments, the Ad E2a, E1A, E4, and major late promoter (MLP) promoters were inhibited by Rep expression (Casper et al., 2005; Jing et al., 2001; Nada and Trempe, 2002; Needham et al., 2006). In agreement with these transfection experiments, Timpe et al. demonstrated in AAV and Ad coinfection experiments that the presence of AAV caused reduced expression of all Ad mRNA transcripts with the exception of mRNA transcripts from the E3 gene; however, the authors suggested that reduced expression of early gene products was not the sole reason for the reduced Ad DNA production (Timpe et al., 2006).

Looking at Ad protein expression that is disrupted during AAV replication in Ad infected cells might indicate possible mechanisms of AAV-mediated disruption of Ad replication. The substantial reduction in the level of mRNA transcripts from the Ad E4 gene indicates that the E4 gene was one of the most strongly inhibited by AAV coinfection (Timpe et al., 2006). The Ad E4 gene encodes several proteins whose functions are not fully understood. Adenovirus E4orf3 and E4orf6 are important for disrupting the cellular DNA double-strand break (DSB) response, and without both of these proteins not only is the DSB response intact but Ad viral replication is hindered (Berk, 2007). An important component of the DSB response is the MRN-complex composed of Mre11, Rad50, and NBS1 (Williams et al., 2007). The E4orf6 along with
E1B-55K proteins are involved in proteosome-mediated destruction of the MRN-complex components (Harada et al., 2002; Querido et al., 2001; Stracker et al., 2002). The E4orf3 protein is involved in the altered localization of MRN-complex components (Araujo et al., 2005; Evans and Hearing, 2005; Liu et al., 2005; Stracker et al., 2002). Without disruption of the MRN-complex, Ad DNA is concatamerized since the cell recognizes the Ad genomes as DNA double-strand breaks (Stracker et al., 2002).

Adeno-associated virus replication is not hindered by the cellular DNA damage response. On the other hand, Ad replication is inhibited by the absence of Ad proteins that disrupt the cellular DNA damage response. In fact, replication of AAV DNA can occur in the absence of a helper-virus, albeit inefficiently, when cells are treated with genotoxic agents, like hydroxyurea (Yalkinoglu et al., 1988). Also, UV treated cells were able to induce limited AAV DNA replication (Yakobson et al., 1989; Yalkinoglu et al., 1988). Furthermore, AAV is not hindered by concatamerization since rescue of integrated AAV DNA in the host chromosome or in a plasmid does occur (Cheung et al., 1980; Laughlin et al., 1986; Samulski et al., 1982). The rescue and replication of AAV DNA can tolerate large deletions in one of the ITRs that flank the genome (Samulski et al., 1983; Senapathy et al., 1984). In light of this, we attempted to determine if AAV can alter or inhibit adenovirus’ ability to disrupt the DSB response pathway.

The MRN-complex is an important complex with a critical, multifunctional role in the DNA double-strand break repair (DSBR) process (Williams et al., 2007). During DSBR, the MRN complex recruits ataxia-telangiectasia mutated (ATM) protein to areas of damage and, once activated by autophosphorylation, the ATM protein phosphorylates (activates) downstream effector and other signaling proteins (Branzei and Foiani, 2008;
Lee and Paull, 2007). As stated previously, Ad disrupts the MRN-complex. When looking at AAV coinfection effects on the MRN-complex in Ad infected cells, there was an increase in protein levels of these key proteins, although it was not to the levels of mock-infected cells. Also, phosphorylation of NBS1 at S343 was observed to a greater extent in Ad and AAV coinfected cells versus cells infected with Ad alone. The MRN-complex is required for the activation of the ATM protein (Carson et al., 2003). The phosphorylation state of ATM protein as well as one of its target proteins, Chk2, was assessed. Ataxia-telangiectasia mutated protein was activated as seen by phosphorylation at S1981; however, activation of ATM was greater in Ad and AAV coinfected cells compared to Ad infected cells. The ATM target protein Chk2 was also phosphorylated at T68 in Ad and AAV coinfected cells while no phosphorylation at T68 was detected in Ad infected cells. This indicates that activation of the DSBR pathway occurs in Ad and AAV coinfected cells to a greater extent than Ad infected cells. Areas of co-localization of p-NBS1 S343 and p-ATM S1981 with Ad and AAV replication centers observed in immunofluorescence experiments indicates that the DSB response proteins have access to viral DNA. Also, although the signal was weak, p-Chk2 T68 was noted to localize inside the nucleus both inside and outside AAV and Ad replication centers. Phosphorylated Chk2 localizes throughout the nucleus in cells with DSB (Bekker-Jensen et al., 2006; Lukas et al., 2003). Experiments indicated that activation of the DSB pathway in Ad and AAV coinfected cells was related to intact AAV, expressing Rep proteins. Since disruption of the Ad E4 gene in Ad infected cells causes Ad DNA to concatamerize, and since AAV coinfection reduces the expression of Ad E4 gene, experiments were conducted to determine if concatamerization of Ad DNA occurs with Ad and AAV
coinfected cells. Concatamerized Ad DNA was not detected in coinfected cells. However, activation of the DSBR pathway might still disrupt Ad replication by unknown mechanism even in the absence of concatamerized Ad DNA (Berk, 2007; Evans and Hearing, 2003; Mathew and Bridge, 2007).
Adeno-associated virus (AAV) was discovered as a contaminating virus in adenovirus preparations (Atchison et al., 1965). Following the discovery of AAV1 other serotypes were identified: AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, and AAV11 (Bantel-Schaal and zur Hausen, 1984; Gao et al., 2004; Gao et al., 2002; Georg-Fries et al., 1984; Hoggan et al., 1966; Mori et al., 2004; Parks et al., 1967; Rutledge et al., 1998). Gao et al. used a polymerase chain reaction (PCR) technique to scan 259 human and 474 non-human primate tissue samples to identify latently infected proviral AAV sequences. Over 100 additional potential AAV viral isolates were identified; however, little is known about these isolates since only the portion of the AAV genome containing the cap gene was identified and sequenced (Gao et al., 2005).

Most studies of AAV have been with serotype 2; however, with the potential to use AAV as a gene therapy vector, researchers are also looking more extensively at other serotypes, to expand the tropism of the vector. Heparin sulfate proteoglycan has been identified as the primary receptor for AAV2, and fibroblast growth factor receptor 1 and integrin αvβ5 have been identified as co-receptors for AAV2 (Qing et al., 1999; Summerford et al., 1999; Summerford and Samulski, 1998). In contrast, AAV4 and AAV5 utilize α2,3 O-linked sialic acid and α2,3 N-linked sialic acid, respectively, as receptors (Kaludov et al., 2001; Walters et al., 2001). Once AAV2 attaches, it is thought that the virus is internalized by clathrin-coated vesicles (Bartlett et al., 2000; Duan et al.,
1999). The virus is then transported to the nucleus where the virus is uncoated and the viral genome is released (Weitzman, 2006).

Without helper virus coinfection of the AAV-infected cell, the viral genome integrates into the long arm of chromosome 19 at position 19q13.42 which is designated AAVSI (Dutheil and Linden, 2006; Kotin et al., 1992; Kotin et al., 1991; Samulski et al., 1991). The AAVSI site is located in a gene designated MBS85 (Tan et al., 2001). This site-specific integration requires Rep68/78 protein expression (Surosky et al., 1997). Once helper virus infects an AAV-infected cell, the genome is rescued out of the host chromosome and AAV replication can resume (Dutheil and Linden, 2006). Some of the known helper viruses are: adenovirus, herpes simplex virus (I and II) (Buller et al., 1981), cytomegalovirus (McPherson et al., 1985), human papillomavirus (HPV) (Walz et al., 1997), and human herpesvirus 6 (HHV-6) (Thomson et al., 1994).

**Adeno-associated virus serotype 2 genome**

Adeno-associated virus is a linear single-stranded DNA virus whose genome encodes two major open reading frames (ORF) and is flanked at both ends by inverted terminal repeat sequences (Figure 1) (Cotmore and Tattersall, 2006). The genome of AAV2 is 4,679 nucleotides long (Cassinotti et al., 1988; Ruffing et al., 1994; Srivastava et al., 1983). During AAV genome replication either strand is packaged into a progeny virus with equal efficiency, and viral particles containing either strand are capable of infecting cells and producing progeny virus (Samulski et al., 1987). To distinguish the complementary strands they were designated ‘plus’ or ‘minus’ strand based on thymidine content (Berns and Adler, 1972). The AAV2 genome contains three promoter regions designated (based on relative position in the genome) as $P_5$, $P_{19}$, and $P_{40}$ and all the
mRNAs generated from these promoters use the polyadenylation signal at map unit 96 (one map unit is equivalent to 1% of the genome) (Green and Roeder, 1980a; Laughlin et al., 1979; Lusby and Berns, 1982; Srivastava et al., 1983). The transcripts generated from all three promoters use the minus strand as the template (Carter et al., 1972; Carter and Rose, 1972; Rose and Koczot, 1971). There is an intron located within the genome with a 5’ splice site at nucleotide 1907 (Figure 1, ‘D’) and two different 3’ splice acceptor sites at nucleotides 2201 (Figure 1, ‘A1’) and 2228 (Figure 1, ‘A2’) (Becerra et al., 1988; Trempe and Carter, 1988).

**Figure 1. Adeno-associated virus serotype 2 genome and transcript products.**

The single-stranded linear genome of AAV2 is depicted at the top of the figure. The ITR hairpins are indicated at each end of the genome. The *rep* and *cap* open reading frame regions are indicated above the genome. The promoter regions (●), polyadenylation signal (■) and the mRNA splice signals (▼) are depicted and labeled on the genome. The 5’ donor splice signal is designated ‘D’ and the 3’ acceptor splice signals are designated ‘A1’ and ‘A2’. The mRNAs generated are depicted below the genome as a line and the size of the transcript is indicated at the right of the mRNA. Spliced out
portions are depicted with a bump in the mRNA. The protein products are indicated as solid black or gray rectangles below the respective mRNA and the names of the proteins are indicated at the left of the figure.

The left ORF contains the *rep* gene that encodes the four non-structural proteins named after their function (replication) and apparent molecular weight: Rep78, Rep68, Rep52, and Rep40 (Hermonat et al., 1984; Mendelson et al., 1986; Tratschin et al., 1984a). Two transcripts, 4.2 kb and 3.9 kb, are generated from the *P₅* promoter and two transcripts, 3.6 kb and 3.3 kb, are generated from the *P₁₉* promoter (Mendelson et al., 1986; Srivastava et al., 1983). The two larger Rep proteins, Rep78 and Rep68, are generated from the 4.3 kb and 3.9 kb mRNA transcripts, respectively (Mendelson et al., 1986). The two smaller Rep proteins, Rep52 and Rep40, are generated from the 3.6 kb and 3.3 kb mRNA transcripts, respectively (Mendelson et al., 1986). The 3.9 kb and the 3.3 kb mRNAs are spliced forms of the 4.2 kb and the 3.6 kb transcripts, respectively (Green and Roeder, 1980b; Laughlin et al., 1979; Srivastava et al., 1983). The 5’ donor splice site is at nucleotide 1907 and the 3’ acceptor splice sites are located either at nucleotide 2201 or 2228. It is not known which of the 3’ splice sites is used to generate the 3.3 kb and 3.9 kb spliced mRNAs or whether both are used to generate two different versions of the 3.3 kb and the 3.9 kb spliced mRNAs (Qiu et al., 2006). The splicing of the Rep78 (4.3 kb) and Rep52 (3.6 kb) mRNAs to get the Rep68 (3.9 kb) and Rep40 (3.3 kb) mRNAs is such that the region that encodes the last 93 amino acids of Rep78 and Rep52 is spliced out and replaced with 17 or nine amino acids depending on the 3’ splice site used. Figure 1 depicts the mRNAs and proteins generated from the *P₅* and *P₁₉* promoters.
The right ORF controlled by the $P_{40}$ promoter contains the cap gene which encodes the three structural proteins designated: VP1, VP2, and VP3 (Cotmore and Tattersall, 2006). The cap gene is in a different reading frame than the rep gene (Srivastava et al., 1983). There is a 2.6 kb mRNA transcript transcribed from the $P_{40}$ promoter that is differentially spliced to form two 2.3 kb mRNAs (Becerra et al., 1988; Cassinotti et al., 1988; Trempe and Carter, 1988). The three structural proteins are translated from the 2.3 kb mRNAs (Jay et al., 1981). The two 2.3 kb mRNAs are not generated in equal concentrations so there is a major product using the 2228 nucleotide splice site and a minor product using the 2201 splice site (Trempe and Carter, 1988). The minor product encodes for the VP1 protein while the major mRNA product encode the VP2 and VP3 proteins (Becerra et al., 1988; Jay et al., 1981; Trempe and Carter, 1988). The major product encodes for VP2 using a less efficient start codon (ACG) and encodes for VP3 using the classical AUG start codon (Becerra et al., 1985). The difference in transcript amount (VP1 vs. VP2/VP3) as well as a weak start codon (VP2 vs. VP3) causes VP3 to be the major product compared to VP1 and VP2. The ratio of VP1:VP2:VP3 translation is approximately the same ratio as VP1:VP2:VP3 virion content ratio (Buller and Rose, 1978). The virion contains 60 structural proteins in a ratio of 1:1:10 of VP1:VP2:VP3 (Xie et al., 2002). Figure 1 depicts the transcripts and proteins generated from the $P_{40}$ promoter.

Located at both ends of the genome are the inverted terminal repeat (ITR) sequences. The ITRs are 145 nucleotides long and the first 125 nucleotides fold back on themselves to form a ‘T’ like structure (Figure 1) (Lusby et al., 1980). The ITR has defined regions designated: A, A’, B, B’, C, C’, and D. The A, B, and C regions are
base paired with A’, B’, and C’ regions, respectively (Figure 2) (Lusby et al., 1980). The B and C sections of the hairpin is in either orientation, designated ‘flip’ or ‘flop’, with respect to the genome, and are flipped/flopped during replication of the genome (Lusby et al., 1981; Lusby et al., 1980; Samulski et al., 1982).

**Figure 2. Adeno-Associated Virus Inverted Terminal Repeat.**

![Diagram](image)

Both the Flip and Flop orientations are shown. The A, A’, B, B’, C, C’, and D regions are indicated. The RBE is highlighted with a white rectangle and the RBE’ is indicated with a bracket. The trs site is highlighted with a gray rectangle and the place where nicking occurs is indicated with an arrow head (▲).

The ITR acts as the origin of DNA replication and is important for packaging the genome as well as integration of the viral genome into the host chromosome during latent infection (McLaughlin et al., 1988; Senapathy et al., 1984). Cellular polymerase δ uses the ITR from the 3’ end of the AAV genome (which is folded back onto the genome base pairing with a portion of the A-stem) as a primer for DNA synthesis (Lusby et al., 1980; Nash et al., 2007; Ward, 2006). Sites located on the ITR important for the origin function are the Rep binding element (RBE), the terminal resolution site (trs site), and the RBE’ (Ryan et al., 1996; Snyder et al., 1993). The RBE, is an imperfect (GAGC)$_4$ tandem repeat sequence in the A-stem of the ITR to which Rep68 and Rep78 bind (McCarty et al., 1994a; Ryan et al., 1996). The trs site (3’-CCGTTTG -5’) in the A-stem region near
the RBE site (Brister and Muzyczka, 1999), is important for completing AAV replication. Rep68 or Rep78 nicks between the two thymidines opening the hairpin, allowing the nicked 3’ end to be used as a primer for DNA polymerase δ for DNA replication (Nash et al., 2007; Ward, 2006). Ryan et al. identified a CTTTG sequence, later designated RBE’, that when mutated affected the binding affinity of Rep68 to the ITR (Ryan et al., 1996). The CTTTG sequence, between palindromes within the B-C portion of the hairpin, is the same distance from the trs site whether the ITR is in the flip or flop orientation (Ryan et al., 1996). Rep68 and Rep78 bind with higher affinity to the entire ITR than just the A-stem portion (containing the RBE) alone (McCarty et al., 1994b; Ryan et al., 1996). Figure 2 depicts important sites/regions of the ITR.

**Adeno-associated virus serotype 2 non-structural proteins**

The four Rep proteins of AAV2 are the best characterized of the Rep proteins among all of the AAV serotypes. Rep78, Rep68, Rep52, and Rep40, have enzymatic functions required for viral genome integration, replication, and regulation of gene expression (Dutheil and Linden, 2006; Nuesch, 2006). The four Rep proteins arise from the use of different promoters and alternate splicing of the mRNA transcripts (Mendelson et al., 1986) (Figure 1). Rep40 and Rep52 are truncated versions of Rep68 and Rep78, respectively; the first 224 amino acids of Rep78/68 are absent in Rep52/40 (Srivastava et al., 1983). Rep78 differs from Rep68, and Rep52 differs from Rep40, at the C-terminal end where the last 93 amino acids of Rep78/52 are replaced in Rep68/40 with 17 or nine amino acids (Srivastava et al., 1983). Since there is a large degree of sequence overlap between the two large Rep proteins they have many of the same enzymatic activities and are almost interchangeable with regards to their replicative functions (Nuesch, 2006).
Similarly, with the large degree of sequence overlap between the two small Rep proteins they were also found to have many of the same enzymatic activities and are almost interchangeable with regards to their replication functions (Nuesch, 2006).

Rep68 and Rep78 exhibit sequence-specific DNA binding, which plays an important role during synthesis of the viral genome, transcriptional regulation, and integration of the viral genome into the AAWSI site (Linden et al., 1996). Using Rep deletion mutants, it was demonstrated that the N-terminal domain of Rep68 and Rep78 is the region important for the sequence specific DNA binding activity (Owens et al., 1993). Rep68/78 binds to the imperfect (GAGC)$_4$ tandem repeat sequence, designated the RBE, located in the ITR (Figure 2) (McCarty et al., 1994a; Ryan et al., 1996). Rep68 and Rep78 also have a site-specific, stand-specific endonuclease activity important for viral genome replication and integration of the viral genome into the AAWSI site (Im and Muzyczka, 1990; Im and Muzyczka, 1992). This nicking activity works in conjunction with Rep68/78’s RBE binding ability (Ryan et al., 1996). The binding of Rep68/78 to the RBE positions the protein to nick the target sequence, 3’-CCGGTTG -5’ (trs site), between the two thymidines (Brister and Muzyczka, 2000). During the nicking process, Rep68 or Rep78 is covalently linked to the 5’end of the DNA via a transesterification process (Snyder et al., 1990). Experiments where distance between the trs site and the RBE was increased or where the RBE sequence was inverted greatly reduced Rep68/78’s nicking ability (Brister and Muzyczka, 2000). The RBE and trs site are located within the ITR (Figure 2) and within the AAWSI site (Linden et al., 1996; McCarty et al., 1994a; Ryan et al., 1996; Urcelay et al., 1995; Weitzman et al., 1994). Additionally, Rep68 and Rep78 have a DNA ligase activity; however, the implications of this activity have not
been fully elucidated (Im and Muzyczka, 1992; Smith and Kotin, 2000). Rep68/78 contain a nuclear localization signal which consists of two groups of basic amino acids between residues 492 to 509 (Cassell and Weitzman, 2004; Kleinschmidt et al., 1995; Yang et al., 1992).

Common to all four Rep proteins are helicase and ATPase activities (Collaco et al., 2003; Im and Muzyczka, 1990; Im and Muzyczka, 1992; Smith and Kotin, 1998). Rep68/78 helicase activity is important during replication of the viral genome (Ward, 2006). In order for Rep68/78’s nicking activity to occur with a double stranded substrate the Rep68/78’s helicase activity is needed to open up the \textit{trs} site (Ward, 2006). Rep40/52 helicase activity is important for packaging of the viral genome into a preformed capsid (Chejanovsky and Carter, 1989; Yoon-Robarts et al., 2004).

The differences between the two large Rep proteins and between the two small Rep proteins deal mainly with protein interactions. Rep78 and Rep52 differ from Rep68 and Rep40 with respect to their interaction with protein kinase A (PKA) and PrKX (a homolog of PKA catalytic subunit); the domain of Rep78/52 required for this interaction is the C-terminus which also contains a zinc binding domain (Chiorini et al., 1998; Di Pasquale and Stacey, 1998; Horer et al., 1995). The C-terminus unique to Rep68 and Rep40 is phosphorylated at Ser 535 which affects its interaction with cellular proteins 14-3-3\textepsilon and 14-3-3\textgamma (Han et al., 2004).

\textit{Replication of the AAV Genome}

Replication of AAV’s single stranded genome begins with the ITR folding back on itself forming a hairpin structure. A cellular DNA polymerase uses the hairpin with a free 3’ end as a primer for DNA synthesis (Ward, 2006). The cellular polymerase \textdelta
synthesizes the genome through the other ITR (Nash et al., 2007; Ward, 2006). Once the ssDNA is converted to dsDNA (replicative form monomer or RF monomer), Rep protein transcription and translation can occur. The Rep proteins are needed to open up the ITR used as a primer for the cellular polymerase (Ward, 2006). Rep68/78 bind to the RBE and nick at the trs site; Rep68/78 covalently links to the ITR (Snyder et al., 1990). The ITR is unwound and the cellular DNA polymerase is able to synthesize the primer ITR (Ward, 2006). Once synthesis is completed the genome is packaged into a preformed viral capsid. It is not entirely clear how packaging occurs but it requires Rep52 and Rep40 with functional helicase activity (Chejanovsky and Carter, 1989; King et al., 2001). One intermediate that develops during AAV genome replication is the replicative form dimer (RF dimer). This occurs when the ITR of the RF monomer which is not used as a primer disassociates, allowing the single strands of DNA to reform hairpins (Ward, 2006). The reformed hairpin with a free 3’ end is used as a primer for the cellular polymerase and synthesizes the AAV genome again (Ward, 2006). If the cellular polymerase reaches the original hairpin primer before the Rep proteins can nick the trs site and synthesizes through the original primer hairpin all the way back to the other replicated ITR, then a RF dimer is formed (Ward, 2006).

In vitro replication experiments have been used to identify cellular proteins involved in replication of the viral DNA. Ni et al. noted that in vitro AAV genome replication could occur from extracts of HeLa cells that were at a high density. The HeLa cell extracts were fractionated and crude fractions, as well as components of the fractions, were tested for their ability to replicate the AAV genome. Experiments suggested either DNA polymerase δ or ε was involved in the AAV genome replication and that
proliferating cell nuclear antigen (PCNA), replication factor C (RFC), replication protein A (RPA), and an unidentified component(s) from one fraction of the fractionated extracts were capable of supporting AAV genome replication (Ni et al., 1998). Proliferating cell nuclear antigen is a DNA sliding clamp which tethers DNA polymerases to DNA, increasing the polymerase’s processivity (Maga and Hubscher, 2003). Replication factor C is a multimeric protein important for loading PCNA onto DNA (Indiani and O'Donnell, 2006). Replication protein A is a single stranded DNA (ssDNA) binding protein that protects ssDNA from forming hairpin structures and digestion by nucleases (Fanning et al., 2006). Nash et al. used fractionated Ad infected 293 cell extracts in in vitro replication assays to identify proteins important for AAV genome replication. In agreement with Ni et al., PCNA and RFC were important components for the in vitro replication. Nash et al. found that when comparing DNA polymerase δ, ε, and α in the in vitro replication assay only DNA polymerase δ was capable of replicating AAV DNA. In contrast to Ni et al., it was demonstrated that RPA was not important for in vitro AAV DNA replication nor was the adenovirus ssDNA binding protein E2a; however, additional unidentified component(s) from the fractionated extracts were important for in vitro AAV DNA replication (Nash et al., 2007).

**Rep protein transcription regulation**

One of the functions of the Rep proteins is transcriptional regulation. When no helper virus is present, low level transcription of the Rep proteins occurs and further transcription of the AAV genome is repressed by the Rep proteins; however, once a helper virus is present, the transcriptional repression by Rep protein is relieved (Beaton et al., 1989; Horer et al., 1995; Pereira et al., 1997; Pereira and Muzyczka, 1997b).
Rep proteins repress transcription of other viral promoters as well including those of the helper virus (Casper et al., 2005; Horer et al., 1995; Prasad et al., 2003; Su et al., 2000). Additionally, the larger Rep proteins repress several cellular promoters (Batchu et al., 1994; Batchu et al., 2001; Hermonat, 1991; Hermonat, 1994; Prasad et al., 2003).

**Cellular proteins that interact with Rep proteins**

There are several cellular proteins that have been identified that interact with the Rep proteins, many of which are transcriptional regulatory proteins. High mobility group 1 (HMG1) protein interacts and augments Rep78 and Rep68 enzymatic activities (Costello et al., 1997). The transcription factor, Sp1, helps Rep68/78 interact with AAV’s $P_{19}$ and $P_{40}$ promoters (Pereira and Muzychka, 1997a; Pereira and Muzychka, 1997b). Topors, which binds p53 and topoisomerase I, is a cellular protein that interacts with the larger Rep proteins and regulates AAV gene expression (Weger et al., 2002). TATA box binding protein (TBP) interacts with Rep78 and prevents it from binding to its target in the $P_{97}$ promoter of HPV-16, which may be a mechanism that Rep78 inhibits transcription at that promoter (Hermonat et al., 1998; Su et al., 2000). Inhibition of the Ad major late transcription promoter (AdMLP) by Rep68/78 may also be accomplished by Rep68/78 interaction with TBP at the TATA box of AdMLP (Needham et al., 2006). Rep78 also interacts with c-Jun and inhibits its transcriptional activation of HPV-16 $P_{97}$ and c-$jun$ gene promoter (Prasad et al., 2003). Positive cofactor 4 (PC4), a transcription cofactor, interacts with Rep78 and Rep68 and affects transcription at all three AAV promoters (Weger et al., 1999). Rep68 interacts with 14-3-3 isotype $\gamma$ and isotype $\epsilon$ when S535 of Rep68 was phosphorylated. Using a point mutation virus in which Rep68 S535 was replaced with an alanine, the authors noted that the interaction reduced AAV
viral genome replication (Han et al., 2004). Rep78 was shown to stabilize Cdc25A as well as prevent Cdc25A phosphatase activity on its substrates Cdk2 and Cdk1, which the authors suggest is one part of the mechanism by which Rep78 arrests cells in S phase (Berthet et al., 2005). Recently, using affinity chromatography, Nash et al. were able to identify 188 proteins that were pulled down in Rep-containing complexes from Ad and AAV coinfected cells (Nash et al., 2009). One of the proteins identified was nucleophosmin, which supports the results of the manuscript discussed in a later section (Bevington et al., 2007; Nash et al., 2009).

**Nucleophosmin (B23 or NPM) interacts with viral proteins**

Nucleophosmin (B23, NPM, or NPM1) is a highly conserved protein (among rat, mouse, and human) (Chang and Olson, 1989; Okuda, 2002). It exists in two isoforms, with the more abundant B23.1 localizing primarily in the nucleolus (Wang et al., 1993). B23.2 is an alternatively spliced form of B23.1 with 36 amino acids at the C-terminus of B23.1 replaced with two amino acids (Chang and Olson, 1989; Chang and Olson, 1990; Okuwaki et al., 2001a). Nucleophosmin is involved in a variety of cellular activities that include: ribosome biogenesis (Okuda, 2002; Savkur and Olson, 1998; Yung et al., 1985), histone chaperone (Okuwaki et al., 2001b), duplication of centrosomes (Okuda, 2002; Okuda et al., 2000), shuttling proteins to the nucleus and nucleolus (Borer et al., 1989; Szebeni et al., 1995; Szebeni et al., 1997), and has chaperone protein characteristics (Szebeni et al., 2003; Szebeni and Olson, 1999). With regards to enzymatic function, both forms of NPM (B23.2 to a lesser extent) have ribonuclease activity utilizing tRNA and mRNA as substrates; however, NPM has a specificity for rRNA (Herrera et al., 1995; Savkur and Olson, 1998). Only the larger isoform of NPM has DNA and RNA binding
activity with nonspecific binding to single-stranded DNA, double-stranded DNA, and RNA substrates (Dumbar et al., 1989; Herrera et al., 1996; Wang et al., 1994).

Nucleophosmin interacts with human immunodeficiency virus proteins, Rev and Tat, which are viral proteins involved in mRNA processing and viral transcription regulation, respectively (Fankhauser et al., 1991; Li, 1997; Nekhai and Jeang, 2006). Rex protein, involved in mRNA transport and splicing of human T-cell leukemia virus, is another viral protein that interact with NPM (Adachi et al., 1993; Kashanchi and Brady, 2005). Nucleophosmin may import, or help import, these three viral proteins into the nucleus and nucleolus (Adachi et al., 1993; Fankhauser et al., 1991; Li, 1997; Szebeni et al., 1995; Szebeni et al., 1997). Nucleophosmin interacts with hepatitis delta antigen (large and small) proteins and modulates hepatitis delta viral RNA replication by an unknown mechanism (Huang et al., 2001). Adenovirus infection and Ad structural protein V alters cellular localization of NPM (Matthews, 2001; Walton et al., 1989). Additionally, an in vitro model using Ad core proteins identified nucleophosmin as a protein that stimulates replication (Okuwaki et al., 2001a).

**Cell cycle**

The cell cycle is divided up into four stages: gap1 (G1), DNA synthesis (S), gap 2 (G2), and mitosis (M). Quiescent cells are in G0. Cyclin dependent kinases (Cdk) are serine/threonine kinases that along with their partner protein, cyclin, form complexes that promote and regulate progression through the stages of the cell cycle (Vermeulen et al., 2003). Cdk4-cyclin D and Cdk6-cyclin D regulate progression through G1 phase, and Cdk2-cyclin E regulates entrance into S phase (Vermeulen et al., 2003). One of the important substrates for Cdk4-cyclin D and Cdk6-cyclin D are the retinoblastoma (Rb)
family proteins (Kozar and Sicinski, 2005). The Rb family of proteins consists of pRb, p107, and p130 (Du and Pogoriler, 2006). The binding of Rb family proteins to E2F family transcription factors prevent the activation of E2F target genes (Du and Pogoriler, 2006). The E2F transcription factors are important for the expression of many proteins including other cell cycle proteins, cyclin A and cyclin E, as well as DNA replication proteins, like PCNA (Du and Pogoriler, 2006). Cdk4/6-cyclin D both phosphorylate pRb, which releases the E2F1 transcription factor (Kozar and Sicinski, 2005). The target genes of E2F1 are genes important for the S phase, which include other cell cycle regulatory proteins like cyclin A and cyclin E (Pardee et al., 2004). The Cdk2-cyclin E complex further phosphorylates pRb to allow cells to enter S phase, while Cdk2-cyclin A regulates progression through S phase (Kaldis and Aleem, 2005). Cdk1 (Cdc2) appears to be able to replace Cdk2 to interact with cyclin E, allowing cells to enter S phase (Kaldis and Aleem, 2005). Cdk1-cyclin A is responsible for progression through G2, while Cdk1-cyclin B regulates progression through mitosis (Kaldis and Aleem, 2005). Cdk7-cyclin H along with MAT1 form the Cdk-activating complex (CAK) which activates Cdk-cyclin complexes by phosphorylation during the cell cycle (Lolli and Johnson, 2005). There are other Cdk-cyclin complexes that are not involved in cell cycle regulation. One example is the Cdk9-cyclin T complex that phosphorylates RNA polymerase II stimulating elongation of mRNA transcripts during transcription (Garriga and Grana, 2004).

Other important proteins involved in the regulation of the cell cycle are the Cdc25 family of proteins. The Cdc25 proteins are phosphatases that activate cyclin-dependent kinase proteins by removing the phosphate on threonine and tyrosine at the Cdk
activation domain (Rudolph, 2007). Additionally, Sp1 is a transcriptional regulator that binds GC-rich regions to promote transcription of a variety of genes (Wierstra, 2008). A variety of proteins modify Sp1, including the Cdk2-cyclin A complex that phosphorylates Sp1 (Wierstra, 2008). The Cdk2-cyclin A complex phosphorylates Sp1, increasing its transcriptional activity (Fojas de Borja et al., 2001). Also, Sp1 is involved in the regulation of expression of a number of cell cycle proteins: Cdk2, cyclin D1, cyclin D2, cyclin D3, cyclin E, and cyclin B1 (Wierstra, 2008). Negative regulators of the cyclin-dependent kinases, called cyclin-dependent kinase inhibitors (CKI), are grouped into two families called CIP/KIP family and the INK4 family (Mainprize et al., 2001). The CIP/KIP family, including p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, inhibit Cdk4/6-cyclin D and Cdk2-cyclin E/A arresting cells at the G1-to-S transition (De Clercq and Inze, 2006). The p21^{Cip1} and p27^{Kip1} proteins inhibit the Cdk1-cyclin A complex, which is important for exiting mitosis (De Clercq and Inze, 2006). The INK4 family includes p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}, and mostly inhibits binding of cyclin D to Cdk4 and Cdk6 (De Clercq and Inze, 2006).

**DNA Damage Response**

Repair of DNA damage is a complex process involving sensing, signaling, and effector proteins. The proteins involved depend on the type of damage and the phase of the cell cycle although there is some signaling overlap (Branzei and Foiani, 2008; Pommier et al., 2005). One set of signaling proteins involved in different repair pathways are the phosphoinositide 3-kinase related kinases ataxia-telangiectasia mutated (ATM) protein, ATM/Rad3 related (ATR) protein, and DNA-PK (Branzei and Foiani, 2008). These proteins are involved in different repair processes. Ataxia-telangiectasia
mutated protein and DNA-PK are primarily activated with DNA double-stranded breaks from ionizing radiation or chemically induced, and ATR is primarily activated by ssDNA and stalled replication forks (Branzei and Foiani, 2008; Dery and Masson, 2007; Pommier et al., 2005). Recruitment of these signaling proteins to damage sites on the DNA is primarily by the MRN-complex for ATM, by Ku80 for DNA-PK, and by ATRIP for ATR (Branzei and Foiani, 2008). The rest of this section will be focused on ATM activation.

The MRN-complex is composed of Mre11, Rad50, and NBS1, and is required for activation of the ATM protein, a serine and threonine kinase (Lee and Paull, 2007). The ATM protein exists as an inactive dimer in normal cells, and once activated the ATM protein autophosphorylates itself at S1981 and separates into active monomers (Bakkenist and Kastan, 2003). Activated ATM localizes to the double-strand break (Bekker-Jensen et al., 2006). Once activated, ATM phosphorylates many other proteins that are involved in cell cycle control and the DNA damage response, which includes (but not limited to): NBS1, Chk2, RPAp34, p53, H2AX, SMC1, BRCA1, and Artemis (Lee and Paull, 2007).

Check kinase 2 (Chk2), a serine and threonine kinase, is phosphorylated by ATM on T68 primarily in response to DNA double-stranded breaks induced by ionizing radiation (Matsuoka et al., 2000; Pommier et al., 2005). The phosphorylation of Chk2 at T68 induces oligomerization of Chk2 and autophosphorylation on T383 and T387 (Lee and Chung, 2001; Pommier et al., 2005; Schwarz et al., 2003). Activated Chk2 can then induce S phase arrest by phosphorylating Cdc25A at S123, which causes Cdc25A (a phosphatase) degradation that in turn prevents Cdk2 activation (Falck et al., 2001). Similarly, progression into mitosis is blocked involving a similar cascade where activated
Chk2 phosphorylates Cdc25C (a phosphatase) at S216 causing Cdc25C to be sequestered (instead of degraded), which prevents Cdk1 activation (Matsuoka et al., 1998).

**Adeno-associated virus and cellular response**

There have been several publications that address cell cycle effects and cellular response to infection with AAV. Infection of primary fibroblasts with AAV arrested the cells in G0/G1 and G2/M phases (Hermanns et al., 1997). Infection of primary fibroblasts resulted in a change in the mobility of Rb-family proteins pRb, p130, and p107 by Western blot, which was consistent with the pattern the hypophosphorylated form of these proteins exhibited on SDS-PAGE electrophoresis (Hermanns et al., 1997). This altered mobility of the Rb-family of proteins was accompanied by increased protein levels of p21^{Waf1}, an inhibitor of G1 phase Cdk-cyclin complexes that are responsible for pRb phosphorylation (Hermanns et al., 1997). The increase in p21^{Waf1} was also seen on infection of primary keratinocytes with AAV2 (Alam et al., 2006). Using a retroviral system to individually express the four Rep proteins it was noted that Rep68 and Rep78 were capable of inducing G1 and G2 phase cell cycle arrest, while Rep78 was also able to induce S phase arrest (Saudan et al., 2000). In Rep78 transfection experiments, Berthet et al. suggested that Rep78-mediated S phase block required both Cdc25A inactivation as well as DNA damage signaling protein ATM and Chk2 activation (Berthet et al., 2005).

There are also studies that have looked at the effects of infection with inactivated AAV or recombinant AAV virus (rAAV) that replace the rep and cap genes with reporter genes. Using different p53 knock-out cell lines and control cell lines, Raj et al. found that UV-inactivated AAV can induce apoptosis in cells that lack p53 or cause G2 arrest in cells with functional p53, and this response might be due to a cellular DNA damage
response (Raj et al., 2001). The G2 arrest in cells with functional p53 was accompanied by increased levels of p53 and p21\textsuperscript{Waf1} and decreased levels of Cdc25C (Raj et al., 2001). In rAAV transduction experiments, it was noted that there was an increase in transduction efficiency in ATM deficient cell lines and this increase was not due to differences in binding or nuclear transport of the recombinant virus (Sanlioglu et al., 2000; Zentilin et al., 2001). The authors also showed that Ku86 and Rad52, proteins involved in DSBR, interacted with rAAV genome (Zentilin et al., 2001). Furthermore, Grifman et al. used a 293 cell line (transformed with Ad \textit{E1A} and \textit{E1B} genes) with inducible expression of Ad E4orf6 and found that rAAV transduction was enhanced with E4orf6 expression (Grifman et al., 1999). The authors also noted that rAAV transduction of this 293 cell line with induced E4orf6 expression was accompanied by reduced cyclin A protein levels; conversely, overexpression of cyclin A was found to reduce rAAV transduction efficiency (Grifman et al., 1999).

There have been no reports in the literature on AAV and Ad coinfection effects on cell cycle proteins and only a couple of reports on cell cycle-related proteins. There has been suggestive evidence with AAV and Ad coinfection experiments that AAV infection inhibits adenovirus-mediated inactivation of Rb family proteins and inhibits adenovirus-mediated degradation of p53 (Batchu et al., 2002; Batchu et al., 1999). Studies using a different AAV helper virus, HPV, showed that using HPV31b-positive cervical carcinoma cells infected with AAV2 caused decreased p21 protein levels as well as increased Cdk2-cyclin associated kinase activity. However, no changes in the cell cycle were noted upon AAV infection of these cells (Alam et al., 2006).
**Adenovirus**

Adenoviruses capable of infecting humans belong in the *Mastadenovirus* genus of the *Adenoviridae* family (Berk, 2007). There are 51 human serotypes subgrouped into six species based on hemagglutination characteristics (Berk, 2007). Adenovirus serotype 5 (Ad5), the most extensively studied serotype, contains a genome of 35,935 nucleotides (Chroboczek et al., 1992). The Ad genome is linear, double-stranded DNA encoding transcription units split into three phases of expression: early (E1A, E1B, E2 early, E3, and E4), delayed early (E2 late, IVa2, and IX) and late (L1, L2, L3, L4, and L5) (Berk, 2007).

**Adenovirus and the cell cycle**

Adenovirus utilizes cellular DNA synthesis machinery in order to replicate its own viral genome; therefore, Ad must induce quiescent cells into S phase (Berk, 2007). Initial studies performed to determine which adenovirus gene(s) were responsible for cell cycle induction of quiescent cells identified the gene products of *E1A* (Braithwaite et al., 1983). Two of the protein products encoded by the *E1A* gene are designated E1A 12S (also known as small E1A) and E1A 13S (also known as large E1A) (Berk, 2007). The E1A 12S and the E1A 13S transcripts are formed from alternative splicing, with the larger version of E1A containing an additional 46 amino acids in the middle region of the protein (Perricaudet et al., 1979). The E1A 13S protein contains several conserved regions: N-terminal region, conserved region 1 (CR1), CR2, CR3, and CR4 (Kimelman et al., 1985; van Ormondt et al., 1980). The CR3 domain is absent in E1A 12S protein (Berk, 2005).
Two regions of the E1A protein are important for inducing cells to enter S phase, the CR2 domain alone or the E1A N-terminal region in association with the CR1 domain (Berk, 2007; Howe et al., 1990; Lillie et al., 1987; Stein et al., 1990; Zerler et al., 1987). The CR2 domain was found to bind to the Rb family of proteins: pRb, p107, and p130 (Barbeau et al., 1992; Berk, 2005; Dyson et al., 1992; Whyte et al., 1988). The binding of E1A to members of the Rb family of proteins displaces the Rb family proteins from E2F transcription factor proteins, which allows the E2F transcription factors to activate expression of important S phase genes (Berk, 2007). The N-terminal region and CR1 domain of the E1A proteins together also induce cells to enter S phase (Howe et al., 1990; Lillie et al., 1987; Zerler et al., 1987). The mechanism by which this is accomplished is less clear, but may involve chromatin remodeling proteins based on identified proteins that interact with this region of E1A proteins (Berk, 2007). Other important protein-protein interactions of the E1A proteins include inhibition of several CDK inhibitory (CKI) proteins (described in earlier section). E1A proteins inhibit p16^{Ink4a}, p21, and p27^{Kip1} by an unknown mechanism (Alevizopoulos et al., 1998; Alevizopoulos et al., 2000; Chattopadhyay et al., 2001; Mal et al., 1996).

**Adeno-associated virus and adenovirus**

Adenovirus has been the most extensively studied helper virus for AAV. The gene products required from adenovirus are: E1A, E1B, E2a, E4orf6, and VA1 (Janik et al., 1981; Laughlin et al., 1982). Indeed, AAV helper plasmids that effectively provided helper function for AAV replication were designed containing some of the important Ad early genes (E2a, E4, and VA1), and utilizing a cell line which contained the other important Ad early gene (HEK 293 cell line containing E1A and E1B) (Collaco et al.,
1999; Xiao et al., 1998). Overall, Ad E1B and E4 appear important for AAV DNA replication, Ad E2a and VAI RNA appear important for AAV post-transcriptional and translational events, respectively, and E1A appears important for AAV promoter induction (Carter, 1990).

Various studies have tried to elucidate the role of each Ad element important for helper function. Adenovirus E1A protein induces AAV \( P_5 \) promoter (Chang et al., 1989) as well as the AAV \( P_{19} \) promoter (Tratschin et al., 1984b). Adenovirus E1A protein helps activate the AAV \( P_5 \) promoter by relieving the cellular transcription factor YY1 induced repression (Shi et al., 1991). The Ad E2a protein also induces the AAV \( P_5 \) promoter (Chang and Shenk, 1990). The Ad E2a protein aids the cellular polymerase processivity in an \textit{in vitro} AAV DNA replication assay, although cellular RPA can substitute less efficiently (Ward et al., 1998). Adenovirus VAI RNA increases translation of mRNA from AAV \( P_{19} \) and \( P_{40} \) promoters (West et al., 1987). In the absence of E1B, rescue and replication of chromosomally integrated AAV DNA was delayed (Ostrove and Berns, 1980; Samulski and Shenk, 1988). Samulski and Shenk showed that in the absence of either E1B or E4orf6 the number of mRNAs was reduced even though transcription rates were unchanged, indicating some post-transcriptional effect (Samulski and Shenk, 1988).

\textit{Adenovirus evades host DNA double-strand break (DSB) response}

Adenovirus disrupts the host DNA repair response. In experiments where both Ad E4orf3 and E4orf6 protein expression was eliminated, the Ad viral genomes were concatamerized and viral replication was significantly reduced (Berk, 2007; Weiden and Ginsberg, 1994). Using either an adenovirus mutant lacking the entire E4 region or an
E4orf3/6 double mutant to infect DSBR mutant cell lines, demonstrated that DNA-PKcs, Mre11, and NBS1 were involved in adenovirus concatamerization (Boyer et al., 1999; Stracker et al., 2002). Also, DNA-PKcs was found to co-elute with either E4orf3 or E4orf6, indicating potential interaction of E4orf3 or E4orf6 with DNA-PKcs directly or as part of a complex (Boyer et al., 1999). Carson et al. compared phosphorylation of DNA repair pathway proteins between Ad infection and an Ad E4 deletion virus infection. It was found that, in the absence of E4 expression, Chk1 was phosphorylated at S345, Chk2 at T68, ATM at S1981, p53 at S15, and 53PB1 at S25 (the authors noted significantly less although present phosphorylation of Chk2, ATM and 53PB1 with Ad) (Carson et al., 2003).

Adenovirus E4orf3 and E4orf6 complement each other in disrupting the DSB response. The E4orf3 protein alters the localization of the MRN-complex proteins. Immunofluorescence experiments showed that expression of the E4orf3 protein caused the MRN-complex proteins to change from a diffuse pattern in the nucleus to “track-like” structures (co-localizing with E4orf3) that partially overlap PML oncogenic domains (PODs); also, MRN-complex proteins localized to cytoplasmic aggresomes with the expression of E4orf3 (Araujo et al., 2005; Evans and Hearing, 2005; Liu et al., 2005; Stracker et al., 2002). All three MRN-complex proteins, Rad50, NBS1, and Mre11, co-localized with E4orf3 outside Ad replication centers; however, while NBS1 initially localized outside Ad replication centers later in the infection NBS1 localization started to overlap with Ad replication centers (Evans and Hearing, 2005).

Adenovirus E4orf6 and E1B-55K can both further disrupt the MRN-complex. Adenovirus E4orf6 and E1B-55K interact with cellular proteins to form an E3 ubiquitin
ligase complex (Harada et al., 2002; Querido et al., 2001). Levels of the MRN-complex proteins decrease over time during an Ad infection; Mre11, Rad50, and NBS1 are degraded in a proteosome-dependent manner dependent on the E4orf6-E1B-55K ubiquitin ligase complex (Stracker et al., 2002). The disruption of the MRN-complex has significant consequences on the cells ability to response to DSB. Carson et al. showed that disruption of the MRN-complex prevented ATM activation and prevented phosphorylation and activation of downstream DSB response proteins (Carson et al., 2003). Adenovirus disrupts the cellular DSB response in order to effectively replicate inside a cell (Berk, 2007).

**Host Cell Response to Ad and AAV Coinfection**

There were two approaches used to assess the host cell response during an Ad and AAV coinfection discussed within this document. One approach, discussed in the first chapter, was to identify cellular proteins that interact with Rep proteins and then conduct experiments to characterize the interaction by both *in vitro* assays and during an Ad and AAV coinfection. Another approach, discussed in the second chapter, was to determine cellular protein expression and phosphorylation differences during an Ad and AAV coinfection compared to an Ad infection. Cell cycle proteins and cell cycle-related proteins expression differences were addressed during a coinfection since infection with the individual viruses have different effects on the cell cycle. Also of interest were DSB response protein expression and phosphorylation differences during an AAV and Ad coinfection compared to Ad infection, since AAV down-regulates Ad proteins important for disrupting the DSB response.
Chapter 1: Manuscript

Adeno-associated virus interactions with B23/Nucleophosmin:

Identification of sub-nucleolar virion regions.

Joyce M. Bevington, Patrick G. Needham, Kristin C. Verrill, Venkatesh Basrur, Roy F. Collaco and James P. Trempe

**Abstract**

Adeno-associated virus (AAV) is a human parvovirus that normally requires a helper virus such as adenovirus (Ad) for replication. The four replication proteins (Rep78, 68, 52 and 40) encoded by AAV are pleiotropic effectors of virus integration, replication, transcription and virion assembly. Using Rep68 column chromatography and mass spectrometry we have identified the nucleolar, B23/Nucleophosmin (NPM) protein as a Rep interacting partner. Rep-NPM interactions were verified by co-immunofluorescence and chemical crosslinking studies. We have found that there is demonstrable, but limited co-localization between Rep and NPM in co-infected cells. In contrast, there was significant co-localization between NPM and AAV Cap proteins. *In vitro* experiments using purified MBPRep78 and NPM show that NPM stimulates MBPRep78 interactions with the AAV ITR as well as endonuclease activity. These studies suggest that NPM plays a role in AAV amplification affecting Rep function and virion assembly.
Introduction

Adeno-Associated Virus (AAV) is a non-pathogenic member of the Parvovirus family and the Dependovirus genus (Muzyczka and Berns, 2001). As a Dependovirus, AAV needs another virus, such as adenovirus, to efficiently replicate inside a host cell. AAV has a linear single-stranded DNA genome of 4,780 nucleotides (Muzyczka and Berns, 2001). The genome contains two translation open reading frames (ORF) encoding three structural and four non-structural proteins and is flanked at both ends by inverted terminal repeat (ITR) sequences that serve as origins of replication (Lusby, Fife, and Berns, 1980; Srivastava, Lusby, and Berns, 1983). The ORF on the left side encodes four non-structural proteins, or replication (Rep) proteins designated Rep78, Rep68, Rep52, and Rep40 based on their apparent molecular weight in SDS-PAGE gels (Mendelson, Trempe, and Carter, 1986). Rep78 and Rep68 are translated from mRNAs originating from a transcription promoter at map unit 5 (p5). Rep52 and Rep40 are translated from mRNAs originating from a transcription promoter at map unit 19 (p19). Rep68 and Rep40 differ from Rep78 and Rep52 as a result of mRNA splicing that replaces 92 amino acids from the carboxyl terminus with 9 amino acid residues. Rep78/68 are required for viral DNA replication, regulation of AAV gene expression and site-specific integration into human chromosome 19, which occurs in the absence of helper virus infection (Kotin et al., 1990). The smaller Rep proteins, Rep52/40, play roles in virus assembly (Chejanovsky and Carter, 1989; King et al., 2001). Rep78 and Rep68 both interact with a Rep-binding site (RBS) found in the A-stem of the AAV ITR. Both larger Rep proteins also possess ATPase, helicase, and site-specific strand-specific endonuclease activities
that are important for viral replication (Chiorini et al., 1994; Im and Muzyczka, 1990; Im and Muzyczka, 1992). Rep52 and Rep40 are not endonucleases but share Rep78/68’s ATPase and helicase activities (Collaco et al., 2003; Im and Muzyczka, 1992; Smith and Kotin, 1998). Rep78 and Rep68 also have DNA ligase activity (Smith and Kotin, 2000). Since there is extensive sequence identity, the two large or two small Rep proteins are nearly interchangeable in terms of function (Collaco et al., 2003; Im and Muzyczka, 1990; Im and Muzyczka, 1992; Smith and Kotin, 1998). Three structural, or capsid (Cap or VP), proteins are encoded on the right side of the genome. A transcription promoter at map unit 40 (p40) directs the transcription of differentially spliced mRNAs that are translated into the three structural proteins VP1-3.

AAV and Ad replicate and assemble their genomes in the nucleus of the coinfected cell. AAV Rep and Cap proteins co-localize with the Ad, E2a, single-stranded DNA binding protein in replication centers (Hunter and Samulski, 1992; Weitzman, Fisher, and Wilson, 1996). AAV capsid proteins also localize in the nucleolus at early stages of infection and Rep protein expression is required for capsid proteins to leave the nucleolus (Wistuba et al., 1997). Furthermore, Rep proteins transiently exist in the nucleolus (Wistuba et al., 1997). While searching for cellular factors that interact with AAV Rep proteins, we observed in vitro associations with the abundant nucleolar protein B23/Nucleophosmin (NPM). NPM is a nucleolar protein with many functions (Okuda, 2002). NPM is involved in ribosome biogenesis (Savkur and Olson, 1998; Yung, Busch, and Chan, 1985), duplication of centrosomes (Okuda, 2002; Okuda et al., 2000), shuttling proteins to the nucleus (Szebeni, Herrera, and Olson, 1995; Szebeni et al., 1997), and has chaperone protein characteristics (Szebeni et al., 2003; Szebeni and Olson, 1999). Two
forms of the protein, referred to as B23.1 and B23.2 arise from differential splicing of mRNA. B23.1, and to a lesser extent B23.2, have ribonuclease activity that can cleave tRNA and mRNA but has specificity for rRNA (Herrera, Savkur, and Olson, 1995; Savkur and Olson, 1998). Only B23.1 nonspecifically binds to single-stranded DNA, double-stranded DNA, and RNA (Dumbar, Gentry, and Olson, 1989; Herrera et al., 1996; Wang et al., 1994). The B23/NPM gene is often targeted in chromosomal translocations associated with acute myeloid leukemia (AML) resulting in expression of oncogenic NPM fusion proteins (Redner, 2002; Yoneda-Kato et al., 1996). NPM exerts other effects on cell proliferation in that it associates with Rb, p53 HDM2 and p14ARF (Bertwistle, Sugimoto, and Sherr, 2004; Colombo et al., 2002; Kurki et al., 2004; Takemura et al., 1999). These associations are believed to play pivotal roles in cellular DNA damage response and cancer (Kurki, Peltonen, and Laiho, 2004).

Using co-immunofluorescence and coimmunoprecipitations, we demonstrate that a portion of the AAV Rep protein in the coinfectected cell associates with NPM in intact nucleoli and in punctate extra-nucleolar structures in the infected nucleus. At early stages of infection, Cap proteins are found in punctate replication/assembly structures that are affiliated with NPM. Using purified Rep78/68 and NPM proteins, we demonstrate that NPM stimulates Rep-specific binding to the AAV ITR and site-specific endonuclease activities. Our observations that NPM associates with the AAV Cap and Rep proteins expands the cast of NPM-interacting partners and provide new insights into the AAV replication cycle.
Results

Identification of cellular Rep-interacting partners

The wide array of functions performed by the Rep proteins during AAV infection suggests that these relatively small proteins must interact with cellular proteins to facilitate the virus amplification cycle. Most of the studies that have identified Rep interacting partners have used yeast two-hybrid methodologies. We chose a different approach resulting in the identification of several novel partners. Rep68, purified from E.coli, was covalently attached to CnBr-activated sepharose to form a Rep-column. HeLa nuclear extracts were prepared and fractionated over the column followed by several wash steps to eliminate non-specific binding. Proteins were eluted from the column in 1M KCl and separated by SDS-PAGE. The gel was stained with Coomasi, gel fragments excised and treated with trypsin to elute peptides for mass spectrometry identification (Table 1).

Table 1.

<table>
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<tr>
<th>Rep68 interacting partners</th>
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<tr>
<td>HMG-1</td>
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<tr>
<td>SET/TAF-I</td>
</tr>
<tr>
<td>Acidic Leu-rich nuclear phosphoprotein 32 family member (Anp32)</td>
</tr>
<tr>
<td>Nucleolin (C23)</td>
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<tr>
<td>Nucleophosmin (B23/NPM)</td>
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* Hela nuclear extracts were passed over a Rep68 column. Adherent proteins were eluted with KCl, separated by SDS-PAGE and identified by mass spectrometry.

One of the proteins that interacted with the Rep68 column was nonhistone HMG-1. Detection of HMG-1 validates our approach because it has already been identified as a Rep-interacting partner (Costello et al., 1997). The SET/TAF1 and Anp32 proteins are...
members of the acidic leucine-rich nuclear phosphoprotein 32 (Anp32) family of protein phosphatase 2A (PPP2, formerly PP2A) inhibitors (Santa-Coloma, 2003). SET/TAF1 has also been identified as a Rep-interacting partner that stimulates AAV replication (Pegoraro et al., 2006). SET/TAF-I is also a phosphatase inhibitor that is reported to be necessary for Ad DNA replication in vitro (Matsumoto et al., 1995). Nucleolin (C23) and NPM are nucleolar proteins. C23 interacts with NPM and has roles in ribosomal synthesis, can act as a cell surface receptor, and along with NPM, shuttle proteins from the cytoplasm to the nucleus (Li et al., 1996; Srivastava and Pollard, 1999). A role for C23/nucleolin in AAV amplification has been suggested because it was co-purified with intact AAV2 capsid (Qiu and Brown, 1999).

NPM is not known to play any role in AAV replication. However NPM is involved in the replication cycles of other viruses. NPM is redirected to adenovirus replication centers during adenovirus infection and NPM is also important in in vitro DNA replication of the Ad genome (Matthews, 2001; Okuwaki et al., 2001a; Walton et al., 1989). The nucleolus is important for the replication of the related Parvovirus, minute virus of mice (MVM) (Walton et al., 1989). NPM associates with HTLV-1 Rex, HIV-1 Rev and hepatitis delta antigen (Adachi et al., 1993; Fankhauser et al., 1991; Huang et al., 2001; Li, 1997). Given NPM’s role in the replication cycle of other viruses and its involvement in the cellular DNA damage response, we investigated its interactions with AAV proteins.

**NPM colocalization with Cap and Rep proteins**

During the early-middle stages of AAV and Ad coinfection, Rep proteins localize to punctate replication centers in the nucleus and colocalize with Cap proteins (Hunter
and Samulski, 1992; Weitzman, Fisher, and Wilson, 1996; Wistuba et al., 1997). As replication progresses the Rep and Cap proteins spread throughout the nucleus (Weitzman, Fisher, and Wilson, 1996; Wistuba et al., 1997). Co-immunofluorescence experiments were conducted to determine if Rep and NPM proteins colocalize in AAV and Ad coinfected HeLa cells. Immunofluorescence with anti-Rep showed staining throughout the nucleus as well as in punctate nuclear regions (Fig. 1A). Merging Rep- and NPM-stained images showed limited areas of colocalization (Fig. 1C). A minority of the cells that are Rep positive have some degree of co-localization between Rep and NPM at early stages of infection. Staining with AAV anti-Cap showed similar punctate structures (Fig. 1D). However merged Cap- and NPM-stained images showed a much greater degree of co-localization (Fig. 1F). All of the cells that had punctate Cap staining also had co-localized NPM staining. Moreover, all of the punctate, Cap-stained structures also showed NPM staining. As a negative control for these experiments, coinfected cultures were stained for NPM and Ad hexon protein (Fig. 1, G-I). Ad hexon is the most abundant outer structural protein. We also stained for AAV Cap and hexon (Fig. 1, J-L). There was no detectable co-localization of NPM with hexon and only diffuse nuclear and cytoplasmic co-localization between Cap and hexon.
Figure 1: Co-localization of NPM and AAV proteins. HeLa cells were coinfected with Ad5 (10 m.o.i.) and AAV2 (250 m.o.i.) and fixed 20 to 30 hours post-infection. Fixed cells were stained for AAV Rep (panel A), Cap (panel D) and NPM (panels B and E). Merged images (panels C and F) show co-localization as yellow or white spots. Fixed cells were also stained for: Ad hexon protein (panels G and J), NPM protein (panel H),
Ad hexon (panel J) and AAV Cap (panel K). These images are also merged (panels I and L). Nuclei were stained with DAPI and are depicted in blue in the merged images (C, F, I, and L). Scale bar is equivalent to 15 microns. Confocal microscopy was performed on AAV- and Ad-infected HeLa cells. Immunofluorescent staining of Cap (panels M and N) and Rep (panels O and P) are shown in green. NPM staining (panels M-P) is shown in red. Panels M and N and O and P are of the same field respectively and are separated by 1.5 microns. Yellow staining is the result of image merging and is indicative of co-localization.

The results shown in Fig. 1, panels A-L, were obtained with a standard immunofluorescence microscope. To more conclusively demonstrate that Rep and Cap co-localize with NPM, confocal microscopy was used. Fig. 1, panels M and N are from the same field and the images are separated by 1.5 microns. Co-localization of Cap and NPM is demonstrated by the yellow color in these images. It is clear that all of the nucleoli and punctate structures demonstrate colocalization. Panels O and P show Rep and NPM staining with colocalization in the swollen nucleoli and in the punctate structures. These results are consistent with the results shown in the first part of Fig. 1 but provide more convincing evidence of colocalization.
Figure 2. Nucleolar remodeling during AAV and Ad coinfection. AAV and Ad coinfected HeLa cells (B-D), Ad infected HeLa cells (F-H) and uninfected cells (A, E) were fixed 20 p.i. NPM was stained with Alexa568 (A-D) or FITC (E-H). AAV Cap proteins were stained with Alexa488 (B-D) and Ad hexon was stained with Alexa568 (F-H). Alexa568 is a red fluorophore whereas FITC and Alexa488 are green fluorophores. Nuclei were counterstained with DAPI. Swollen nuclei and small punctate subnuclear structures are evident in panels B-D. Panels B-D and F-H are merged images. Scale bar is equivalent to 15 microns.

NPM staining in AAV and Ad co-infected cells was found in two structures, numerous small punctate dots throughout the nucleus and large rounded structures that appear to be swollen nucleoli (Fig. 2, B-D). These structures suggest that nucleoli are remodeled in the coinfected cell. The punctate structures were often smaller than normal nucleoli and we have observed as many as fifty per nucleus (Fig. 2D). Cells infected with Ad alone will often have multiple nucleoli-like structures but they are not as numerous as those seen in AAV and Ad coinfected nuclei. Swollen nucleoli have been described previously (Wistuba et al., 1997). These nucleoli are enlarged in Rep-expressing, infected
cells and had a more intense fluorescence as compared to uninfected (Rep-negative) cells (Wistuba et al., 1997). Cap co-localization in the swollen nucleoli was observed throughout the structure (Fig. 1, F, M and N). Nearly all of the punctate structures stain with both NPM and Cap antibodies suggesting that these may be sites of capsid assembly. Ad-infected cells did not show the abundant sub-nucleolar, NPM-stained structures (Fig. 2F-H).

**AAV Cap, Rep and NPM association detected by chemical crosslinking and immunoprecipitation.**

To further investigate if there is a physical interaction between AAV proteins and NPM, we coimmunoprecipitated these proteins with specific antibodies. Our initial attempts to detect an association between NPM and the AAV Rep proteins using co-immunoprecipitations from AAV and Ad coinfected cells were unsuccessful therefore we used dithiobis (succinimidyl) propionate (DSP) to chemically crosslink weakly interacting proteins. Nuclei were isolated from virus-infected cultures and crosslinked with DSP. Nuclear extracts were prepared and immunoprecipitated with antisera against Rep, Cap and NPM. The crosslinks were reversed and proteins separated by gel electrophoresis and immunoblotted.
Figure 3. Coimmunoprecipitation of NPM and AAV proteins. Ad- or Ad- and AAV-coinfected Hela cells were harvested 22 h post infection, nuclei were isolated and chemically crosslinked with 2 mM DSP. Antibodies to Rep and NPM were used for immunoprecipitation, the crosslinked were reversed and the precipitates separated by SDS-PAGE. The separated proteins were analyzed by western blot using NPM, Rep or Cap antibodies. The extracts (Ext.) used were from Ad-infected (Ad) or AAV- and Ad-coinfected (Co) cells. The use of the DSP crosslinking agent (XL) is indicated by ‘+’ or ‘-‘. Lanes 1 and 2 in each panel are crude extracts loaded on the gel without prior immunoprecipitation.

When extracts were immunoprecipitated with anti-Rep, NPM was coimmunoprecipitated from the coinfectected culture, but only when chemically crosslinked (Fig. 3A, lane 5).
NPM was not precipitated from the uncrosslinked extracts (Fig. 3A, lane 4). Conversely, Rep proteins were co-immunoprecipitated with anti-NPM from crosslinked extracts whereas no Rep coimmunoprecipitation was observed without crosslinking (Fig. 3B, compare lanes 4 and 5). To detect Cap and NPM interactions, infected extracts were immunoprecipitated with anti-NPM. This experiment showed that NPM antibody co-immunoprecipitated AAV structural proteins from both untreated and crosslinked coinfectected nuclear extracts (Fig. 3, lanes 4 and 5). We have been unable to coimmunoprecipitate NPM with either a polyclonal anti-Cap antibody obtained from denatured VP3 or the A20 monoclonal antibody that recognizes native capsid (results not shown). These results demonstrate a close association, if not a physical interaction, between NPM and AAV proteins.

**NPM stimulates MBPRep78 binding to the AAV ITR RBS**

Immunofluorescence and chemical crosslinking showed colocalization and close association between Cap and NPM proteins. Since Rep proteins associate with the maturing virion via noncovalent interactions, and covalently, via linkage to the 5’ end of the viral DNA, it is not possible to determine if there is a direct interaction between NPM and the Rep proteins. In vitro EMSA analyses were performed to investigate potential Rep-NPM interaction. AAV Rep78/68 proteins interact with the RBS found in the A-stem of the AAV ITR element (Muzyczka and Berns, 2001). An A-D stem DNA fragment was radiolabeled and incubated with purified MBPRep78 and NPM. Figure 4 shows that NPM stimulated MBPRep78 binding to a DNA probe containing its cognate binding site. Both a His-tagged and a GST-tagged NPM stimulated MBPRep78 binding. Thus, the increased binding does not depend on the tag used to purify NPM. (Figure 4A;
compare lanes 5 and 6 to lane 4). Although NPM stimulated binding, no new complexes were observed. This suggests that NPM stimulated binding in a transient manner and that the chaperone activity of NPM may be involved in this binding.

**Figure 4.** NPM stimulates MBPRep78 interaction with the AAV ITR. A 65-bp DNA fragment from the A-D component of the AAV ITR was radiolabeled and incubated with NPM and MBPRep78 (designated Rep78 in the figure). Protein-DNA complexes were separated by nondenaturing gel electrophoresis and exposed to x-ray film. **A.** 3.4 nM MBPRep78 was used in lanes 4, 5, and 6, 14.3 nM GSTNPM was used in lanes 2 and 5, and 13.8 nM HisNPM was used in lanes 3 and 6. **B.** 8.9 nM MBPRep78 was used in lanes 3 to 8, and 4.7, 9.4, 18.7, 37.5, 74.9, and 93.5 nM GSTNPM was used in lanes 4, 5, 6, 7, 8, and 2, respectively. **C.** 3.4 nM MBPRep78 was used in lanes 3 to 8. 1.8, 3.5, 7, 14, 28, and 28 nM of BSA was used in lanes 4, 5, 6, 7, 8, and 2, respectively. Equimolar amounts of BSA:MBPRep78 and GSTNPM:MBPRep78 were used in the respective lanes (lanes 4 to 8) of Figure 4 B and C.

NPM didn’t show any detectable binding to AAV DNA by itself (Figure 4A, lane 2 and 3) even though it nonspecifically binds to DNA (Dumbar, Gentry, and Olson, 1989; Wang et al., 1994). A probable reason for this is that conditions used in this experiment were not optimal for NPM binding to DNA since NaCl concentration in the buffer was 0.05M and NPM binds to DNA at lower salt concentrations (Dumbar, Gentry, and Olson, 1989; Herrera et al., 1996; Wang et al., 1994).
To further demonstrate the enhanced binding of MBPRep78, a dose-response assay was performed with increasing amounts of NPM or BSA. Fig. 4B shows that with increasing concentrations of NPM, MBPRep78 interaction with the AAV RBS increases. Whereas BSA did not stimulate binding (Fig. 4C). Similar molar ratios of MBPRep78 to BSA were used in EMSA assays to verify that NPM stimulation, rather than an unspecified stimulatory effect, results in increased MBPRep78 binding. This verifies that increased binding of MBP-Rep78 is dependent upon NPM.

**Increased Rep-mediated nicking at the AAV trs site with NPM**

Rep78 or Rep68 interaction with the AAV ITR is required for site-specific nicking of the covalently closed end of the viral genome. Upon binding and oligomerization, Rep78 or Rep68 make a site-specific, strand-specific nick in the terminal resolution site \((trs)\) enabling completion of DNA synthesis at the ends of the viral DNA. To determine if NPM affects Rep-mediated nicking, endonuclease assays were performed with increasing amounts of NPM or BSA. Purified MBPRep78 and GSTNPM were incubated with a radiolabeled 182 nt ITR endonuclease substrate.
Figure 5. NPM stimulates MBPRep78 endonuclease activity. MBPRep78 (1.34 nM, designated Rep78) was incubated with radiolabeled ITR with ATP to induce endonuclease activity. The reactions were separated by denaturing polyacrylamide gel electrophoresis and exposed to x-ray film. A. Lanes 4, 5, 6, 7, and 2 contain to 0.8, 4, 20, 40, and 61 nM of HisNPM, respectively. B. Lanes 3, 4, 5, 6, and 1 contain 0.8, 4, 20, 40, and 60 nM of BSA, respectively.

Fig. 5A shows that increasing amounts of NPM stimulates nicking, yielding the appropriate, 73 nt product. Equimolar concentrations of BSA had no effect on MBPRep78 nicking (Fig. 5B). This result is consistent with the EMSA analyses because stimulation of binding to the A-D oligonucleotide would be expected to result in increased nicking activity.
Discussion

The minimal coding capacity of the Parvovirus genome dictates that numerous cellular proteins are required to support virus amplification. Several cellular proteins have been identified that interact with AAV proteins. Most of the proteins that interact with Rep play roles in mRNA transcription. Rep78/68 interact with: Sp1 (Hermonat, Snatin, and Batchu, 1996; Pereira and Muzyczka, 1997), HMG-1 (Costello et al., 1997), the transcriptional co-activator PC4 (Weger et al., 1999), TATA binding protein (Hermonat et al., 1998; Needham et al., 2006), the p53 and topoisomerase binding protein, Topors (Weger, Hammer, and Heilbronn, 2002), a putative protein kinase, protein kinase X (PKX), and protein kinase A (PKA) (Chiorini et al., 1998; DiPasquale and Chiorini, 2003). The biological effect of the PKX association is inhibition of the steady-state levels of cAMP-responsive-element-binding protein (CREB) and cyclin A protein. Nucleolin, also known as C23, co-purifies with AAV2 and was found to associate with intact AAV2 capsid by immunoprecipitation and immunofluorescence techniques (Qiu and Brown, 1999). C23/nucleolin is a ubiquitous nucleolar protein implicated in nuclear transport, organization of nucleolar chromatin, packaging of pre-RNA, rDNA transcription, and ribosome assembly (Srivastava and Pollard, 1999). Thus there are only a limited number of cellular proteins known to interact with AAV-encoded proteins.

To identify additional cellular proteins that interact with Rep78/68, we attached purified Rep68 to activated sepharose creating a Rep affinity chromatography resin. HeLa nuclear extracts were passed over the column and eluted with high salt. Mass spectrometry analyses identified several new potential Rep partners. One of the proteins
identified was HMG-1. This observation verifies the validity of our approach because HMG-1 has already been identified as a Rep-interacting partner (Costello et al., 1997).

Two of the identified proteins are involved in protein phosphatase activity: protein SET/TAF-1 and Anp32. The SET/TAF1 and Anp32 proteins are members of the acidic leucine-rich nuclear phosphoprotein 32 family of protein phosphatase 2A (PPP2, formerly PP2A) inhibitors (Santa-Coloma, 2003). SET/TAF1 was first identified as a gene fused to the CAN gene in a patient with acute undifferentiated leukemia (von Lindern et al., 1992). SET was subsequently found to be identical to template activation factor I (TAF1), a cellular protein necessary for DNA replication of the Ad genome in vitro (Matsumoto et al., 1995). C23/nucleolin and B23/NPM were also identified. Recently, SET/TAF1 was shown to be a Rep-interacting partner that regulates AAV DNA replication (Pegoraro et al., 2006). We focused on NPM because of its wide array of functions in normal and transformed cells.

B23/NPM is a nucleolar protein with many proposed functions (Okuda et al., 2000). It exists in two forms, B23.1 and B23.2, which are nearly identical except at the C-terminal end (Chang and Olson, 1989; Chang and Olson, 1990). The protein sequence is highly conserved among rat, mouse, and human. B23.2, which is the alternatively spliced form of B23.1, replaces 36 amino acids at the C-terminus of B23.1 with 2 amino acids (Chang and Olson, 1989; Chang and Olson, 1990). The more abundant B23.1 protein exists predominantly in the nucleolus (Okuwaki, Tsujimoto, and Nagata, 2002; Wang, Umekawa, and Olson, 1993). NPM has a variety of proposed functions including ribosome biogenesis (Okuwaki, Tsujimoto, and Nagata, 2002; Savkur and Olson, 1998; Yung, Busch, and Chan, 1985), histone chaperone (Okuwaki et al., 2001b), duplication of
centrosomes (Okuda, 2002; Okuda et al., 2000), shuttling proteins to the nucleus (Borer et al., 1989; Szébeni, Herrera, and Olson, 1995; Szébeni et al., 2003; Szébeni et al., 1997), and has general chaperone protein characteristics (Szébeni and Olson, 1999).

B23.1, and to a lesser extent B23.2, have ribonuclease activity that cleaves tRNA and mRNA but has specificity for rRNA (Herrera, Savkur, and Olson, 1995; Savkur and Olson, 1998). Only B23.1 nonspecifically binds to single-stranded DNA, double-stranded DNA, and RNA (Dumbar, Gentry, and Olson, 1989; Herrera et al., 1996; Wang et al., 1994). NPM has also been implicated in the initial cellular response to environmental stressors. DNA damaging agents such as UV light induces expression (Weber et al., 2000) and nuclear re-localization of NPM (Kurki, Peltonen, and Laiho, 2004) thus stimulating DNA repair and escape from apoptosis (Wu, Chang, and Yung, 2002). NPM is part of a large protein complex that includes the p53 tumor suppressor HDM2 and p19Arf (Colombo et al., 2002; Itahana et al., 2003; Korgaonkar et al., 2005; Kurki et al., 2004). NPM increases the stability and transcriptional potential of p53 via its chaperone activity and inhibition of Mdm2 ubiquitin ligase activity (Colombo et al., 2002; Kurki et al., 2004).

Our co-immunofluorescence and chemical cross-linking studies demonstrate that NPM is found in close proximity to AAV Rep and Cap proteins. Intra-nuclear Cap and NPM co-localization was prevalent with domains showing high concentrations of Cap enriched in NPM. However Rep and NPM co-localization was not as prevalent as that observed with Cap proteins. Immunofluorescence using confocal microscopy further substantiated the hypothesis that AAV proteins co-localize with NPM. From our chemical cross-linking experiments, the relatively short length of the DSP crosslinker
(12Å) suggests that NPM exists in close proximity to Cap or Rep proteins. Since Rep proteins associate with the virion or viral DNA by noncovalent and covalent interactions respectively (Dubielzig et al., 1999; Prasad and Trempe, 1995), it is not possible to verify that NPM directly interacts with both viral proteins in the coinfected cell. That AAV Cap proteins are co-immunoprecipitated with anti-NPM from non-crosslinked extracts suggests there is a stronger affinity between these proteins than between NPM and Rep. Our inability to coimmunoprecipitate NPM with Cap-specific antibodies suggests that NPM interactions may block the structural epitopes required for immunoprecipitations.

We performed \textit{in vitro} studies with purified Rep and NPM to further investigate whether these proteins interact. NPM stimulated MBPRep78 interactions with the AAV ITR and Rep endonuclease activity. NPM by itself did not interact with the ITR even though it has DNA binding ability. NPM purified as either a His-tagged and GST-tagged fusion protein stimulated ITR binding. Neither version of the protein appeared to become part of the MBPRep78-ITR complex in that no new species were found in the EMSA. Stimulation of MBPRep78 binding to the ITR would be expected to result in increased endonuclease activity as we observed here. Although NPM stimulated MBPRep78-ITR binding and endonuclease activities, it did not stimulate Rep-specific ATPase activity (data not shown). These experiments suggest that NPM stimulates Rep binding via its chaperone activity (Szebeni and Olson, 1999). In the infected cell, NPM may also interact with Rep in a transient manner.

It will be interesting to determine which region of the Rep proteins is responsible for the interaction with nucleophosmin (it is presumably not the C-terminus since both
Rep 68 and 78 interact) and if mutant studies will yield an idea of the significance of the Rep-nucleophosmin interaction.

Early in infection Rep proteins are primarily nuclear and are observed in a punctate pattern. As infection progresses, Rep proteins pass transiently through the nucleolus followed by colocalization with AAV DNA as demonstrated by in situ hybridization. Cap proteins also transiently pass through the nucleolus. AAV DNA, Rep and Cap proteins co-localize in punctate replication/encapsidation centers (Hunter and Samulski, 1992; Weitzman, Fisher, and Wilson, 1996; Wistuba et al., 1997). Expression of the AAV Cap gene in the absence of Rep or AAV DNA replication in HeLa cells resulted in Cap enrichment in the nucleolus (Weger et al., 1997; Wistuba et al., 1997). It has been proposed that Rep expression may be required for assembled capsid to escape the nucleoli (Wistuba et al., 1997). An interesting observation from the immunofluorescence studies is the apparent nucleolar remodeling that occurs in AAV and Ad coinfected cells. In addition to the appearance of swollen nucleoli, there were numerous punctate structures that were smaller than nucleoli from uninfected cells. The near total congruence of Cap and NPM staining in these structures suggest that capsid assembly and/or genome encapsidation may occur at these sub-nucleolar structures.

It is unclear which of the numerous functions of NPM is involved in AAV amplification. Our in vitro studies suggest that the NPM chaperone activities may play a role in Rep protein function in vivo. A transient interaction between Rep and NPM may also be reflected in the minimal colocalization between these proteins observed in our co-immunofluorescence studies. The prevalence of NPM and Cap co-localization suggest that these proteins are more intimately involved. The accumulation of Cap proteins in the
nucleolus in the absence of Rep protein or AAV DNA suggest that capsid assembly occurs in the nucleolus (Wistuba et al., 1997). Our results, and those described above, support a model of AAV assembly in which Cap proteins accumulate and assemble in the nucleolus where they associate with NPM and perhaps other nucleolar proteins. Passage of Rep proteins through the nucleolus allows for exit of Rep-Cap-NPM complexes from the nucleolus and into the nucleoplasm where viral DNA encapsidation occurs. Little is known about AAV capsid assembly \textit{in vivo} (Timpe et al., 2005). It will be informative to determine if NPM plays a role in AAV assembly.
Methods

Cells and Virus preparation: HeLa cells (American Type Culture Collection) were grown as a monolayer at 37°C (5% CO₂ atmosphere) in Eagle’s minimum essential medium (MEM) supplemented with 10%(v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 25 U/mL penicillin, 25 μg/mL streptomycin, 2.5 μg/mL amphotericin B, and 100 μg/mL gentamicin. AAV2 and Ad5 were prepared by the method previously described (Casper et al., 2005; Winters and Russell, 1971).

Protein Purification: An amino-terminal His₆ tagged NPM (B23.1 version) in a pQE30 prokaryotic expression vector (pHis-B23.1) in SG13009 cells was kindly provided by Dr. Mark Olson (Umekawa et al., 2001). A 5 mL overnight Luria-Bertani (LB) starter culture was used to inoculate a 1L LB culture and incubated at 37°C until an A₆₀₀ of ~0.6 was reached. The culture was induced with 1mM IPTG for 2.5 hours at 37°C. The cells were pelleted at 9000 x g for 15 minutes at 4°C and then washed once with 30 mL phosphate buffered saline (PBS). Cells were pelleted again at 9000 x g for 10 min. at 4°C. Cells were lysed in 20 mL of lysis buffer (50 mM NaPO₄, 500 mM NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 40 μg/mL lysozyme, pH 8.0) on ice for 20 minutes. Cells were sonicated using a Fisher Sonic Dismembrator 550 on ice level 3 with one minute on and 10 seconds off three times. The lysate was pelleted for 10 minutes 12000 x g at 4°C. The supernatant was applied to 0.5 mL column of Ni-NTA Superflow (Qiagen) equilibrated in lysis buffer. The column was washed with nine column volumes of cold wash buffer (50 mM NaPO₄, 500 mM NaCl, 50 mM imidazole, 1 mM β-mercaptoethanol, pH 8.0). HisNPM was eluted off the column with cold elution buffer.
(50 mM NaPO₄, 500 mM NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol, pH 8.0).
The purified protein was dialyzed overnight in 20 mM Tris pH 8.0, 10% glycerol, 200 mM NaCl.

An amino-terminal gluathione-S-transferase (GST) tagged B23.1 (GSTNPM) prokaryotic expression vector (pGST-B23.1) was constructed as follows. The B23.1 cDNA was excised from pHis-B23.1 with BamH I and inserted into the BamH I multi-cloning site of the pGEX-6P-2 (Amersham Biosciences) vector. For protein expression, BL21 Star (DE3) was transformed with the plasmid. Purification of GSTNPM was as follows. A 5 mL overnight LB starter culture was used to inoculate a 250 mL LB culture and incubated at 37°C until an A₆₀₀ of ~0.6 was reached. The culture was induced with 1 mM IPTG for 2.5 hours at 37°C. The cells were pelleted at 10,000 x g for 10 minutes at 4°C and then washed once with 30 mL PBS. The pellet was resuspended in 5 mL cold PBS. The cells were lysed using a Fisher Sonic Dismembrator 550 on ice level 4 for 30 seconds. Triton X-100 was added for a final concentration of 1%. The lysate was pelleted for 5 min. 4°C at 16000 x g. The supernatant was added to 1 mL of 50% glutathione-agarose (Sigma) bead slurry. The 50% glutathione-agarose bead slurry was prepared following manufacturers instructions. The protein extract was mixed with the slurry for 2 min. at 4°C. The beads were pelleted at 500 x g for 1 min. at 4°C. The bound protein was washed twice with 50 mL cold PBS + 1 M NaCl and then twice with 50 mL cold PBS. GSTNPM was eluted with 500 μL cold 50 mM Tris pH 7.5 + 5 mM reduced glutathione. Glycerol was added for a final concentration of 10%.

An amino-terminal His₆ tagged Rep68 (HisRep68) in a pQE70 prokaryotic expression vector was kindly provided by Dr. R. J. Samulski (Young et al., 2000).
Purified bacterially expressed HisRep68 was prepared as described previously (Casper et al., 2005).

An amino-terminal maltose binding protein (MBP) tagged Rep78 (MBPRep78) in a pPR997 prokaryotic expression vector was kindly provided by Dr. R. M. Kotin (Chiorini et al., 1994). Purified bacterially expressed MBPRep78 was prepared as described previously (Needham et al., 2006).

**Rep-Column Chromatography:** HisRep68 was attached to the activated CNBr-Sepharose (Sigma catalog # C5338) by the procedure described by the manufacturer with slight modifications. 1.5 mg of HisRep68 in 1 mL volume was dialyzed for 4 hours in 100 mL of 100 mM NaHCO₃, 500 mM NaCl, pH 8.3. The buffer was discarded and dialysis continued for 2 hours in 100 mL fresh buffer. The CNBr-resin was prepared by swelling in 1 mM HCl, washed with distilled water and then with coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3). HisRep68 from dialysis was added immediately to 300 μL of the prepared resin and allowed to rotate overnight at 4°C. The following day the resin was washed twice in cold coupling buffer and then mixed for 2 hours with blocking buffer (100 mM Tris-Cl, 500 mM NaCl, pH 8.0). The blocking buffer was washed away and the resin was equilibrated in HeLa extract Buffer D (20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol). HeLa nuclear extracts were prepared following the method described (Dignam, Lebovitz, and Roeder, 1983). To look for Rep interacting proteins, 400 μL of HeLa nuclear extract (in Buffer D) was mixed with 100 μL of the Rep-column resin and rotated at 4°C for 2 hours. The resin was allowed to settle and the supernatant was removed. The resin was washed three times with 1 mL of Buffer D + 300 mM KCl and the supernatant saved. This was followed by a wash of 250 μL of
Buffer D + 500 mM KCl and 250 μL of Buffer D + 1 M KCl. Samples of these fractions were separated by SDS-PAGE gels and silver stained. Fractions of interest were separated by SDS-PAGE and stained with colloidal Coomassie G-250. Bands were cut from the gel and sent for Mass Spectrometry analysis.

**Protein identification by LC-tandem MS:** The proteins were separated on SDS-PAGE and visualized with Colloidal Coomassie stain (Invitrogen). Protein bands were excised and destained with 30% methanol for 3 h at RT. *In gel* proteolysis with modified, sequencing grade trypsin (Promega, Madison, WI) was carried out essentially as described previously (Basrur et al., 2003). Briefly, gel slices were further washed with 150 μL of 50% acetonitrile in 0.1 M ammonium bicarbonate buffer, pH 8.0, for 30 min. Trypsin (0.5 μg, Promega) was added in a minimal volume of 0.1 M ammonium bicarbonate buffer and the digestion was carried out for 16 h at 37 °C with an additional aliquot of trypsin (0.25 μg) added after 12 h. Peptides were extracted sequentially with 150 μL of 60% acetonitrile containing 0.1% TFA for 30 min, and 100 μL of acetonitrile containing 0.1% TFA. All extracts were pooled and concentrated using Vacufuge to a final volume of 15 μL. Two μL1 of the digest was separated on a reverse phase column (Aquasil C18, 15 μm tip x 75 μm id x 5 cm Picofrit column, New Objectives, Woburn, MA) using acetonitrile/1% acetic acid gradient system (5-75% acetonitrile over 35 min followed by 95% acetonitrile wash for 5 min) at a flow rate of ~250 nL/min. Peptides were introduced into an in-line, ion trap mass spectrometer (LCQ Deca XP Plus, ThermoFinnigan) equipped with a nano-spray source. The mass spectrometer was set for analyzing the positive ions and acquiring a full MS scan and a collision induced dissociation (CID) spectrum on the most abundant ion from the full MS scan (relative
collision energy ~30%). Dynamic exclusion was set to collect 3 CID spectra on the most abundant ion and then exclude it for 3 min. Database search against an indexed, non-redundant human protein database was performed using TurboSEQUEST software (BioworksBrowser v 3.0, ThermoFinnigan). CID's which showed \( X_{corr} \) and \( \mu C_n \) values of >2.0 and >0.2, respectively, for a +2 charged peptide, were considered positive. All CID spectra were also verified manually using the MS-Digest and MS-Product provision of Protein Prospector (http://prospector.ucsf.edu).

**Immunofluorescence**: Antibodies used in these experiments: rabbit polyclonal antibody affinity purified against all four Rep proteins (Trempe, Mendelson, and Carter, 1987) and Cap proteins (data unpublished), anti-nucleophosmin (Zymed cat.#32-5200), goat polyclonal anti-hexon (American Research Products, Inc. cat.#12-6235-1), goat anti-mouse AlexaFlour568 (Molecular Probes cat.#A-11031), donkey anti-goat AlexaFlour568 (Molecular Probes cat.#A-11057), donkey anti-rabbit AlexaFlour488 (Molecular Probes cat.#A21206), donkey anti-mouse FITC and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes). HeLa cells were plated 24 hours prior to infection at 1x10^4 cells/well in each well of an 8-chamber slide (LAB-TEK Brand, Nalge Nunc International). Cells were infected or mock infected for 1 hour in 200 \( \mu \)L of 2%(v/v) FBS MEM medium (supplemented with L-glutamine) with Ad5 and AAV2 at a multiplicity of infection (m.o.i.) of 10 and 250, respectively. After 1 hour, an equivalent amount of 18%(v/v) FBS MEM medium (supplemented with L-glutamine and antibiotics) was added to the cells. At 20-30 hours, cells were washed twice with PBS. Cells were then fixed and permeabilized with 100% cold methanol for 10 minutes on ice and then air-dried for 10 minutes. The slides were washed twice with 2% bovine serum.
albumin (BSA) in PBS (BSA-PBS) and blocked in the same solution for 20 min. The slide chambers were removed leaving the gasket. The slides were incubated in RIPA buffer (50mM Tris pH 8, 150mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% nonidet P40) for 10 minutes and then washed three times with BSA-PBS. Slides were incubated with primary antibody in BSA-PBS for 1 hour followed by three washes with BSA-PBS. Slides were incubated with secondary antibody in BSA-PBS for 1 hour followed by three washes with BSA-PBS. Nuclei were stained with 150 nM DAPI for 5 minutes followed by three washes with BSA-PBS. A final three washes with just PBS was performed before removing the slide gasket and mounting the slide with DAKO Fluorescent Mounting Medium (DAKO Corporation). All antibody and wash incubations were done at room temperature. A Nikon eclipse E800 fluorescent microscope was used for visualization of colocalization. A BioRad Radiance 2000 Laser Scanning system mounted on an Olympus BX51WI microscope was used to confirm colocalization.

**Crosslinking and Immunoprecipitation:** HeLa cells were grown in 150 mm plates to 95% confluence and infected. The infection was performed in serum free medium with Ad5 and/or AAV2 at a m.o.i. of 10 and 100, respectively. After two hours incubation at 37°C, the medium was replaced with 10% (v/v) FBS MEM supplemented with L-glutamine and antibiotics. Twenty-two hours later the cells were scraped from the plate and spun down at 1500 x g for 5 min at 4°C. The pelleted cells were washed twice with 5 ml cold PBS + 5 mM MgCl₂. The washed pellets were re-suspended and incubated on ice for 10 minutes in 500 μL cold Buffer A (10 mM HEPES (pH 7.8), 10 mM KCl, 1.5 mM MgCl₂ 0.4% Triton-X 100, 0.34 M sucrose, 10% (v/v) glycerol, 1 μM leupeptin, 1 μg/mL pepstatin A, 1 mM benzamidine, 1 mM PMSF). The lysate was pelleted at 1500 x g for 5
min at 4°C. The supernatant (cytoplasmic extract) was carefully removed, the nuclear pellet was re-suspended in 500 μL cold Buffer A + 200 mM NaCl and vortexed for 5 seconds. Dithiobis (succinimidy) propionate (DSP) (Pierce Chemical Company) dissolved in dimethyl sulfoxide (stock = 50 mM) was added to the re-suspended pellet to a final concentration of 2 mM and vortexed for 5 seconds. This treated nuclear fraction was rotated for 20 minutes at room temperature. The crosslinking was stopped by the addition of 50 mM glycine and further rotation for 10 minutes at room temperature. EDTA was added to a final concentration of 20 mM; the crosslinked nuclear fraction was vortexed vigorously for 30 seconds and incubated on ice for one hour. The lysed crosslinked extract was sonicated using a Fisher Sonic Dismembrator 550 on ice (level 4, one second pulses, for 45 seconds). SDS was added (final concentration of 1% (wt/v)) and the lysed extract was denatured at 65°C for 10 minutes. Meanwhile, for each immunoprecipitation, 75 μL of Immunopure protein A agarose bead slurry (Pierce) was washed with 500 μL of IPP buffer (50 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 150 mM NaCl, 0.5% NP-40). The beads were rotated at 4°C for 5 min, pelleted at 500 x g for 2 min and the supernatant was aspirated. 500 μL of IPP buffer was added to these equilibrated beads followed by 100 μL of the earlier heat treated, denatured extracts. The extracts were pre-cleared by rotation for 1 h at 4°C. The beads were then pelleted at 500 x g for 2 min and the supernatant containing the cleared extracts was carefully added to 75 μL of freshly equilibrated beads (washed as above). Appropriate antibodies were added and the immunoprecipitation was performed by rotation at 4°C for 16 hours. The antibodies used in the immunoprecipitations were: anti-NPM, anti-Rep and rabbit anti-Cap raised against denatured VP3 (described above). The beads were pelleted (500 x g
for 2 minutes at 4°C) and washed three times with IPP buffer. The proteins were eluted from the beads and crosslinks broken by the addition of loading buffer (1.5 X SDS-PAGE Sample buffer, 10% (v/v) β-mercaptoethanol) and heating to 100°C for 10 min. Eluted proteins were run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Immobilon- Fisher Scientific Co.). Transferred proteins were Western blotted as indicated and detected by chemiluminescence.

**EMSA Probe Preparation:** The A-D stem sequence used for Figure 4 B is as follows:

TCCAGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTCGCTCGCTCGCTCACTGAGGC and Phos-

GCCTCAGTGAGCGAGCGAGAGGAGTTGGCCACTCCCTCTCTCGCTCGCTCGCTCACTGAGGC and Phos-

AGGGGTTCCTGGA (Invitrogen Custom Primers). The A-D stem sequence used for Figure 4 A and C is as follows:

CCTAGTGATGGAGTTGGCCACTCCCTCTCTCGCTCGCTCGCTCACTGAGGC and Phos-

GCCTCAGTGAGCGAGCGAGAGGAGTTGGCCACTCCCTCTCTCGCTCGCTCGCTCACTGAGGC and Phos-

AGGGGTTCCTGGA (Invitrogen Custom Primers). Both AD stem sequences contain the Rep binding element (RBE) and the trs nicking site (Chiorini et al., 1995; Muzyczka, 2001). The oligos were annealed following the manufacturers recommendations. Briefly, the oligos were annealed together in a 50 µL reaction (100 mM Tris pH 7.5, 500 mM NaCl, 50 mM EDTA), which was heated in sand bath at 65°C for 10 minutes and then allowed to cool slowly to room temperature. One pmole of annealed oligos was incubated with 20 U of T4 polynucleotide kinase and 50 µCi [γ-32P] ATP. The reaction went 16 hours 37°C and purified over a 1 mL G-25 Sephadex column in TE.
**Electrophoretic mobility shift assay (EMSA):** EMSAs were performed following method previously described (Needham et al., 2006). Briefly, the radiolabeled A-D substrate was incubated in a 20 \( \mu \)L reaction with purified MBPRep78 and/or purified GSTNPM (B23.1) or BSA in binding buffer (10mM Tris pH 7.5, 50mM NaCl, 4% (v/v) glycerol, 1mM MgCl\(_2\), 0.44 mM EDTA, 0.5 mM dithiothreitol (DTT), 12.5 \( \mu \)g polydeoxyinosinic-deoxycytidylic (Sigma) per mL, 50 \( \mu \)g of BSA per mL) for 20 minutes at room temperature. The mixture was run on a non-denaturing 4% polyacrylamide TBE gel and vacuum dried. The gels were exposed to BioMax MR Film (Kodak).

**Endonuclease activity assay:** The hairpin probe was prepared as follows. The AAV ITRs were excised from psub201 with Xba I and Pvu II and calf intestinal phosphatase (CIP) treated. One pmole of the purified CIP treated fragment was incubated with 20 U of T4 polynucleotide kinase and 50 \( \mu \)Ci [\( \gamma \)-\(^{32}\)P] ATP. The reaction went 16 hours 37ºC and purified over a 1 mL G-25 Sephadex column in TE. The labeled fragment was heated in a boiling water bath for five minutes and then snap cooled on ice forming the hairpin probe. The endonuclease activity assay was performed as described previously with slight modifications (Im and Muzychka, 1992; Li et al., 2003). Briefly, the labeled hairpin probe (5 fmoles) was incubated with the purified MBPRep78 (1.34 nM) and/or purified HisNPM or BSA in a 20 \( \mu \)L reaction containing: 25 mM HEPES-KOH pH 7.5, 10 mM NaCl, 5.5 mM MgCl\(_2\), 0.5 mM ATP, 0.2 mM DTT, 0.25% Tween 20, and 10 \( \mu \)g of BSA per mL. The reactions were incubated at 37ºC for 1 hr. The reaction was stopped with 20 \( \mu \)g of proteinase K incubated for 30 min. at 37ºC. The products were phenol/chloroform extracted and then ethanol precipitated. The pellet was resuspended in 2 \( \mu \)L of 10x agarose loading buffer and 18 \( \mu \)L of dH\(_2\)O and run on a 10% denaturing
polyacrylamide gel containing 50% urea and vacuum dried. The gels were exposed to BioMax MR Film (Kodak). The cleavage product is 73 nucleotides.
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References


Chapter 2

Cell Cycle and DNA Damage Response to AAV and Ad Coinfection
Introduction

Adenovirus (Ad) and AAV, individually, have different effects on the cell cycle during an infection. Although both viruses require the induction of the cell into S phase, only Ad is capable of doing so. Adenovirus E1A proteins induce cells to enter S phase (Braithwaite et al., 1983). The E1A 12S and E1A 13S proteins bind to Rb family members and displace them from E2F transcription factor proteins (Barbeau et al., 1992; Berk, 2005; Dyson et al., 1992; Whyte et al., 1988). The E2F transcription factor proteins are responsible for transcription of several proteins important during the cell cycle. A number of cyclins, including cyclins A and E, as well as PCNA are targets of the E2F family transcription factors (Du and Pogoriler, 2006). The Cdk4/6-cyclin D complexes normally are responsible for phosphorylating pRb and inducing the release of E2F, which allows E2F to function (Kozar and Sicinski, 2005). Binding of Ad E1A 12S or 13S to pRb and disrupting the pRb-E2F interaction bypasses the need for pRb phosphorylation by Cdk4/6-cyclin D (Berk, 2005). Adeno-associated virus requires Ad to induce cells into S phase in order to utilize the cell’s replication proteins (Weitzman, 2006). As discussed in the ‘Literature’ section, AAV can have different effects on the cell cycle. Infection of primary fibroblasts with AAV arrests cells in G0/G1 and G2/M phases (Hermanns et al., 1997). Retrovirally-expressed Rep68 and Rep78 arrested cells in G1 and G2, while Rep78 also arrested cells in S phase (Saudan et al., 2000). During a coinfection of AAV and Ad there is AAV-mediated repression of Ad protein expression, including E1A (Timpe et al., 2006). While there are several studies on the effects of the individual virus on the host cell there are currently no published studies on the effects of
coinfection on the cell cycle proteins and only a couple of reports on the effects of coinfection on cell cycle-related proteins. The major aim of this chapter is to address the question of host cell response in terms of cell cycle and cell cycle-related protein expression changes during an Ad and AAV coinfection compared to Ad infection.

As discussed earlier, AAV competes with Ad during a coinfection for cellular components. Adeno-associated virus reduces Ad production by up to 50-fold during a coinfection (Timpe et al., 2006). Adenovirus protein expression is also reduced during a coinfection, including the Ad E4 gene products (Timpe et al., 2006). Although reduction of Ad gene products contributes to reduced Ad viral replication during an Ad and AAV coinfection, this does not appear to be the sole reason behind the reduced viral production (Timpe et al., 2006). The Ad E4 gene contains multiple open reading frames that encode several proteins that are named based on position in the E4 gene (Tauber and Dobner, 2001). Two of these proteins, Ad E4orf3 and Ad E4orf6, are important for Ad viral replication since they disrupt the cellular DSB response (Berk, 2007). Ataxia-telangiectasia mutated protein (ATM), a serine-threonine kinase, is primarily involved in DSB repair signaling (Branzei and Foiani, 2008; Dery and Masson, 2007; Pommier et al., 2005). Activation of ATM is through recruitment of ATM to sites of DNA damage by the MRN-complex (Branzei and Foiani, 2008). There are multiple targets for ATM phosphorylation, including Chk2 and NBS1 (Lee and Paull, 2007). The loss of Ad E4orf3- or E4orf6-mediated disruption of the DSB pathway hinders Ad replication (Berk, 2007; Evans and Hearing, 2003; Mathew and Bridge, 2007). Indeed, infection of HeLa cells with an Ad E4 deletion mutant resulted in activation of the DSB response with increases in the phosphorylated forms of ATM S1981 and Chk2 T68 compared to wild...
type infection (Carson et al., 2003). Since AAV is capable of down-regulating Ad viral proteins, including \textit{E4} gene products, the effects of an AAV and Ad coinfection on the DSB response were examined.
Results

Ad and AAV Coinfection Effects on Cell Cycle Protein Expression

Adenovirus and AAV have different effects on the cell cycle when cells are infected with the individual viruses. As discussed in the introduction, Ad causes cells to enter S phase, which is necessary for the virus to replicate (Berk, 2007). Expression of Rep68/78 proteins induces G1 and G2 phase arrest while Rep78 expression causes S phase arrest (Saudan et al., 2000). There have been several studies looking at wild type or recombinant AAV transduction, as well as Rep protein effects on cell cycle regulatory proteins; however, there have been no published studies on the combined effects of AAV and Ad coinfection on cell cycle regulatory proteins. To address this, preliminary studies looking at the expression differences of several cell cycle proteins were undertaken to determine if there are any differences in expression between Ad infection and Ad and AAV coinfection (Figure 1).
Figure 1. Cyclin and Cdk Protein Expression.

Nuclear extracts from HeLa cells that were mock-infected (M), Ad-infected (Ad), or AAV & Ad coinfeected (Ad & AAV) were run on SDS-PAGE gels and protein expression was detected by chemiluminescence Westerns. Protein expression was examined 24 hrs post-infection. The change in protein expression is indicated next to each representative Western. Change in expression is expressed in terms of Ad & AAV coinfection compared to Ad infected extracts. Band intensity of Ad infected and Ad and AAV coinfected extracts were normalized against mock infected extracts. The results were averaged and represented as percent change in expression. The ‘*’ indicates if an outlier was not included in the average. GraphPad Prism® software was used to evaluate for statistical significant (p<0.05) changes using unpaired T test. (A) Cyclin and Cdk proteins involved in cell cycle regulation were examined and a representative blot was shown. Cell cycle phases to which each Cdk and cyclin protein is important are indicated below the protein name. (B) Transcriptional regulators cyclin T1 and Cdk9 protein expression was examined. (C) A representative actin blot to confirm equal loading of proteins is shown.

HeLa cells were mock-infected, Ad infected, or Ad and AAV coinfeected and after 24 hours post-infection nuclear extracts were made. Equal quantities of nuclear protein extracts were separated by SDS-PAGE electrophoresis and immunblotted to determine
protein expression differences. In most cases, extracts from two separate infection experiments were examined and at least two nuclear extraction methods were used; the different extraction methods were employed due to difficulty in extracting certain proteins.

There were no statistically significant changes in protein expression comparing Ad infected to Ad and AAV coinfected extracts for Cdk1, Cdk2, Cdk4, Cdk6, Cdk7, cyclin A, cyclin B1, and cyclin H (Figure 1A). In primary fibroblasts Hermanns et al. saw a decrease in cyclin A expression (Hermanns et al., 1997). Although not statistically significant (with a p-value of 0.467), cyclin A expression was reduced in Ad and AAV coinfected cells compared to Ad infected cells (Figure 1A, compare lanes 2 and 3).

Looking at the transcription regulatory Cdk-cyclin complex Cdk9-cyclinT1, there was no statistically significant difference in protein expression (Figure 1B). For the Cdk6 blot there was a second band that appears in the Ad and AAV coinfection with a higher molecular weight than Cdk6 that appears to be a cross-reacting band, but might represent a modified form of Cdk6. Several of the proteins examined had considerable variability that might be attributed to use of non-synchronized cells or normal variability. Increasing the number of Westerns analyzed might help to identify other trends. Further studies looking at effects of down-stream proteins as well as experiments using primary cell lines would help to confirm these results.

The Cdk-cyclin complexes affect many other cellular proteins as part of their regulatory function. A few of these cell cycle-related proteins were examined by Western blot to identify any changes in protein expression or mobility in Ad and AAV coinfected cells versus those infected with Ad alone (Figure 2).
Nuclear extracts from HeLa cells that were mock-infected (M), Ad-infected (Ad), or AAV & Ad coinfectected (Ad & AAV) were run on SDS-PAGE gels and protein expression was detected by chemiluminescence Westerns. (A) At 24 hrs post-infection, protein expression of cell cycle-related proteins were examined. The change in protein expression is indicated next to each representative Western. Change in expression is expressed in terms of Ad & AAV coinfection compared to Ad infected extracts. Band intensity of Ad infected and Ad and AAV coinfectected extracts were normalized against mock infected extracts. The results were averaged and represented as percent change in expression. The '*' indicates if an outlier was not included in the average. GraphPad Prism® software was used to evaluate for statistical significant (p<0.05) changes using unpaired T test. (B) Sp1 was examined at different time points (indicated above the lanes) for Ad infected (Ad) and Ad and AAV coinfectected (Ad & AAV) HeLa cells. A control lane of uninfected cells (No) is shown in lane 1. A representative actin blot to confirm equal loading of proteins is shown. For the Sp1 Western, two mobility patterns were observed with the slower migrating band indicated with a ‘►’ and the faster migrating band indicated with an open arrowhead to the left of the blot.

The difference between Ad infected and Ad and AAV coinfectected cells for PCNA, Cdc25A, and pRb protein expression were not statistically significant (Figure 2A, compare lanes 2 and 3). In Ad infected extracts there were two bands that show up when blotting for Sp1 (Figure 2A, lane 2). The faster migrating band might represent an unmodified form of Sp1 (Figure 2A, open arrowhead) and the slower migrating band might represent a post-translationally modified form of Sp1 (Figure 2A, solid arrowhead). The Sp1 protein is extensively modified by phosphorylation, glycosylation,
sumoylation, and acetylation (Wierstra, 2008). In the Ad and AAV coinfected extracts (Figure 2A, lane 3) the faster migrating band seen in Ad infected extracts (Figure 2A, lane 2) disappears and the slower migrating band increases in intensity. Subsequent experiments using phosphatase treatments have shown that the slower migrating band is phosphorylated (Collaco, unpublished).

A time course experiment was performed to analyze these effects on the Sp1 protein. HeLa cells were infected with Ad alone or with Ad and AAV. Nuclear extracts were prepared at six hour intervals post-infection. The extracts were run on SDS-PAGE gels and the immunoblots were analyzed for protein expression (Figure 2A). The time course experiment (Figure 2B) is in agreement with the 24 hour Sp1 Western (Figure 2A). With Ad infection alone both a slow and a fast migrating band is seen. In the Ad infection the faster migrating band intensifies at the 12 hour time point then decreases in intensity as time goes on; whereas, the slower migrating band first appears at the 12 hour time point and intensifies overtime (Figure 2B, lanes 2-5). In the AAV and Ad coinfection the faster migrating band decreases in intensity as time goes on while the slower migrating band first appears at the 12 hour time point and intensifies over time (Figure 2B lanes 6-9). These experiments indicate that Sp1 is more extensively modified in AAV and Ad coinfected cells compared to Ad infected cells. Modification of Sp1 affects its DNA binding ability, transactivation level, and protein levels (Wierstra, 2008). The Sp1 protein is important for the activation of transcription at the AAV $P_{19}$ and $P_{40}$ promoters (Pereira and Muzyczka, 1997a; Pereira and Muzyczka, 1997b).
Ad and AAV Coinfection Effects on the DNA Damage Response

Adenovirus must circumvent the cellular DNA damage response in order to replicate efficiently. One of adenovirus’ targets is the MRN-complex. Adenovirus through E4orf6, E4orf3, and E1B-55K disrupts the MRN-complex by altering localization of components of the complex and by targeted degradation of MRN-complex proteins (Evans and Hearing, 2005; Stracker et al., 2002). Adeno-associated virus requires Ad E4 gene products for efficient transduction of AAV gene products; however, AAV down-regulates Ad E4 gene product expression (Timpe et al., 2006). The effects of Ad and AAV coinfection versus Ad infection on MRN-complex protein expression was examined (Figure 3).

Figure 3. Mre11-Rad50-NBS1 Complex Protein Expression and Phosphorylation.

Nuclear extracts from HeLa cells that were mock-infected (M), Ad-infected (Ad), or AAV & Ad coinfected (Ad & AAV) were run on SDS-PAGE gels and protein expression/phosphorylation was detected by chemiluminescence Westerns. (A) At 24 hrs post-infection, protein expression of MRN-complex proteins were examined. The change in protein expression is indicated next to each representative Western. Change in expression is expressed in terms of Ad & AAV coinfection compared to Ad infected extracts. The ‘*' indicates if an outlier was not included in the average. (B) Protein expression of Rad50, NBS1, and Mre11 as well as the phosphorylation state of NBS1 at S343 were examined at different time points (indicated above the lanes) for Ad infected (Ad) and Ad and AAV coinfected (Ad & AAV) HeLa cells. A control lane of uninfected cells (No) is shown in lane 1. Representative blots are shown.
HeLa cells were mock-infected, Ad infected, or Ad and AAV coinfectected and nuclear extracts were prepared 24 hours post-infection. Equal concentrations of the nuclear extracts were separated on a SDS-PAGE gel and immunoblotted to determine protein expression differences. In most cases, extracts from two separate infection experiments were examined and the results were averaged to estimate percent change in expression. A representative blot and relative change in protein expression between Ad infection and Ad and AAV coinfection is shown in Figure 3. Consistent with what Carson et al. saw, there was a decrease in MRN-complex proteins with Ad infected cells compared to mock-infected cells (Figure 3A, compare lanes 1 and 2) (Carson et al., 2003). Increases in Rad50, NSB1, and Mre11 protein levels were seen with Ad and AAV coinfectected extracts compared to Ad infected extracts; however, the levels did not rise to mock-infected levels (Figure 3A, compare lanes 2 and 3).

A time course experiment was performed to analyze MRN-complex protein levels. HeLa cells were infected with Ad or coinfectected with Ad and AAV. Nuclear extracts were prepared at six hour intervals post-infection. The extracts were separated on SDS-PAGE gels and the immunoblots were analyzed for protein expression (Figure 3B). The protein levels of MRN-complex proteins in Ad infected cells were decreased at the 12 hr time point consistent with the Carson et al. experiments (Figure 3B, compare lanes 2-5) (Carson et al., 2003). In Ad and AAV infected cells, protein levels of MRN-complex proteins also decrease but to a lesser degree than Ad alone infected cells (Figure 3B, compare lanes 6-9 to lanes 2-5). At the 12 hr time point the presence of p-NBS1 S343 was present in both conditions; however, a greater amount of NBS1 was phosphorylated at S343 in Ad and AAV coinfectected cells versus Ad infected cells (Figure 3B, compare lanes 6-9 to lanes 2-5).
3B, compare lanes 6-9 to lanes 2-5). The presence of a greater amount of MRN-complex proteins and phosphorylated NBS1 when comparing AAV and Ad coinfected cells versus Ad infected cells indicates the disruption of the DNA damage response brought on by Ad proteins might be disrupted by AAV infection. This disruption of the DNA damage response inhibition might be an additional method for AAV to reduce Ad replication since Ad replication is disrupted by the intact DNA damage response.

Although there was an increase in MRN-complex proteins in coinfected versus Ad infected cells this might not have an effect on Ad replication since one of Ad’s methods of disrupting the DNA damage response is by altering the localization of MRN-complex proteins. In response to DSB, p-NBS1 S343 normally localizes to the areas of DSB in the nucleus (Lukas et al., 2003). Immunofluorescence experiments were conducted to determine if the altered localization of MRN-complex proteins by Ad was disrupted with an AAV coinfection. Co-localization between p-NBS1 S343 and Ad and AAV replication centers was addressed. Unfortunately, Mre11 and Rad50 immunofluorescence experiments were not obtained since the antibodies used did not work well in immunofluorescence experiments that were attempted.
HeLa cells were Ad infected (A-D) or Ad and AAV coinfected (E-H). After 20-24 hrs post-infection, the cells were fixed and localization of p-NBS1 (S343) in Ad infected and Ad and AAV coinfected cells was determined by immunofluorescence. DAPI stain was used to identify the cellular nuclei (A, E, blue). Ad E2a (B, red) immunofluorescence is used to identify Ad replication centers and localization p-NBS1 (S343) (C) is shown in green. Merged image of panels B and C is depicted in panel D (co-localization in yellow). Rep (F, green) immunofluorescence is used to identify Ad and AAV replication centers and localization p-NBS1 (S343) (G) is shown in green. Merged image of panels F and G is depicted in panel H (co-localization in yellow). Scale bar is equivalent to 16 μm.

HeLa cells were Ad infected or Ad and AAV coinfected and fixed after 20-24 hrs post-infection. Antibodies against AAV Rep proteins were used to identify Ad and AAV replication centers since AAV and Ad replication centers overlap during a coinfection (Figure 4F, red) (Weitzman et al., 1996). Adenovirus replication centers are identified by using an antibody against Ad E2a (Figure 4B, red). In agreement with the Western (Figure 3B), there was p-NBS1 (S343) detected in Ad infected cells (Figure 4C, green). Phosphorylated NBS1 (S343) localized both to areas of Ad replication centers and areas outside Ad replication centers (Figure 4D, yellow). At the time point used other authors
have noted that NBS1 localized to both Ad replication centers and areas outside Ad replication centers with cells infected with Ad (NBS1 starts to overlap with Ad replication centers as time goes on) (Evans and Hearing, 2005). This is in agreement with what is seen here between p-NBS1 S343 and Ad replication centers. In Figure 4F, Rep is diffusely localized in the nucleus with bright spots/rings corresponding to nucleoli. Rep transiently localizes to the nucleolus (Wistuba et al., 1997). There are areas of diffuse co-localization (Figure 4H) between Rep (Figure 4F) and p-NBS1 (Figure 4G). The diffuse localization of p-NBS1 S343 overlapping Rep localization indicates that p-NBS1 (S343) localization is not altered away from replication centers but is co-localized with both Ad and AAV replication centers. The presence of at least one of the MRN-complex proteins in Ad replication areas points to the possibility that activated DSB response has access to Ad replication centers during an Ad and AAV coinfection. Assessing the other MRN-complex protein localization would help reinforce this observation.

One of the signaling proteins involved in DSBR process is ATM which is dependent on the MRN-complex for efficient activation (Uziel et al., 2003). Disruption of the MRN-complex by Ad infection subsequently disrupted the DSB response by the host cell. Carson et al. showed that Ad5 infecting HeLa cells had only a slight activation of ATM and Chk2 and no Chk1 activation at the 24 hr or later time points (Carson et al., 2003). When using an Ad E4 deletion mutant there was increased levels of activation of ATM, Chk1, and Chk2 (based on presence of phosphorylated forms) (Carson et al., 2003). These activated forms were also seen earlier in the infection than seen in the Ad5 infection (Carson et al., 2003). Since Ad in the presence of AAV coinfection has reduced
expression of Ad E4 gene products (Timpe et al., 2006), experiments were conducted to
determine if Ad and AAV coinfection had a similar DSBR activation to an Ad E4
deletion mutant infection (Figure 5).

Figure 5. DNA Double Stranded Break Repair Signaling Protein Expression and
Phosphorylation.

Nuclear extracts from HeLa cells that were mock-infected (M), Ad infected (Ad), or
AAV & Ad coinfected (Ad & AAV) were run on SDS-PAGE gels and protein
expression/phosphorylation was detected by chemiluminescence Westerns. (A) At 24 hrs
post-infection, DSBR signaling protein expression and phosphorylation was examined
and representative blots are shown (B) The phosphorylation state of ATM and Chk2 were
examined at different time points (indicated above the lanes) for Ad infected and Ad and
AAV coinfected HeLa cells. Representative p-ATM (S1981) blot and p-Chk2 (T68)
blots are shown. A representative actin blot to confirm equal loading of proteins is also
shown.

HeLa cells were mock-infected, Ad infected, or Ad and AAV coinfected and after
24 hours post-infection nuclear extracts were prepared. Equal concentrations of the
nuclear extracts were separated on a SDS-PAGE gel and immunoblotted to determine
protein expression and phosphorylation differences (Figure 5). In Ad and AAV infected
cells there was increased phosphorylation of ATM at S1981 compared to Ad alone
infected cells (Figure 5A, compare lanes 2 and 3). In Ad infected cells phosphorylation
of Chk2 was not detected; whereas, in Ad and AAV coinfected cells phosphorylation at
T68 of Chk2 was observed (Figure 5A, compare lanes 2 and 3). Phosphorylation at T68 of Chk2 induces Chk2 oligomerization and activation (Ahn et al., 2002; Xu et al., 2002). Also, phosphorylation of Chk2 at T432 was detected in both Ad infected and Ad and AAV coinfected cells; however, there was a greater amount of phosphorylation detected in Ad and AAV coinfected cells versus Ad infected cells (Figure 5, compare lanes 2 and 3). These results support the notion that indeed DSB response, which is normally repressed in Ad infected cells, is activated in Ad and AAV coinfected cells.

Phosphorylated ATM localizes to sites of DSB as well as areas of ssDNA (Bekker-Jensen et al., 2006). If activation of DSB response disrupted Ad replication you would expect to see p-ATM at sites of Ad replication as well as AAV replication centers since Ad and AAV replication centers coincide. Immunofluorescence experiments were performed to determine if co-localization occurs between p-ATM S1981 and Ad and AAV replication centers.
HeLa cells were Ad infected (A-D) or Ad and AAV coinfected (E-H). After 20-24 hrs post-infection, the cells were fixed and localization of p-ATM S1981 in Ad infected and Ad and AAV coinfected cells was determined by immunofluorescence. DAPI stain was used to identify the cellular nuclei (A, E, blue). Ad hexon (B, red) immunofluorescence is used to identify Ad infected cells and localization of p-ATM S1981 (C) is shown in green. Merged image of panels B and C is depicted in panel D (co-localization in yellow). Rep (F, green) immunofluorescence is used to identify Ad and AAV replication centers and localization of p-ATM S1981 (G) is shown in red. Merged image of panels F and G is depicted in panel H (co-localization in yellow). Scale bar is equivalent to 16 μm.

HeLa cells were Ad infected or Ad and AAV coinfected and fixed 20-24 hrs post-infection. Antibodies against AAV Rep proteins were used to identify AAV and Ad replication centers since they co-localize during an coinfection (Weitzman et al., 1996) (Figure 6F, green). Due to cross-reactivity problems of available antibodies we were unable to identify Ad replication centers in Ad infected extracts; however, Ad-positive cells were identified using an antibody against the Ad structural protein hexon (Figure 6B, red). Even though in Ad infected cells p-ATM S1981 could be detected by Western
(Figure 5A and B) it could not be detected in the immunofluorescence experiment (Figure 6C, green). In Ad and AAV coinfected cells, p-ATM S1981 (Figure 6G, red) was detected in Rep-expressing cells (Figure 6F, green). There was considerable co-localization between p-ATM S1981 and Rep proteins (Figure 6H); in areas where p-ATM S1981 (Figure 6G, red) is concentrated in nuclear foci there is some co-localization (Figure 6H, yellow). Confocal experiments are necessary to demonstrate whether the co-localization seen between Rep and p-ATM S1981 are in the same plane.

One of the targets of activated ATM is Chk2, which phosphorylates Chk2 at T68. Although DSB might be localized to specific sites in the nucleus p-Chk2 T68 localizes throughout the nucleus (Bekker-Jensen et al., 2006; Lukas et al., 2003). If in Ad and AAV coinfected cells the DSB response was activated through ATM and its target Chk2 then you would expect diffuse nuclear localization of p-Chk2 T68. Immunofluorescence experiments were performed to determine localization of p-Chk2 T68 in Ad and AAV infected cells.
Figure 7. Presence of Phosphorylated Chk2 (T68) in AAV & Ad Coinfected Cells.

HeLa cells were Ad infected (A-D) or Ad and AAV coinfected (E-H). After 20-24 hrs post-infection, the cells were fixed and localization of p-Chk2 (T68) in Ad infected and Ad and AAV coinfected cells was determined by immunofluorescence. DAPI stain was used to identify the cellular nuclei (A, E, blue). Ad E2a (B, red) immunofluorescence is used to identify Ad replication centers and localization of p-Chk2 (T68) (C) is shown in green. Merged image of panels B and C is depicted in panel D (co-localization in yellow). Rep (F, red) immunofluorescence is used to identify Ad and AAV replication centers and localization of p-Chk2 (T68) (G) is shown in green. Merged image of panels F and G is depicted in panel H (co-localization in yellow). Scale bar is equivalent to 16 μm.

HeLa cells were Ad infected or Ad and AAV coinfected and fixed after 20-24 hrs post-infection. Antibodies against AAV Rep proteins were used to identify Ad and AAV replication centers (Figure 7F, red). Adenovirus replication centers in Ad infection are identified using an antibody against Ad E2a (Figure 7B, red). In agreement with the Western (Figure 5A and B), no p-Chk2 T68 was detected by immunofluorescence in Ad infected cells (Figure 7C, green). In Ad and AAV coinfected cells, p-Chk2 T68 was detected in Rep expressing cells (Figure 7, compare panels F and G). The weak p-Chk2
signal precludes a definitive assessment of p-Chk2 localization (Figure 7H, yellow); however, p-Chk2 is present throughout the nucleus even outside AAV replication centers (Figure 7, compare panels G to F). The diffuse localization of p-Chk2 T68 is consistent with what occurs in cells with focal sites of DNA damage (Bekker-Jensen et al., 2006; Lukas et al., 2003).

The immunofluorescence experiments demonstrated the presence of phosphorylated forms of NBS1, ATM, and Chk2 in Ad and AAV coinfected cells. Both p-ATM S1981 and p-NBS1 S343 co-localized with Ad and AAV replication centers in coinfected cells. Phosphorylated NBS1 S343 was also present both inside and outside Ad replication centers in Ad infected cells. As with the Western, the levels of p-NBS1 was reduced in Ad infected cells compared to Ad and AAV coinfected cells. Phosphorylated ATM S1981 was not detected in Ad infected cells by immunofluorescence even though the presence of p-ATM S1981 was observed in Ad infected cells by Western. Unfortunately, p-Chk2 T68 antibody did not work as well for immunofluorescence as it did for Westerns so definitive assessment of p-Chk2 T68 localization is difficult; however, p-Chk2 T68 is present throughout the nucleus even outside Ad and AAV replication centers in Ad and AAV coinfected cells. This is consistent with localization of p-Chk2 T68 seen in focal DSB experiments (Lukas et al., 2003).

A recombinant AAV (rAAV) virus was used to determine if the activation of ATM, NBS1, and Chk2 by phosphorylation is related to AAV Rep protein expression. The recombinant virus replaces the rep and cap genes of the AAV genome with a gene encoding for firefly luciferase (rAAVluc).
Figure 8. Activation of DSBR response related to AAV Rep protein expression.

HeLa cells were infected by one of the following conditions: mock-infected, single virus type infection, or Ad coinfected with either wild type AAV or recombinant AAV containing the firefly luciferase gene instead of the rep and cap genes. Nuclear extracts were run on SDS-PAGE gels and protein expression/phosphorylation was detected by chemiluminescence Westerns. (A) Representative blots of ATM, phosphorylated ATM S1981, and ATR. (B) Representative blots of MRN-complex proteins Rad50, NBS1, and phosphorylated NBS1 S343. (C) Representative blot of phosphorylated Chk2 at T68. (D) Control Westerns of Rep78 and actin. Antibodies used in each Western are indicated to the right of each Western. Above each lane indicates the presence (+) or absence (-) of a given virus during the infection.

Single virus type infection was compared to mock-infected cells to confirm individual virus type contributions (Figure 8, lanes 2, 3, and 4). Coinfected cells with Ad and either wild type AAV or rAAVluc were used to determine whether Rep protein expression or the presence of the recombinant AAVluc DNA contributed to the change in protein expression and presence of phosphorylated DSBR proteins. The expression of ATR, another DSB response signaling protein, was fairly constant across all conditions (Figure 8B, compare lanes 2-6 to lane 1). Activation of ATM demonstrated by the
presence of phosphorylated ATM at S1981 was present in Ad and AAV coinfected cells to a greater amount than either Ad alone or Ad and rAAVluc coinfected cells (Figure 8B, compare lane 6 to lanes 2 and 5). Phosphorylated ATM S1981 was not detected in mock-infected, AAV infected, or rAAVluc infected cells (Figure 8B, lanes 1, 3, and 4, respectively). Activation of Chk2, a down-stream target of activated ATM was only detected in Ad and AAV coinfected cells (Figure 8B, compare lane 6 to lanes 1-5). The MRN-complex proteins were also looked at. Activation of NBS1, demonstrated by the presence of phosphorylated NBS1 at S343 was present in Ad and AAV coinfected cells to a greater amount than either Ad alone or Ad and rAAVluc coinfected cells (Figure 8A, compare lane 6 to lanes 2 and 5). Activation DSBR proteins demonstrated by the presence of phosphorylated forms of ATM, NBS1, and Chk2 was dependent on the presence of Rep protein expression. This is consistent with what Berthet et al. observed with Rep78 protein transfection experiments where Rep78 expression induced the activation of ATM and Chk2 (Berthet et al., 2005).

As noted earlier disruption of both Ad E4orf3 and E4orf6 disrupts adenovirus replication and induces the host cells DNA damage response (Stracker et al., 2002). Disruption of both E4orf6 and E4orf3 also caused the adenovirus DNA to be concatamerized (Weiden and Ginsberg, 1994). The MRN-complex proteins are thought to be involved in the concatamerization via the DSB process (Stracker et al., 2002). However, activation of the DSBR pathway in itself disrupts Ad genome replication regardless of Ad genome concatamerization (Berk, 2007; Evans and Hearing, 2003; Mathew and Bridge, 2007). Experiments were conducted to determine if the Ad genome was concatamerized with an AAV coinfection.
HeLa cells were mock-infected, Ad infected, or Ad and AAV coinfected and after 24 and 48 hours post-infection Hirt extracts were prepared to extract the viral DNA. Equal volumes of viral DNA were treated with a restriction enzyme to cut up potentially concatamerized DNA (this allowed the fragments to be separated on a standard agarose gel). The restriction enzyme was chosen such that if the Ad genomes were concatamerized in a head-to-tail, tail-to-tail, or head-to-head fashion a different size band would appear compared to digested non-concatamerized Ad DNA. The fragments were separated on an agarose gel and transferred to a nytran membrane. The membrane was probed with two different probes to detect any fragments of concatamerized Ad DNA (Figure 9).
Figure 9. Determining if Ad genome concatamerization occurs.

At 24 hrs and 48 hrs, Hirt extracts from HeLa cells that were mock-infected (M), Ad infected (Ad), or AAV & Ad coinfected (Ad & AAV) were digested, run on agarose gels, transferred to nytran membrane, and probed. Both cut (c) and uncut (u) Hirt extracts were run on the gels. (A) Fragments were probed using a 3’ probe against the Ad E4 region. Digested linear DNA would produce a 5,469 bps sized fragment. Digested concatamerized DNA would produce a 6,807 bps and/or 10,932 bps sized fragment depending on orientation of concatamerization. (B) Fragments were probed using a 5’ probe against the Ad E1A region. Digested linear DNA would produce a 1,338 bps sized fragment. Digested concatamerized DNA would produce a 6,807 bps and/or 2,682 bps sized fragment depending on orientation of concatamerization. The left side of each film depicts size markers in kilobases. The open arrow and the solid arrow at the right side of the films indicate uncut and digested linear fragment, respectively.

Uncut viral DNA (Figure 9, lanes 2, 4, 7, 9) was run next to the respective digested DNA (Figure 9, lanes 3, 5, 8, and 10) for both time points as a control. Mock-infected extracts were also digested as a negative control (Figure 9, lanes 1, 6). The fragments created when the non-concatamerized adenovirus genome is digested with Xba I are 18,003, 9,250, 5,469, 1,878, and 1,338 bps. The 3’ probe in Figure 9A, targets the E4 gene which is located on the 3’ end of the Ad genome. When the genome is digested with Xba I the fragment that is detected by the 3’ probe is 5,469 bps in size (Figure 9A,
solid arrow). If the Ad genome where concatamerized the size fragments created after Xba I digestion detected by the 3’ probe would be 6,807 bps and/or 10,932 bps depending of the orientation of the genomes when joined. The 5’ probe in Figure 9B, targets the E1A gene which is located on the 5’ end of the Ad genome. When the genome is digested with Xba I the fragment that is detected by the 5’ probe is 1,338 bps in size (Figure 9B, solid arrow). If the Ad genome where concatamerized the size fragments created after Xba I digestion detected by the 5’ probe would be 6,807 bps and/or 2,682 bps depending of the orientation of the genomes when joined. The only band detected of both digested Ad infected (Figure 9, lanes 3, 8, solid arrow) and digested Ad and AAV coinfectected extracts (Figure 9, lanes 5, 10, solid arrow) was that of the non-concatamerized Ad genome. This was seen for both probes used. The band detected in uncut extracts (Figure 9A and B, open arrow) was that of non-concatamerized Ad genome. There is the possibility that during the linking of the viral DNA fragments that the ends of the genome might be lost which would create a digested fragment approximately the size of non-concatamerized DNA. However, further experiments conducted using pulse field gel electrophoresis confirmed that there was no detectable Ad genome concatamerization by ethidium bromide staining (Trempe, unpublished). Although Ad E4 gene products are decreased in presence of an AAV coinfection, there does not appear to be any detectable concatamerized DNA.
Discussion

Adenovirus pushes the cell into S phase to highjack the DNA synthesis machinery. Several Ad proteins are important for deregulating the cell cycle. The Ad E1A protein bypasses Cdk4/6-cyclin D to initiate cell cycle progression by disrupting the association of pRb with E2F transcription factors. The release of the E2F transcription factor causes the positive regulation of certain genes needed to transition into the S phase as well as genes required in the S phase for DNA synthesis. Adeno-associated virus is not capable on its own to push cells into S phase nor does it have the necessary proteins like a polymerase to replicate its own genome like other viruses. Adeno-associated virus requires a helper-virus to provide the cellular environment for efficient replication of the virus.

In the preliminary experiments discussed in this section there were no statistically significant (p < 0.05) differences in cell cycle protein expression noted between Ad alone infection and Ad and AAV coinfection (Figure 1). A decrease in cyclin A expression was noted by Hermanns et al. in AAV infected cells (Hermanns et al., 1997). Although not statistically significant cyclin A expression was found to decrease upon coinfection versus Ad infection. Further experiments looking at targets of Cdk-cyclin A complexes would help determine if there is an effect on cyclin A. A Cdk-cyclin not involve in cell cycle regulation was also examined and there was also no statistically significant difference with Cdk9-cyclin T1 expression comparing Ad infected with Ad and AAV coinfected cells. Examination of some cell cycle-related proteins found no statistically significant change in expression between Ad and AAV coinfected versus Ad infected
cells for PCNA, pRb, and Cdc25A (Figure 2). The Sp1 transcription factor was found to be modified to a greater extent with AAV and Ad coinfect ed extracts versus Ad infected extracts. The significance might be related to Sp1’s involvement in AAV $P_{19}$ and $P_{40}$ regulation, which Sp1 helps activate these promoters (Pereira and Muzyczka, 1997a; Pereira and Muzyczka, 1997b). The increased amount of modified Sp1 might also be related to activation of the DSB response. It has been recently demonstrated that Sp1 was phosphorylated at S56 and S101 in an ATM-dependent manner (Iwahori et al., 2008; Olofsson et al., 2007). Iwahori et al. suggests that the role of phosphorylated Sp1 during DSB repair might not be related to Sp1’s role in transcription regulation but that it might be involved in DNA recombination (Iwahori et al., 2008). The phosphorylation of Sp1 in an ATM dependent manner was also seen in HSV-1 infected cells (Iwahori et al., 2007).

Production of AAV occurs at the expense of Ad in coinfect ed cells. Indeed, production of infectious Ad particles was reduced by up to 50-fold in coinfected cells (Timpe et al., 2006). Decreased expression of Ad proteins contributes to the reductions but other unknown mechanisms appear to be involved as well (Timpe et al., 2006). One of the effects AAV has on Ad is the reduction of Ad $E4$ gene expression (Timpe et al., 2006). Adenovirus E4orf6 and E4orf3 are important for disruption of the cellular DSB response since Ad does not replicate effectively if the DSB response is intact (Berk, 2007). However, activation of the DSB response may not have a deleterious effect on AAV replication. Helper-independent replication of AAV occurs when cells are treated with DNA damaging agents such as hydroxyurea or UV treatment although it isn’t as efficient as helper-dependent replication (Yakobson et al., 1989; Yalkinoglu et al., 1988). Integration of the AAV genome into the $AAVS1$ site of the host chromosome often
contains multiple head-to-tail concatamers of the AAV genome (Cheung et al., 1980; Samulski et al., 1991). Experiments looking at rescue of AAV integrated into host chromosomes found that rescue of AAV can occur even if its genome is concatamerized (Laughlin et al., 1986). Also, studies on recombinant AAV vectors used for gene therapy found that the rAAV vectors existed as an episome of concatamerized rAAV (Afione et al., 1996; Duan et al., 1998). Rescue and replication of the AAV genome can occur also if the AAV genome is contained within a plasmid (Samulski et al., 1982). Concerning the AAV ITRs which might get disrupted due to recombination events, there can be considerable loss of one of the ITRs and rescue and replication of the genome can still occur (Samulski et al., 1983; Senapathy et al., 1984). A strategy that AAV might use to help it compete with Ad replication is to activate the damage response pathway and disrupt Ad attempts to subvert the pathway.

One of the targets of Ad is the MRN-complex. Adenovirus disrupts the complex by causing its degradation and altering the localization of the complex-proteins away from replication centers (Evans and Hearing, 2005; Stracker et al., 2002). Experiments were performed to determine the effects of Ad and AAV coinfection versus Ad infection on the MRN-complex protein expression. Consistent with what was seen previously, there was a decrease in MRN-complex proteins, Mre11, NBS1, and Rad50, with Ad infected compared to mock-infected cells (Carson et al., 2003). Although not to the levels of mock-infected cells increases in MRN-complex proteins were seen when Ad and AAV coinfected were compared to Ad infected cells (Figure 3). Greater activation of one of the complex proteins, NBS1, by phosphorylation at S343 was seen with an AAV and Ad coinfection compared to an Ad infection (Figure 3). Localization of p-
NBS1 S343 was addressed to determine if the phosphorylated form of the protein co-localized with replication centers. There were areas of co-localization between p-NBS1 S343 and Rep proteins (Figure 4). Replication centers of AAV and Ad overlap with Rep localizing to AAV and Ad replication centers (Weitzman et al., 1996). The co-localization of p-NBS1 with AAV and Ad replication centers indicates that MRN-complex proteins might have access to Ad replication centers. It would be interesting to determine if Mre11 and Rad50 also co-localized to these replication centers as well.

Down-stream targets of the MRN-complex were assessed to further demonstrate DSB response activation. One of the targets is ATM. Phosphorylated ATM at S1981 is greater in Ad and AAV coinfection compared to Ad infection (Figure 5). The phosphorylation state of Chk2, an ATM target protein, was also assessed. Phosphorylated Chk2 T68 was present in Ad and AAV coinfection and absent in Ad infection (Figure 5). Adeno-associated virus coinfection reduces Ad E4 gene products. The AAV and Ad coinfection results resemble experiments comparing Ad E4 deletion mutant infected HeLa cells to Ad infected HeLa cells. Carson et al. noted that there was increased levels p-ATM S1981, p-Chk2 T68, and p-Chk1 S345 in Ad E4 deletion mutant infection compared to Ad infection (Carson et al., 2003). Later experiments also showed that Chk1 was phosphorylated at S345 in AAV and Ad coinfection (Collaco, unpublished). Another target of ATM is Sp1 which is phosphorylated in response to DSB (Iwahori et al., 2008; Olofsson et al., 2007). There was a difference in banding pattern noticed when comparing Ad and AAV coinfection to Ad infection. Two bands were present in Ad infection while the faster migrating band reduced in intensity and the slower migrating band increased in intensity in Ad and AAV coinfected extracts (Figure 5).
2). This slower migrating band appears to represent a phosphorylated form based on subsequent phosphatase treated extracts (Collaco, unpublished). Whether this phosphorylated form is related to ATM activation is hard to assess at this point. Determining if S56 and S101 are phosphorylated on Ssp1 might help determine if this modified form is in reaction to the DSB response in this instance (Iwahori et al., 2008).

Phosphorylated ATM is recruited to sites of DSB (Bekker-Jensen et al., 2006). If indeed AAV is disrupting Ad’s ability to knock-out the DSB response you would expect p-ATM S1981 to co-localize with in AAV and Ad replication areas. Indeed, immunofluorescence experiments demonstrated areas of co-localization between p-ATM S1981 and Rep proteins (Figure 6). Antibodies against Rep were used to identify Ad and AAV replication centers since they co-localize (Weitzman et al., 1996). To further examine the activation of the DSB response p-Chk2 T68 localization was assessed. Ataxia-telangiectasia mutated protein phosphorylates Chk2 at T68 in response to DSB (Matsuoka et al., 2000; Pommier et al., 2005). Even if the DSB is localized to a specific area in the nucleus p-Chk2 T68 localizes throughout the nucleus in order to fulfill its signaling function (Bekker-Jensen et al., 2006; Lukas et al., 2003). The weak p-Chk2 T68 prevents definitive assessment of localization; however, p-Chk2 T68 is present throughout the nucleus even outside AAV replication centers in Rep expressing cells (Figure 7). This indicates that the DSB response is activated through at least ATM and Chk2 in AAV and Ad coinfection and this response is abrogated or at least reduced in Ad infections.

In order to assess whether activation of the DSB response is related to Rep protein expression experiments comparing coinfection of Ad with either wild type AAV or a
recombinant AAV (rAAVluc), where the rep and cap genes are replaced by a firefly luciferase gene, were conducted. The rAAVluc virus is incapable of replication. The Ad and rAAVluc coinfection acted similar to Ad alone infection (Figure 8). The presence of p-NBS1 S343 was increased with Ad and AAV coinfection compared to either Ad infection or Ad and rAAVluc infection. The level of p-NBS1 S343 was comparable between Ad infection and Ad and rAAVluc coinfection. Similarly, p-ATM S1981 was present in Ad and AAV coinfection and was absent in either Ad infection or Ad and rAAVluc infection. The reason neither Ad infection nor Ad and rAAVluc coinfection showed any bands for p-ATM S1981 is probably related to the earlier time point used in these experiments. In experiments performed by Carson et al., there was reduced p-ATM S1981 present at the 16 hr time point compared to the 24 hr time point in Ad infected HeLa cells (Carson et al., 2003). Also, p-Chk2 T68 is in agreement with the other experiments. Phosphorylated Chk2 T68 was only detected in Ad and AAV coinfected cells. Both Ad infected and Ad and rAAVluc infected cells did not have detectable levels of p-Chk2 T68. These experiments indicate that the activation of the DSB response is related to Rep protein expression in the context of an AAV coinfection. This is also in agreement with Rep transfection experiments conducted by Berthet et al. where expression of AAV Rep78 was related to ATM and Chk2 activation (Berthet et al., 2005).

Adenovirus E4 deletion mutants were defective for replication and had concatamerized viral DNA (Berk, 2007; Weiden and Ginsberg, 1994). The MRN-complex proteins are thought to be involved in the concatamerization of the viral genome (Stracker et al., 2002). Experiments were conducted to determine if AAV mediated
reduction of Ad E4 gene expression and AAV mediated activation of the DSB response in AAV and Ad coinfections resulted in Ad genome concatamerization. The experiments were designed to distinguish between concatamerized Ad viral DNA from nonconcatamerized Ad viral DNA based on the banding pattern of digested Ad viral DNA (Figure 9). There were no bands present that would indicate that the Ad viral DNA was concatamerized in Ad and AAV coinfection. As expected there were also no bands present that would indicate that the Ad viral DNA was concatamerized in Ad infections. These results were later confirmed with pulse field gel electrophoresis experiments (Trempe, unpublished). This however does not preclude the possibility of activation of the DSB response by AAV in Ad and AAV coinfected extracts since there are reports that activation of the DSB response in itself disrupts the Ad genome replication regardless of Ad genome concatamerization (Berk, 2007; Evans and Hearing, 2003; Mathew and Bridge, 2007).
Materials

Cell Lines: HeLa cells (American Type Culture Collection) were grown as a monolayer at 37°C (5% CO₂ atmosphere) in Eagle’s minimum essential medium (MEM) supplemented with 10%(v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 25 U/mL penicillin, 25 μg/mL streptomycin, 2.5 μg/mL amphotericin B, and 100 μg/mL gentamicin.

Virus: Adeno-associated virus serotype 2 and adenovirus serotype 5 were generated and purified by methods previously described (Casper et al., 2005; Winters and Russell, 1971).

Antibodies: Antibodies against the following proteins were used in the experiments:

- Actin [C-11] (Santa Cruz Biotechnology, sc-1615), ATM [H-248] (Santa Cruz Biotechnology, sc-7230), phos-ATM S1981 (Rockland, 200-301-400), ATR [N-19] (Santa Cruz Biotechnology, sc-1887), Chk2 (Cell Signaling, 2662), phos-Chk2 T68 (Cell Signaling, 2661), phos-Chk2 T432 (Cell Signaling, 2667), NBS1 [1D7] (GeneTex, GTX70224), phos-NBS1 S343 (Novus Biologicals, NB100-284), Rad50 [13B3] (GeneTex, GTX70228), Mre11 [12D7] (GeneTex, GTX70212), PCNA [PC10] (Santa Cruz Biotechnology, sc-56), pRb [C-15] (Santa Cruz Biotechnology, sc-50), Sp1 [PEP 2-G] (Santa Cruz Biotechnology, sc-59-G), Cdc25A [M-191] (Santa Cruz Biotechnology, sc-7157), Cyclin A [H-432] (Santa Cruz Biotechnology, sc-751), Cyclin A [AB-3] (Calbiochem, CC17), Cyclin B1 [GNS1] (Santa Cruz Biotechnology, sc-245), Cyclin H [D-10] (Santa Cruz Biotechnology, sc-1662), Cyclin T1 [H-245] (Santa Cruz Biotechnology, sc-10750), Cdc2 p34 (Cdk1) [17] (Santa Cruz Biotechnology, sc-54),
Cdk2 [M2] (Santa Cruz Biotechnology, sc-163), Cdk4 [C-22] (Santa Cruz Biotechnology, sc-260), Cdk6 [C-21] (Santa Cruz Biotechnology, sc-177), Cdk7 [C-19] (Santa Cruz Biotechnology, sc-529), Cdk9 [C-20] (Santa Cruz Biotechnology, sc-484), Ad2 hexon (American Research Products, Inc., 12-6235-1), Ad E2a [B6 cl.10 monoclonal] (kindly provided by Dr. Arnold Berk), AAV2 Rep proteins (Trempe, Mendelson, and Carter, 1987), AAV2 Rep proteins [303.9] (American Research Products, Inc., 03-61069), AAV2 Cap proteins (Trempe, unpublished), and AAV2 VP proteins (Hermanns et al.) (American Research Products, Inc., 03-61058).

The secondary antibodies used in the Westerns were anti-mouse IgG HRP-conjugated (BD Biosciences Pharmingen, 554002), anti-rabbit IgG Peroxidase-conjugated (Sigma-Aldrich, Inc., A4914-1ML), and anti-goat IgG HRP conjugated (Santa Cruz Biotechnology, sc-2020)

The secondary antibodies used in the immunofluorescence experiments were as follows: goat anti-mouse AlexaFlour568 (Molecular Probes, A-11031), donkey anti-goat AlexaFlour568 (Molecular Probes, A-11057), donkey anti-rabbit AlexaFlour488 (Molecular Probes, A21206),

Chemicals: The nuclear stain used in immunofluorescence was 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes, D-1306). The mounting medium used in the immunofluorescence was DAKO Fluorescent Mounting Medium (DAKO Corporation, 53023).

Instruments & Software: A Nikon eclipse E800 fluorescent microscope was used for visualization of immunofluorescence. The AlphaEase®FC version 6.0.0 program from Alpha Innotech Corporation was used to quantify Western chemiluminescence. The
GraphPad Prism® version 4.01 from GraphPad Software, Inc. was used to evaluate statistical significance of the Westerns. ImageJ software was used to prepare immunofluorescence images (Rasband, 1997-2008).
Methods

Nuclear Extracts for Single Time Point: HeLa cells were grown in four 150 mm plates to 80-90% confluency. The conditions used in the experiment were mock-infection, Ad5 infection at a multiplicity of infection (m.o.i.) of five or Ad5 and AAV2 coinfection (5 m.o.i. Ad, 100 m.o.i. AAV). The infections were performed in 12 mL serum free MEM supplemented with 2 mM L-glutamine and after one hour 12 mL of 20% (v/v) FBS MEM supplemented with antibiotics and L-glutamine were added (no aspiration prior to adding medium). Twenty-four hours post-infection the medium was removed and the cells were scraped off the plate in 1x phosphate buffered saline (PBS) (pH 7.3, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4•7H2O, 1.4 mM KH2PO4) containing 5 mM MgCl2. The cells were pelleted at 1000×g for 5 min at 4°C. Nuclear extracts were made by one of the following methods described below: Challberg nuclear extraction, Dignam nuclear extraction, or STM-NP IPP nuclear extraction. The Challberg extraction is similar to the Dignam extraction method except that the Challberg extraction buffer contains a mild detergent.

Challberg Nuclear Extraction: Challberg nuclear extracts were made as described previously with some modifications (Challberg and Kelly, 1979). The pellet was resuspended in 50 mL in cold Wash Buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 0.5 mM MgCl2, 0.5 mM DTT, 0.2 M sucrose) and then pelleted at 1000×g for 5 min at 4°C. The pellet was resuspended in 7 mL of Resuspension Buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 0.5 mM MgCl2, 0.5 mM DTT, phosphatase inhibitor cocktail, 1 μM leupeptin, 1 μg/mL pepstatin A, 1 mM benzamidine, 1 mM PMSF) and incubated on ice for 10 min.
to allow swelling. The swollen resuspended pellet was homogenized with ten strokes of a tight fitting pestle in a Dounce homogenizer. The homogenate was pelleted at \( \sim 2800 \times g \) for 5 min at 4°C. This generated the nuclear pellet. The nuclear pellet was then resuspended in Extraction Buffer (50 mM HEPES [pH 7.5], 5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10% (v/v) glycerol, 400 mM NaCl, 0.1% (v/v) Tween 20, phosphatase inhibitor cocktail, 1 \( \mu \)M leupeptin, 1 \( \mu \)g/mL pepstatin A, 1 mM benzamidine, 1 mM PMSF) and rotated at 4°C for one hr. The extract was pelleted at \( \sim 16000 \times g \) for 20 min at 4°C. The supernatant (nuclear extract) was stored at -80°C.

**Dignam Nuclear Extraction:** Dignam nuclear extracts were made as previously described (Dignam et al., 1983). Cell pellets were resuspended in 5×pellet volume in cold Buffer A (10 mM HEPES [pH 7.75], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 1 mM PMSF) and incubated on ice for 10 min. The cells were pelleted at \( \sim 900 \times g \) for 10 min at 4°C. The supernatant was removed and the pellet with resuspended in 2.5×pellet volume in cold Buffer A. The cells were lysed in a Dounce homogenizer with 10 strokes (pestle A). The lysate was spun down at \( \sim 900 \times g \) for 10 min at 4°C. The supernatant was removed and the pellet was resuspended in 500 \( \mu \)L of cold Buffer A. The resuspended pellet was spun down at \( \sim 25,000 \times g \) for 25 min at 4°C. The supernatant was removed and the nuclear pellet was resuspended in 300 \( \mu \)L of cold Buffer C (20 mM HEPES [pH 7.75], 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF). The resuspended pellet was homogenized again and then rotated at 4°C for 30 min. The lysate was spun down at \( \sim 25,000 \times g \) for 30 min at 4°C. The supernatant (nuclear extract) was dialyzed for 5 hrs with 50×volume of Buffer D (20 mM HEPES [pH 7.75], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF).
Phosphatase inhibitor cocktail was added to the dialyzed extract. The nuclear extract was stored at -80°C.

**STM-NP IPP Nuclear Extraction:** Nuclear extracts were made as described previously with some modifications (Yang et al., 1992). Cell pellets were resuspended in 250 μL cold STM-NP Buffer (10 mM Tris-HCl [pH 8], 0.25 mM sucrose, 10 mM MgCl2, 0.5% NP-40, 0.1 mM DTT, phosphatase inhibitor cocktail, 1 μM leupeptin, 1 μg/mL pepstatin A, 1 mM benzamidine, 1 mM PMSF) and incubated on ice for 30 min. Nuclei were pelleted at ~500×g for 10 min at 4°C. The nuclear pellet was resuspended in 250 μL IPP Buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 20 mM EDTA [pH 8], 0.5% (v/v) Nonidet P 40 (NP-40), 1 mM DTT, phosphatase inhibitor cocktail, 1 μM leupeptin, 1 μg/mL pepstatin A, 1 mM benzamidine, 1 mM PMSF) and incubated on ice with periodic vortexing for 45 min. The lysate was pelleted at ~16000×g for 10 min at 4°C. The nuclear extract was stored at -80°C.

**Nuclear Extracts for Time Course:** HeLa cells were grown in 150 mm plates to 80-90% confluency. The conditions used in the experiment were Ad5-infection (5 m.o.i.) or Ad5 and AAV2 coinfection (5 m.o.i. Ad, 100 m.o.i. AAV). Several 150 mm plates of HeLa cells were used as a ‘mock’ infection control. The infection was performed in 12 mL serum free MEM supplemented with 2 mM L-glutamine and after one hr 12 mL of 20% (v/v) FBS MEM supplemented antibiotics and L-glutamine was added (no aspiration prior to adding medium). At the appropriate time post-infection the medium was removed and the cells were scraped off the plate in cold Hank’s Balanced Salt Solution (HBSS) (Hyclone, SH30030.02). The cells were pelleted at 1000×g for 5 min at 4°C. The cells were washed once with 5 mL cold HBSS and pelleted at 1000×g for 5
min at 4°C. Aspirated cell pellets were frozen on dry ice and stored at -80°C so that they were processed simultaneously. Nuclear extracts were made by the STM-NP IPP nuclear extraction method described above. The nuclear extract was stored at -80°C.

**Recombinant AAV Infections:** Each well of a 6 well dish, which contained HeLa cells at 80-90% confluency, was infected by one of the following conditions: mock infected, Ad infected (5 m.o.i.), AAV infected (100 m.o.i.), rAAVLuc<sub>env</sub> (100 m.o.i.), Ad and AAV coinfectected (5 m.o.i. Ad, 100 m.o.i. AAV), or Ad and rAAVLuc<sub>env</sub> coinfectected (5 m.o.i. Ad, 100 m.o.i. rAAVLuc<sub>env</sub>). The rAAVLuc<sub>env</sub> virus is a recombinant AAV virus that replaces the rep and cap genes with a firefly luciferase gene under a cytomegalovirus (CMV) early promoter. The infections were performed in 1mL serum free MEM supplemented with 2 mM L-glutamine and after one hour the medium was replaced with 10% (v/v) FBS MEM supplemented with antibiotics and L-glutamine. Eighteen hours post-infection the cells were scraped off the plate and pelleted at 2000×g for 5 min at 4°C. The cell pellet was washed once with 1 mL cold PBS + 0.5 mM MgCl₂ and spun down at 2000×g for 5 min at 4°C. Total cellular extracts were made. The cellular pellets were resuspended in 150 μL IPP Buffer with 25 mM DTT instead of 1 mM. The extract was sonicated using the Fisher Sonic Dismembrator (level 4, 1 sec pulses for 6 sec). The lysate was pelleted at ~16000×g for 10 min at 4°C. The pellet was discarded and the supernatant was stored at -80°C.

**Western Blots & Western Blot Analysis:** Equal concentration of proteins were run on a 10% SDS-PAGE gel (29:1 acrylamide:bis-acrylamide) and transferred to a nitrocellulose membrane (Immobilon- Fisher Scientific Co.). For ATM, p-ATM (S1981), Sp1, pRb, NBS1, and Rad50 Westerns, the nuclear extracts were run on 6% SDS-PAGE gels (49:1
acrylamide:bis-acrylamide) and transferred to a nitrocellulose membrane. Transferred proteins were Western blotted by standard procedure and detected by chemiluminescence. For phospho-specific primary antibodies, bovine serum albumin (BSA) instead of milk is used and Tris Buffered Saline (TBS) (pH 7.4, 25 mM Tris, 150 mM NaCl, 2 mM KCl) instead of PBS is used. The chemiluminescence reaction was detected by autoradiography film (Denville Scientific Inc., cat. # E3012) and scanned films were analyzed using the SpotDenso function of the AlphaEase® FC version 6.0.0 program (Alpha Innotech Corporation). The intensity of the band (represented as an IDV value) was normalized against mock-infected extracts. If more than one blot was done for a particular extract than the IDV values for that extract were averaged and the average intensity for that extract was used in the statistical analysis. GraphPad Prism was used to determine statistical significance using the unpaired t test of the difference in expression between Ad and AAV coinfected cell versus Ad infected cells. Percent differences were calculated by the following equation with (IDV = Integrated Density Value):

\[
\%\text{Difference} = \left( \frac{IDV_{\text{Ad} \& \text{AAV}} - IDV_{\text{Ad}}}{\text{Sum Intensity}_{\text{Ad}}} \right) \times 100\%
\]

**Immunofluorescence:** In each well of an 8-chamber slide (LAB-TEK Brand, Nalge Nunc International), \(0.75 \times 10^4\) HeLa cells were plated. Approximately 24 hrs later, the cells were infected mock-infected, Ad5 infected (10 m.o.i.), or Ad5 and AAV2 coinfected (10 m.o.i. Ad, 100 m.o.i. AAV) coinfected in 200 \(\mu\)L 2% (v/v) FBS MEM supplemented with 2 mM L-glutamine. After one hr infection, 200 \(\mu\)L 18% (v/v) FBS MEM supplemented with 2 mM L-glutamine is added (no aspiration prior to adding medium). Twenty to 24 hours post-infection, cells were washed twice with 400 \(\mu\)L PBS
and fixed for 10 min with 400 μL 100% cold methanol. The fixed cells were washed twice with 300 μL 2% (wt/v) BSA in PBS (BSA-PBS) and blocked for 20 min with 300 μL BSA-PBS. The slide chambers were removed leaving the gasket. The cells were incubated with 100 μL RIPA buffer (50mM Tris pH 8, 150mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% nonidet P40) for 10 min and then washed three times with 125 μL BSA-PBS. Primary antibody was diluted in 100 μL BSA-PBS and incubated for one hr at room temperature. After three washes in BSA-PBS, the secondary antibody was diluted in BSA-PBS and incubated for one hr at room temperature in the dark. After three washes in BSA-PBS, the cells were incubated in 150 nM DAPI in BSA-PBS for five min in the dark. The cells were washed three times with BSA-PBS and then three times with PBS. The gasket was removed and the coverslip was mounted with DAKO Fluorescent Mounting Medium. A Nikon eclipse E800 fluorescent microscope was used for visualization of co-localization. If a phospho-specific antibody was used for the primary than 2% BSA in PBS is replaced with 2% BSA in TBS in all steps.

**Concatamerization Experiment:** Each well of a 6 well dish, which contained HeLa cells at ~80% confluency, was mock infected, Ad infected (5 m.o.i. Ad), or Ad and AAV coinfectected (5 m.o.i. Ad, 100 m.o.i. AAV). The cells were harvested at 24 hrs post-infection. The cells were scraped off the plate and pelleted (400×g for 5 min at 4°C). The cell pellet was resuspended in 500 μL of cold PBS + 0.5 mM MgCl₂, the cells were pelleted (400×g for 5 min at 4°C), and the wash was removed. This wash was repeated once. Viral DNA was extracted using the Hirt extraction method (Hirt, 1967). The cell pellet was resuspended in 200 μL Hirt solution (10 mM Tris [pH 8.0]; 1 mM EDTA; 1% SDS) and 40 μg Proteinase K. After 3 hr incubation, 50 μL of 5 M NaCl was added to
the solution and incubated overnight on ice. The lysate was pelleted (16,100×g for 30 min at 4°C) and the pellet was discarded. The supernatant was treated with 5 μL of RNase cocktail (Ambion; 5 μg RNase A and 100 U RNase T1) and incubated at 2 hr at 37°C. The extract was subjected to a phenol:chloroform extraction and ethanol precipitation. To bring up the extract volume to 400 μL, 150 μL of Tris-EDTA (TE) (pH 8, 10 mM Tris, 1 mM EDTA) was added to the extract. The DNA was extracted with an equivalent volume of phenol:chloroform (1:1) (400 μL) and then again with an equivalent volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated with double the volume of ethanol (800 μL) and 40 μL of sodium acetate. The precipitated pellet was washed with 70% ethanol and spun down at 16,100×g for 30 min at 4°C. The viral DNA pellet was resuspended in 20 μL of TE (pH 8.0). Half of resuspended viral DNA was digested with Xba I (Promega). A Southern blot was used to determine the presence or absence of concatamerized viral DNA. The DNA was run on a 1% agarose gel, transferred to a nytran membrane, and crosslinked to the membrane by UV exposure (120 mJ on ‘auto cross link’ setting with Stratagene’s UV Stratalinker 1800). The membranes were prehybridized overnight at 42°C with hybridization solution (25 mM KPO₄, pH 7.4; 5X SSC [pH 7.0; 750 mM NaCl; 75mM Na₃-citrate•2H₂O]; 5x Denhardt’s solution [0.1% wt/v Ficoll 400, 0.1% wt/v polyvinylpyrrolidone, 0.1% wt/v bovine serum albumin]; 50 μg/mL salmon sperm DNA; 50% formamide). A 32P-labeled probe was added to the hybridization solution at ~5x10⁵ cpm of probe to 1 mL of hybridization solution and incubated at 42°C overnight. The membranes were washed twice with 1x SSC + 0.1% SDS at 45°C for 10 min and twice with 0.25x SSC + 0.1% SDS at 45°C for 10 min. The membranes were exposed to HyBlot Cl X-Ray film (Denville Scientific Inc., E3012).
Two probes were used for the experiment, which hybridized to two different regions of the Ad genome. The 3’ probe containing the E4 region of the Ad genome was made by digesting the pVAE4i plasmid with EcoR I (Promega) and Nco I (Promega) and gel extracting (Qiagen; QIAquick Gel Extraction Kit) the 1.6 kb fragment. The 5’ probe containing the $E1A$ region of the Ad genome was made by digesting the pJN20 plasmid with Pvu II (Promega) and Xba I (Promega) and gel extracting (Qiagen; QIAquick Gel Extraction Kit) the 714 b fragment. The pVAE4i plasmid contains the $VA$ and the $E4$ genes of Ad5. The pJN20 plasmid contains the $E1A$ gene of Ad5 (Jones et al., 1983). Both probes were made using a random hexamer labeling kit (Boehringer Mannheim).
Conclusion

1. Nucleophosmin (NPM, B23) interacts with AAV non-structural proteins Rep78 and Rep68.

2. Nucleophosmin increased binding of Rep78/68 to their consensus DNA binding sequence and also increased the DNA nicking activity of Rep78/68.

3. Nucleophosmin interacts directly or indirectly (through interaction with Rep proteins) with AAV structural capsid proteins.

4. Capsid proteins co-localized extensively with NPM within nucleoli and at other small foci within the nucleus. Rep proteins co-localized minimally with NPM early in the infection around the edges of the nucleoli.

5. Nucleolar remodeling occurred in Ad AAV coinfected cells where nucleoli were engorged and smaller nucleoli-like foci appeared.

6. Adeno-associated virus and Ad coinfection was not significantly different compared to adenovirus infection alone with regards to the major cell cycle proteins that were examined.

7. Adeno-associated virus and Ad coinfection resulted in an increase of a hyperphosphorylated form of Sp1 compared to adenovirus infection alone.

8. Adeno-associated virus and adenovirus coinfection induced a marked Rep-dependent DNA damage repair response compared to adenovirus infection alone.

9. Adeno-associated virus and adenovirus coinfection induced DNA damage repair response does not lead to adenovirus genome concatamerization.
Summary

Affinity chromatography was used as a screening tool to identify proteins that interacts Rep protein (Bevington et al., 2007). Nucleophosmin was one protein identified that was examined further. Immunoprecipitation experiments supported the Rep protein interaction (Bevington et al., 2007). Electrophoretic mobility shift assays as well as endonuclease assays were used to assess if there was any functional significance to this interaction. Indeed, NPM affected the enzymatic activity of Rep68/78. There was increased binding and nicking of the substrate by Rep68/78 in the presence of NPM. Furthermore, the immunoprecipitation experiments indicated an interaction between NPM and AAV capsid proteins either directly or indirectly in a complex with Rep proteins (Bevington et al., 2007). Adeno-associated virus capsid proteins co-localized extensively with NPM in nucleolar and small foci within the nucleus. The nucleoli appeared to be remodeled during an Ad and AAV coinfection where the nucleoli were enlarged and many small nucleoli-like foci appeared. This nucleolar enlargement has been described previously (Wistuba et al., 1997). These areas of co-localization between NPM and capsid proteins might represent viral assembly centers and/or genome encapsidation areas. Nash et al. recently reported on cellular proteins complexes containing Rep proteins during an AAV and Ad coinfection and also identified NPM in a complex with Rep proteins (Nash et al., 2009).

In addressing AAV effects on the cell cycle, the cell cycle and cell cycle-related protein levels during an AAV and Ad coinfection were assessed. There were no statistically significant changes in protein levels for Cdk1, Cdk2, Cdk4, Cdk6, Cdk7,
cyclin A, cyclin B1, cyclin H, PCNA, Cdc25A, and pRb. With regards to another Cdk-cyclin not involved in the cell cycle, Cdk9-cyclin T1, there was no statistically significant difference in protein level between AAV and Ad coinfection versus Ad infection. There was a shift in banding pattern for the Sp1 transcription factor that suggests increased modification of the protein with more of the Sp1 modified in AAV and Ad coinfection compared to Ad infection. Modified Sp1, by phosphorylation, has implications in either affecting its transcription activating potential of many promoters (including AAV promoters and cell cycle regulation promoters) and/or relation to activated DSB response pathway (Pereira and Muzyczka, 1997a; Pereira and Muzyczka, 1997b; Wierstra, 2008).

Understanding how AAV affects the DSB response in an AAV and Ad coinfection was addressed next. The MRN-complex, which is normally disrupted by Ad proteins, was activated by NBS1 phosphorylation at S343 to a greater extent in Ad and AAV coinfection compared to Ad infection. Also, this activated p-NBS1 was located in AAV and Ad replication centers, which allows access of the DSB response to viral DNA. Other down-stream proteins also alluded to the activation of the DSB response with increased phosphorylation of ATM at S1981 and the appearance of phosphorylated Chk2 at T68 in AAV and Ad coinfected HeLa cells compared to Ad infected HeLa cells. Again, the activated p-ATM localized to AAV and Ad replication centers. Consistent with what is seen with activated p-Chk2 localization during DSB repair in which it is localized throughout the nucleus (Bekker-Jensen et al., 2006; Lukas et al., 2003); during an AAV and Ad coinfection p-Chk2 T68 localized in the nucleus to both areas of AAV and Ad replication centers as well as areas outside AAV and Ad replication centers. Furthermore, comparing Ad coinfection with wild type AAV or with rAAVluc, the
activation of the DSB response was related to Rep expression in an AAV and Ad coinfection. This is consistent with what Berthet et al. observed, where activation of ATM and Chk2 in cells transfected with a plasmid expressing Rep78 occurred (Berthet et al., 2005). Finally, although the DSB response was activated there was no concatamerized Ad viral DNA detected during an Ad and AAV coinfection; concatamerized Ad genome occurs when cells are infected with a mutated Ad that is defective in disruption of the DSB response (Boyer et al., 1999; Stracker et al., 2002). Activation of the DSB response may still be a contributory method for AAV mediated decrease in Ad replication during a coinfection since earlier reports indicate that activation of the DSB response regardless of Ad genome concatamerization still disrupted Ad replication (Berk, 2007; Evans and Hearing, 2003; Mathew and Bridge, 2007).
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adeno-associated virus Rep protein binding site in the adenovirus E2a promoter. J


Abstract

Adeno-associated virus (AAV) is a small single-stranded linear DNA virus that requires a helper-virus to efficiently replicate inside a host cell. The cellular effects of an AAV coinfection with its helper-virus, adenovirus (Ad), were assessed. Affinity column chromatography and crosslinking experiments identified an interaction between the cellular nucleolar protein, nucleophosmin (NPM, B23), with the AAV replicative proteins, Rep68 and Rep78. Increased substrate binding and nicking by Rep68/78 in the presence of NPM was observed using Rep protein functional assays with bacterially expressed proteins. Immunofluorescence studies demonstrated extensive co-localization between NPM and AAV capsid proteins at nucleoli and at small foci within the nucleus. Co-localization was minimal between Rep proteins and NPM during early infection occurring mostly in and around the edge of the nucleoli. Capsid proteins were pulled out with Rep and NPM proteins in crosslinking experiments indicating either a direct or indirect interaction between capsid proteins and NPM. With AAV and Ad having potentially antagonizing effects on the cell cycle, the cellular effects of an AAV and Ad coinfection were examined. The preliminary results indicated that there were no statistically significant changes in protein levels for the cell cycle and cell cycle-related proteins that were examined when comparing Ad infection to Ad and AAV coinfections. There was an increase in the amount of phosphorylated Sp1 in an Ad and AAV coinfection compared to Ad infection. Whether this is related to Sp1 activation of AAV promoters is unknown. With AAV infection affecting expression of Ad proteins involved in disrupting the cellular DSB response, the effects of an AAV and Ad
coinfection on the DSB response were examined. Increased amounts of the active phosphorylated form of the DSB response proteins, NBS1, ATM, and Chk2, were observed in an AAV and Ad coinfection compared to Ad infection. This activation was induced by the presence of intact AAV virus and not due to increased viral DNA. Concatamerization of the Ad viral DNA was not detected during an AAV and Ad coinfection, which although unexpected does not preclude the possibility that AAV activation of the DSB response contributes to decreased Ad viral replication.