Replication of adeno-associated virus in murine fibroblasts with mouse adenovirus provided helper functions

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FINAL APPROVAL OF DISSERTATION
Doctor of Philosophy in Biomedical Sciences

Replication of Adeno-Associated Virus in Murine Fibroblasts with Mouse Adenovirus Provided Helper Functions

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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REPLICATION OF ADENO-ASSOCIATED VIRUS IN MURINE FIBROBLASTS WITH MOUSE ADENOVIRUS PROVIDED HELPER FUNCTIONS

VIPUL BHRIGU

UNIVERSITY OF TOLEDO, COLLEGE OF MEDICINE

2008
DEDICATION

I dedicate my dissertation to my family especially to my wife, parents, brother and my wife’s parents for their support and encouragement all through my life and my graduate school.
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I sincerely thank my major advisor Dr. James P. Trempe for his support, guidance and encouragement. His scientific acumen, work ethic and balance between work and family will be a constant source of inspiration and guidance throughout my career. I would also like to thank my committee members Dr. Ivana de la Serna, Dr. William Gunning, Dr. Manohar Ratnam, Dr. Randall Worth and Dr. Han-Fei Ding for their guidance and support during my graduate career. I thank Dr. Roy Collaco for his patience, constant support and guidance and for his help with the DNA repair experiments. I would also thank all the past and present members of our laboratory for maintaining a collegial and interactive environment in the laboratory. I extend my appreciation toward all the members of the Department of Biochemistry and Cancer Biology. Last but not the least I thank my family and friends whose love and support has encouraged me through all my academic pursuits.
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INTRODUCTION

Adeno Associated Virus (AAV) is a non-pathogenic, single-stranded DNA parvovirus. It has been classified in the genus Dependovirus because it needs a helper virus for productive replication (Atchison et al., 1965). The most common helper virus for AAV is adenovirus (Ad) (Hoggan et al., 1966) however human cytomegalovirus (CMV) (McPherson et al., 1985), Herpes Simplex Virus (HSV)(Buller et al., 1981), Human Papilloma Virus (HPV) (Walz et al., 1997) and Epstein Barr Virus (EBV) can provide helper functions as well. In the absence of helper virus, AAV establishes a latent infection in cells (Cheung et al., 1980) by preferential integration in the long arm of chromosome 19 (Kotin and Berns, 1989; Kotin et al., 1992; Samulski et al., 1991) at a site known as AAVS1. A 34 bp sequence (Giraud et al., 1994; Linden et al., 1996) located in the first exon of the myosin binding subunit 85 of protein phosphatase 1 has been shown to be the minimal AAVS1 element required to target AAV DNA at this chromosomal position. Helper virus infection can rescue integrated AAV DNA from this site for a productive replication of AAV. During coinfection, adenovirus early gene products facilitate AAV DNA replication, transcription and translation (Chang et al., 1989; Grifman et al., 1999; Samulski and Shenk, 1988). Helper virus independent replication of AAV has also been reported by a small percentage of cells when cells are synchronized by treatment with hydroxyurea, polyamine depletion and mitotic detachment (Yakobson et al., 1987). Treatment of cells with genotoxic agents such as UV, chemical carcinogens, heat shock and metabolic
inhibitors of DNA and protein synthesis also made cells semipermissive for AAV DNA replication (Yalkinoglu et al., 1988).

The helper functions provided by adenovirus are the most extensively characterized. Microinjection of mRNA molecules indicated that only the adenovirus early functions help AAV replicate (Richardson et al., 1980). Genetic analysis of adenovirus mutants has shown that E1A and E1B is required for AAV transcripts to be detected and AAV DNA to replicate (Laughlin et al., 1982; Richardson and Westphal, 1984). E1A pushes cells into S phase of cell cycle thereby promoting the synthesis of cellular DNA replication proteins which in turn help AAV DNA replication. E1B and E4 of adenovirus help transport of AAV mRNA to the cytoplasm from nucleus (Samulski and Shenk, 1988). Adenovirus E4 ORF 6 protein has also been shown to promote conversion of single stranded genome of AAV vectors to double stranded (Ferrari et al., 1996).

AAV serotype 2 (AAV2) has a single-stranded DNA genome which is 4680 nucleotide long (Srivastava et al., 1983). Both ends of the AAV genome have a 145 nucleotide long inverted terminal repeat (ITR) (Lusby et al., 1980) encoding the viral origin of replication (Senapathy et al., 1984) and genome packaging signal. The AAV genome has two translational open reading frames (ORF). The right ORF encodes three structural capsid proteins as a result of alternative splicing (VP1, VP2, and VP3) (Tratschin et al., 1984; Trempe and Carter, 1988a) and the left ORF encodes four non-structural proteins (Rep78, Rep68, Rep52, and Rep40) (Mendelson et al., 1986). The Rep mRNAs are under the control of transcriptional promoters at map unit 5 and 19 (p5 and p19) whereas Cap
mRNAs are under the control of a transcriptional promoter at map unit 40 (p40) (Hermonat et al., 1984). The p5 promoter regulates the expression of Rep 78 and 68, whereas p19 promoter regulates expression of Rep 52 and 40. Expression of all three Cap proteins is under the control of promoter at map unit 40. Rep proteins have pleiotropic effects in the life cycle of AAV. In the absence of helper virus Rep 78 negatively regulates gene expression, DNA replication and is required for site specific integration to establish a latent infection (Beaton et al., 1989; Labow et al., 1986; Samulski, 1993; Tratschin et al., 1986; Weitzman et al., 1994; Young et al., 2000). In the absence of helper virus very little amount of Rep 78 is synthesized. Rep 78 then binds to the promoter at map unit 5 and represses further transcription (Kyostio et al., 1995). When cells are co-infected with adenovirus, its E1A protein transactivates promoter at map unit 5 there by increasing the synthesis of Rep 78. Rep 78 now functions as a transactivator (Labow et al., 1986) of AAV gene expression and induces transcription from the promoters at all the three map units that is 5, 19 and 40 (Pereira et al., 1997). Apart from regulating the gene expression from its own promoters Rep 78 also regulates expression from a number of heterologous promoters (Labow et al., 1987). It has been suggested that Rep binding to the E2a promoter of adenovirus contributes to the inhibition of the E2a gene expression and may affect Ad replication (Casper et al., 2005). Rep mediated inhibition of tat mediated reporter gene expression has been reported (Antoni et al., 1991). Rep binds to HIV LTR and inhibits HIV-1 gene expression and replication. Rep mediated H-ras inhibition occurs by binding of Rep on its promoter (Batchu et al., 1994), thereby
leading to down regulation of H-ras expression. Rep inhibits HPV16 mediated oncogenic transformation by binding to HPV16 p97 promoter (Zhan et al., 1999).

AAV infects a wide range of cells and tissues from a variety of species. However, a large number of non-permissive cell types have also been identified. Even amongst the cells that allow infection, the efficacy of infection differs. A wide range of factors can influence successful infection/transduction of AAV in target cells. For example, cells must first express AAV receptors and co-receptors on their surface for efficient binding of the virus. Heparan sulfate proteoglycan has been identified as the main receptor for AAV-2 (Summerford and Samulski, 1998). In addition fibroblast growth factor receptor 1 (FGFR) (Qing et al., 1999) and αvβ5 (Summerford et al., 1999) have been proposed to be co-receptors required for internalization of virus. After binding, the virus enters the cell via clathrin coated vesicles (Bartlett et al., 2000), is transported to the nucleus (Sanlioglu et al., 2000) and uncoated. The released single stranded genome of AAV undergoes second strand synthesis to generate a transcriptionally active genome (Ferrari et al., 1996; Qing et al., 1997).

The conversion from single stranded DNA to double stranded form is facilitated by host cellular DNA polymerase (Hauswirth and Berns, 1977). There is no evidence of RNA primers or Okazaki fragments during the replication of AAV DNA. Rather the inverted terminal repeat at the ends of the genome forms a hairpin and provides a 3’ free hydroxyl group for cellular DNA polymerase for the synthesis of second strand. The double stranded DNA molecule has a closed 3’ end and is referred to as replicative form monomer (RFm). At the end of the
hairpin, AAV DNA has a 16 base pair sequence which is a tandem repeat of (GAGC)_4 and facilitates binding of two to six molecules of Rep78 to AAV DNA (Dignam et al., 2007; McCarty et al., 1994; Ryan et al., 1996; Snyder et al., 1993). Once bound to DNA Rep 78 nicks AAV DNA by its helicase and endonuclease activity (Brister and Muzyczka, 1999) and this enzymatic activity of Rep produces a new 3’ end for repair synthesis and this process is called as terminal resolution. Either end can then denature and reanneal to form a double hairpinned structure referred to as replicative form dimmer (RF_d) (Ni et al., 1994). The single stranded genome is then formed by strand displacement mechanism from the newly formed 3’ hairpin structure. The single stranded genome is then packaged into the preassembled capsids. The Rep 78 protein remains attached to the encapsidated genome (Prasad et al., 1997) and is removed during virus isolation procedures or upon reinfection.

Although AAV is considered to be non-pathogenic, it inhibits proliferation of transformed cells (Batchu et al., 1999), and represses transformation of mouse fibroblasts by heterologous oncogenes (Khleif et al., 1991). AAV infection inhibits cell cycle progression (Berthet et al., 2005), causes cell death in p53 negative cells (Raj et al., 2001), promotes differentiation and alters expression of several cell-cycle regulated (Winocour et al., 1988) genes. Effects of AAV replication are not limited to the host cell alone; AAV inhibits replication of its helper viruses as well. During co-infection AAV decreases adenovirus DNA replication 2-20 fold (Jing et al., 2001; Timpe et al., 2006). AAV also decreases adenovirus cytotoxicity and production. These AAV effects on host cell and helper virus
come from studies in cell culture. Given the nature of the effects of AAV on host cells and helper virus, and the interest in the use of AAV as vector for gene therapy because of its non-pathogenicity and low immunogenic effects it is essential that effects of AAV on host and helper virus in an animal be studied in detail. To this end we decided to study the effects of AAV replication on mouse adenovirus (MAV) in murine fibroblasts and to characterize MAV helper functions for efficient replication of AAV.

The genome organization of mouse adenovirus (MAV) is similar to that of the human adenovirus (Weber et al., 1994). Further, MAV E1A contains all three conserved regions found in human adenovirus E1A protein, similar to human adenovirus E1A. MAV E1A interacts with cellular proteins such as pRb, p107, (Smith et al., 1996) p130 and Sur2 (Fang et al., 2004) and plays a major role in antagonizing the antiviral effects of interferons (Kajon and Spindler, 2000). These similarities make MAV an excellent model to study AAV, host and helper virus interactions.

As a prelude to studying replication of AAV in a mouse model we analyzed the dynamics of AAV infection in murine fibroblasts with helper functions provided by MAV. We observed that the replication of AAV in presence of MAV was almost 30-100 fold less in NIH 3T3 cells as compared to replication in Ad-5 infected Hela cells. Although the receptor binding of AAV for both cell lines seems comparable, the decreased replication of AAV in 3T3 cells was due to the inefficient transport of AAV from plasma membrane to the nucleus because AAV particles were observed in the perinuclear space and AAV DNA was detected in
cytosolic fraction after infection. Previous studies have shown that in 3T3 cells, transport of rAAV to the nucleus is impaired (Hansen et al., 2000). In our study we observed same phenomenon with wt AAV in the presence of a helper virus though it has been reported that helper virus facilitates the transport of AAV (Xiao et al., 2002). Even after transfection of MAV infected 3T3 cells with plasmid containing the genome of wt AAV (pNTC244) (Laughlin et al., 1983) AAV replication was almost 5 fold less as compared to replication in Ad-5 infected Hela cells. This indicates that in addition to a defect in transport, 3T3 cells do not support replication of AAV DNA as well as Hela cells.
LITERATURE REVIEW

Adeno-associated viruses (AAV) are non-pathogenic members of the Dependovirus genus of the Parvoviridae family. The Parvoviridae family includes small, non-enveloped DNA viruses that contain linear single stranded genomes. Parvoviruses are amongst the smallest of the animal DNA viruses. The virion has a diameter of 18-26nm and is composed entirely of protein and DNA. Each paroviral virion contains a genome of 4-6 kb in size which ends in short palindromic sequences.

Paroviridae consists of two subfamilies a) parovirinae which infects vertebrates and b) densovirinae which infects insects. The parovirinae family is further classified into three genera a) Autonomous parovirus, b) Dependovirus and c) Erythovirus. The autonomous parovirus family includes bovine parovirus, feline panleukopenia virus, H-1 parovirus and Minute Virus of Mice (MVM). Erythovirus includes simian parovirus and B19 parovirus etc. Dependoviruses are unique among animal viruses in that they need an unrelated helper virus such as adenovirus, herpesvirus or human papilloma virus etc. (Muzyczka, 2001) for their productive replication. In the absence of helper virus members of this genus establish a persistent latent infection. However, it has been demonstrated that under certain conditions, such as the treatment of cells with (a) topoisomerase inhibitors such as etoposide or camptothecin (Yalkinoglu et al., 1991), (b) mutagens such as ultra violet radiation (Yakobson et al., 1989) and γ radiation (Alexander et al., 1996), (c) DNA synthesis inhibitors such as hydroxyurea and aphidicolin, protein synthesis inhibitors such as cyclohexamide,
chemical carcinogens, like N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) or heat shock (Bantel-Schaal, 1993; Yalkinoglu et al., 1988), replication of virus can be detected in the absence of helper virus. The complete cycle of helper independent AAV replication also ensues after transfection of hydroxyurea pretreated cells with cloned AAV DNA (Yakobson et al., 1987). A schematic of AAV life cycle is depicted in fig. 1.

**Biphasic Life cycle of AAV**

AAV life cycle in absence or presence of helper virus. The left panel shows that in absence of helper virus AAV infects a cell and integrates its genome (solid blue bar) into chromosome 19. The right panel shows during coinfection with helper virus AAV genome (solid blue bar) and adenovirus genome (solid black bars replicate, lyse the cell and are released.
AAV is a prototype dependovirus which is considered to be non-pathogenic since no disease has been associated with AAV yet. AAV was first identified as a contaminant of adenoviral stocks (Atchison et al., 1965). Dependoviruses also includes avian AAV, bovine AAV, canine AAV, and equine AAV. Eight different serotypes of AAV have been identified (AAV1-8) (Casper et al., 2005; Chiorini et al., 1999; Chiorini et al., 1997; Dawn E. Bowles, 2006; Muramatsu et al., 1996; Rutledge et al., 1998). Recently 53 new AAV capsid DNA sequences have been identified (Gao et al., 2003; Gao et al., 2004; Gao et al., 2002) and cloned from non-human primates and 64 from human tissues. Amongst these members of adeno-associated virus serotype 2 (AAV-2) is the most widely studied and characterized member. AAV infects humans of all ages (During and Leone, 1995) and approximately 85% of the adult population of United States is seropositive for AAV. Although AAV infections appear to be widespread, it has not been implicated with any pathological disease as yet and its normal tissue tropism is poorly defined.

Human target tissues of AAV have not yet been identified however, since it has been found as a contaminant adenovirus in isolates it is thought that it is acquired with adenovirus infection via respiratory tract or gastrointestinal tract. Presence of AAV has been reported in peripheral blood leukocytes (Grossman et al., 1992); when peripheral blood leukocytes propagated in tissue culture were infected with helper adenovirus AAV replication was detected. AAV sequences were also detected by PCR from samples taken from the genital tracts of women suspected of having HSV infection (Friedman-Einat et al., 1997). Wild type AAV
genomes were also found in 14% of muscle biopsy specimens (Tezak et al., 2000): 17% of normal human muscle and 10% of Duchenne muscular dystrophy muscle from a broad age (6months to 43 years) range of population. In this study, AAV messenger transcripts could not be detected thus it was assumed that skeletal muscles serve as reservoir for latent infection of AAV.

A transplacental infection for AAV has been established (Lipps and Mayor, 1980). AAV-1 was detected in the kidneys and lungs of fetuses and newborns, when pregnant mice were injected subcutaneously with AAV type 1 and murine adenovirus as a helper virus.

Based on the reports that in vitro infection of fertilized mouse eggs induced arrest of development at 2 cell stage and in vivo AAV2 infection of pregnant mice led to fetal death and early abortion, studies were conducted to analyze whether AAV may be found in human genital tissue or in curettage material from spontaneous abortion (Botquin et al., 1994; Tobiasch et al., 1994). It was observed that (a) AAV infects the uterine mucosa (possibly persistently) and (b) it can replicate in trophoblast cells. Thus, it might disturb placenta development and may play a role in early miscarriage. However, later in a separate study (Friedman-Einat et al., 1997), AAV sequences were detected only in samples taken from genital tracts of women suspected of having HSV infection. No evidence of AAV replication was found in maternal or embryonal tissues from the first trimester of pregnancy.
**AAV Virion**

The AAV virion consists of a protein coat and a single stranded DNA genome and has a diameter of 20 nm. The virion has an icosohedral symmetry and requires assembly of 60 individual structural proteins. The AAV capsid is made up of three structural proteins that are named VP1 (87Kd) VP2 (73Kd) and VP3 (62Kd), which are present in a ratio of 1:1:10, respectively. The capsid proteins do not appear to be glycosylated but are phosphorylated at tyrosine residues by EGFR-PTK (Zhong et al., 2007). Synthesis of AAV capsid proteins is required during accumulation of single stranded genomes (Tratschin et al., 1984); deletion in the cap gene lead to inhibition of single stranded DNA progeny accumulation. The assembly of AAV capsid takes place in the nucleus. During the later stages of infections Rep, Cap and AAV DNA co-localize in replication centers in the nucleus where the assembly and encapsidation of genome occurs (Timpe et al., 2005). The actual roles of the individual capsid proteins in the assembly process and the absolute requirements for each in the formation of a functional virus particle are unclear. Mutations in the C-terminal region common to all three proteins abolishes virion formation and fails to accumulate single-stranded DNA (Ruffing et al., 1994). Deletion of the N-terminal region unique to the VP1 protein leads to assembly of low-infectivity particles (*lip* phenotype) (Wu et al., 2000) and does not alter amounts of replicating forms of AAV DNA (Tratschin et al., 1984). The *lip* phenotype is caused by the loss of phopholipase activity in the region unique to the VP1 protein of AAV (Girod et al., 2002). There is evidence that expression of either of the less abundant proteins, VP1 or VP2 is
necessary for capsid assembly (Ruffing et al., 1992). Coexpression of VP1 and VP2, VP2 and VP3, and all three capsid proteins and the expression of VP2 alone in Sf9 insect cells resulted in the production of virus like particles resembling empty capsid generated during infection of HeLa cells with AAV-2 and adenovirus. Furthermore individual transfection of AAV capsid protein expressing plasmids showed that VP1 and VP2 localized in the nucleus whereas VP3 was distributed between cytosol and nucleus. However, when VP3 was coexpressed with other structural proteins, its localization to the nucleus was altered indicating that association of VP3 with VP1 or VP2 is required for efficient nuclear transport of VP3. These results suggest a requirement for VP2 in the formation of empty capsids. The VP3 protein does not have a nuclear localization signal unlike VP1 and VP2 (Hoque et al., 1999). Deletion of the N-terminal region of VP2 indicated that the nuclear localization signal of VP2 is located in this region and that the major function of VP2 is translocation of VP3 into the nucleus. However, fusion of SV40 large T antigen nuclear localization signal to VP3 protein leads to formation of virus like particles when VP3 is expressed alone. Recently, it has been demonstrated that VP3 alone is sufficient to form a particle capable of protecting the viral genome (Warrington et al., 2004), VP1 is required for efficient viral infectivity, and VP2 is nonessential. Further, the stoichiometry of the particle can be altered if VP2 is significantly over-expressed in the presence of native levels of VP1 and VP3.
AAV Genome

The AAV-2 genome is a single stranded DNA which is 4679 nucleotides long (Srivastava et al., 1983). AAV encapsidates strands of both polarity with equal frequency. The AAV genome has an inverted terminal repeat of 145 bases at either end of the genome (Lusby et al., 1980). The first 125 bases of these inverted repeats can fold upon themselves to generate a hairpin configuration or a T-shaped structure (Muzyczka, 2001). The inverted terminal repeats in fact consist of three palindromes, one long (nucleotides 42 to 84) and two short (nucleotides 1 to 41 and 85 to 125). In this configuration, only seven bases remain unpaired. Six are required to allow the internal palindromes to fold over and the seventh separate the two internal palindromes. The longer palindrome forms the stem of the T-shaped structure and is designated as the ‘A’ stem. The cross arms of the T-shaped structure are formed by the two shorter palindromes and are designated as ‘B’ and ‘C’ palindromes.

The hairpin T-shaped structure can exist in two orientations referred to as “flip” or “flop”. These orientations result from the inversion of the terminal 125 bases during AAV DNA replication and also from the fact that the two smaller palindromes B and C are not identical (Muzyczka, 2001). A diagram of AAV inverted terminal repeats is depicted in figure 2.
Diagram of AAV inverted terminal repeat.

Schematic of AAV Inverted terminal repeat (Dawn E. Bowles, 2006). RBE represents Rep binding element (sequence in the rectangular box). Trs represents terminal resolution site. A, B, C, A’, B’ and C’ are different palindromes as described in the text.

These inverted terminal repeats serve as the viral origin of replication and the signal for the packaging of the genome into capsid. In the newly released virions 5’ end of the genome remains covalently attached to a copy of the viral Rep78/68 protein which is located on the outside of the particle. The genome has 4 direct repeats of the tetra nucleotides (5’-GAGC-3’) in the viral hairpins and p5 promoters for the binding of Rep78/68 proteins (Chiorini et al., 1995; McCarty et
al., 1994). Rep78/68 via binding to these sequences and regulates viral DNA replication and gene transcription (Tratschin et al., 1986).

AAV 2 has been the basis to derive the AAV genetic map. The map of AAV genome is divided into 100 map units. AAV genome encodes for two different open reading frames. The right open reading frame is called the Capsid (Cap) gene and encodes for three structural proteins, and is located between map positions 40 to 90. The structural proteins are called VP1, VP2 and VP3 with molecular weights of 87Kd, 73Kd and 63Kd respectively. The messenger RNA for these proteins is under the control of a promoter at map position 40 (p40). The frameshift and deletion mutations in this region do not block DNA replication, but the accumulation of progeny single stranded DNA is inhibited (Tratschin et al., 1984). As mentioned earlier, mutations in the N-terminal region of the Cap open reading frame that affect VP1 exclusively lead to packaging of the genome but the infectivity of the particles produced is much lower.

The left open reading frame is called the Replication (rep) gene and encodes for four nonstructural proteins under the control of promoter at map unit 5 and 19 (p5, p19) (Mendelson et al., 1986). Frameshift mutations or large deletions in this gene inhibit AAV DNA replication. The promoter at p5 is responsible for the transcript that gives rise to proteins Rep78, and Rep68 by alternative splicing. Mutation in the p5 promoter region that inhibits synthesis of Rep78 and Rep 68 (Labow et al., 1986) leads to complete inhibition of all AAV promoter driven transcription and AAV DNA replication. The transcripts under the control of promoter at p19 synthesize Rep52 and Rep40 by alternative splicing
Selective elimination of transcripts from p5 promoter (Rep78 and Rep68) inhibits all AAV–directed mRNA accumulation and totally inhibits AAV DNA replication. Mutations that inhibit the expression of transcripts for the p19 promoter (Rep52 and Rep40) allows AAV DNA to replicate but no mature single stranded viral DNA is encapsidated (Chejanovsky and Carter, 1989). Mutations in Rep and Cap open reading frames can be complemented by providing these genes in trans (Beaton et al., 1989). However, the 145 bases of the inverted terminal repeats are required in cis for viral DNA replication and transcription (Senapathy et al., 1984). Inverted terminal repeats are also required for encapsidation of the AAV genome (McLaughlin et al., 1988), integration of genome during latent infection (Yang et al., 1997) and rescue of the genome from an integrated state (Samulski et al., 1983).

**AAV Life Cycle**

Once the virus enters the cell, AAV infection proceeds either towards a lytic life cycle or latent infection depending upon the presence of a helper virus. The role of the helper virus appears to alter the intracellular milieu such that it becomes more conducive for AAV replication. As mentioned earlier if the intracellular environment is altered by stress or by treatment with genotoxic agents, AAV replication can be achieved even in the absence of the helper virus. Irrespective of the pathway AAV chooses, it must pass through the initial stages of the life cycle which involve:

1) Binding to a cell surface receptor, internalization and trafficking from the cell membrane to the nucleus
2) AAV DNA replication

3) AAV protein expression

**Receptor binding, internalization and trafficking**

As for most viruses, infection with AAV is initiated by binding to a cell surface receptor. Heparin sulfate proteoglycan (HSPG) has been identified as the primary receptor for AAV2 (Summerford and Samulski, 1998). Soluble heparin can compete for binding of AAV2 on the cell surface. Heparin can also compete for AAV3 binding to cells, implying that AAV2 and AAV3 may share HSPG as an attachment receptor (Handa et al., 2000; Rabinowitz et al., 2002). The abundance of membrane associated HSPG is one of the critical aspects determining the efficacy of AAV infection in various cell types (Duan et al., 1998). The low abundance of the HSPG on the apical surface of airway epithelial cells has been implicated in the inefficient transduction by the AAV in the lungs. In addition to attachment receptors, AAV also requires coreceptors for efficient infection. One of the coreceptors for AAV2 is fibroblast growth factor receptor 1 (FGFR1) (Qing et al., 1999) and its expression in conjunction with HSPGs has been shown to enhance AAV2 transduction. Integrins are a class of molecules several viruses use to attach to and infect cells. AAV2 is reported to bind to $\alpha_v\beta_5$ integrin, an interaction important for virus internalization (Summerford et al., 1999). AAV2 internalization and transduction were inhibited by anti-$\alpha_v\beta_5$ antibody competition and dramatically increased in CS-1 cells engineered to express $\alpha_v\beta_5$.

In contrast to HSPG for AAV2 and AAV3, sialic acid has been identified as a primary attachment receptor for AAV4 and AAV5 (Kaludov et al., 2001; Walters...
et al., 2001). AAV5 uses primarily N-linked sialic acid for binding, whereas AAV4 preferentially uses O-linked sialic acid for attachment. Platelet derived growth factor receptor (PDGFR) was identified to be coreceptor for AAV5 using bioinformatics (Di Pasquale et al., 2003). Its role as a coreceptor was further confirmed by transfection of a PDGFR expressing plasmid, inhibitor treatment and competition inhibition experiments that altered both viral binding and transduction. Furthermore PDGFR itself is a sialo-glycoprotein containing both N- and O- linked oligosaccharide chains with sialic acid (Daniel et al., 1987; Hosang, 1988) raising the possibility that PDGFR may be capable of acting alone as a receptor for AAV5.

The attachment receptors for AAV1 and AAV6 have not yet been identified, however, they do not appear to involve HSPG (Halbert et al., 2001; Negishi et al., 2004).

Following receptor attachment, AAV enters the cell by endocytosis. The mechanisms of AAV endocytosis have been investigated using fluorescent tagged AAV virions (Bartlett et al., 2000). Cell entry studies for AAV2 and AAV5 have demonstrated particles in clathrin coated vesicles. Using Cy3-labeled AAV particle (Cy3AAV), it was demonstrated that AAV internalizes rapidly by standard receptor-mediated endocytosis from clathrin-coated pits (half-time <10 min) (Bartlett et al., 2000). The lysosomotropic agents ammonium chloride and bafilomycin A₁ prevent AAV-mediated gene transfer when present during the first 30 min after the onset of endocytosis, indicating that AAV escapes from early endosomes yet requires an acidic environment for penetration into the cytosol.
Furthermore the same study demonstrated that expression of dominant negative dynamin inhibited endocytosis of Cy3AAV. However, it did not prevent binding of virus to the cell surface indicating that internalization of AAV occurs by endocytosis.

In a manner similar to ligand and receptor interactions, binding of AAV2 to its coreceptor αvβ5 integrin triggers intracellular signaling pathways that stimulate receptor endocytosis. Integrins are associated with multiple small intracellular signaling molecules, including Rho, Rac and CdC-42, GTPases through which actin fibers facilitate motility and the endocytic pathway (Nobes and Hall, 1995; Parsons, 1996). Accordingly, it was demonstrated that internalization of heparan sulfate proteoglycan-bound AAV2 requires αvβ5 integrin and activation of the small GTP-binding protein Rac1 (Sanlioglu et al., 2000). Following endocytosis, activation of a phosphatidylinositol-3 (PI3) kinase (PI3K) pathway was necessary to initiate intracellular movement of AAV2 to the nucleus via both microfilaments and microtubules. Inhibition of Rac1 using a dominant N17Rac1 mutant led to a decrease in AAV2 mediated PI3K activation, indicating that Rac1 may act proximal to PI3K during AAV2 infection. It was further demonstrated that inhibition of PI3K pathways in Hela cells did not appear to influence the internalization of virus. However, the movement of AAV from cytosol to nucleus was significantly impaired when cells were treated with PI3K inhibitors. These studies suggest that Rac1 and PI3K pathways are linked and both appear to affect distinct stages of AAV transduction.
Further it has been demonstrated that the endocytic pathway of rAAV2 is dependent on dynamin (Duan et al., 1999), a GTPase protein involved in clathrin-mediated internalization of receptors and their ligands from the plasma membrane. Using a recombinant adenovirus expressing a dominant-inhibitory form of dynamin I (K44A), it was demonstrated that rAAV2 infection is partially dependent on dynamin function. Overexpression of mutant dynaminI significantly inhibited AAV-2 internalization and gene delivery, but not viral binding.

Recently, the Notch1 signaling pathway was also shown to be involved in augmenting intracellular trafficking of AAV2 (Ren et al., 2007). RNA profiling of human prostate cancer cell lines with various degrees of AAV transduction indicated a correlation between Notch1 levels and rAAV transgene expression. It was shown that in the absence or low-level expression of Notch1, only binding of virus was found on the cell surface and internalization was impaired. However, increased Notch1 expression in these cells allowed efficient perinuclear accumulation of Cy3AAV capsids. Dynamin levels were not found to be different among these cell lines, but blocking dynamin function abrogated AAV2 transduction in DU145 clones overexpressing full-length Notch1.

Following endocytosis, processing of AAV virions through the endosomal compartments is intricately linked to transduction. It is known that canine parvovirus (CPV) directly injected into the cytoplasm of cells fail to replicate (Vihinen-Ranta et al., 1998). CPV treated at pH 5.0 prior to microinjection was unable to initiate virus production, showing that factors of the endocytic route other than low pH are necessary for the initiation of infection by CPV. Similarly
when directly injected, Alexa568 labeled rAAV2 failed to accumulate in the nucleus of Hela cells (Ding et al., 2005). Acidification inside the late endosomal and lysosomal compartments also appear to be another potential endosomal event critical for AAV transduction (Bartlett et al., 2000; Douar et al., 2001). When the acidification of the endosomal compartment is inhibited by bafilomycin A1, transduction by rAAV2 is significantly decreased.

Vesicle trafficking of AAV is a complex process that leads to particle deposition in many cell locations. In the lung, introduction of AAV2 particles has been described in end stage proteosomes and the nucleus. It was observed that despite the absence of HSPG and other coreceptors for AAV2 the decreased efficacy of AAV2 transduction from the apical surface of airway cells was not due to viral uptake (Duan et al., 2000). Instead, reduced transduction was attributed to the ubiquitination of the AAV2 capsids following endocytosis, which reduced nuclear trafficking of AAV2 particles. Treatment of cells with proteasome inhibitors and ubiquitin ligase (E3) inhibitors significantly increased efficacy of AAV gene delivery. Proteasome inhibitors also increased rAAV-2-mediated gene transfer to the liver ten fold, but they did not affect transduction of skeletal or cardiac muscle (Yan et al., 2002). These findings suggest that tissue-specific ubiquitination of viral capsid proteins interferes with rAAV-2 transduction. Further ubiquitin conjugation of both AAV-2 and AAV-5 capsids was observed by western blot of immunoprecipitated viral capsid proteins from infected HeLa cell lysates. It was also observed that heat-denatured virus particles were preferential
substrates for *in vitro* ubiquitination, suggesting that endosomal processing of the viral capsid proteins is a prelude to ubiquitination.

Thus it appears that following uptake from the cell surface addition, of proteosome inhibitors shifts the trafficking pathway to increase AAV2 in the nucleus. It is generally believed that AAV particles are transported into the nucleus prior to uncoating since fluorescent labeled AAV virions are observed in the nucleus. However, nuclear transport of AAV appears to be a slow and inefficient process. The translocation into nucleus is augmented by proteosome inhibitors (Douar et al., 2001; Yan et al., 2004). The addition of adenovirus proteins is also reported to increase nuclear entry of AAV2 and also may act by changing the trafficking of AAV through the cell (Xiao et al., 2002). This facilitated nuclear translocation of AAV was not blocked by the nuclear pore complex inhibitor thapsigargan. Indeed, coinfection with empty adenovirus capsids also resulted in the appearance of AAV DNA in the nuclei within 40 minutes.

Not much is known about the processes that control nuclear translocation of AAV. It has been suggested that AAV particles could efficiently enter the nucleus of both highly and poorly infectious cell types in a time and temperature dependent manner (Hansen et al., 2001) but is not saturable and seems to occur independently of the nuclear pore complex. The nuclear localization signal in VP2 capsid protein is believed to be important for *de novo* capsid assembly (Hoque et al., 1999) but its role in transport of intact AAV particles to the nucleus is not known. Qiu et al. have demonstrated that a 110-kDa human nucleolin protein was copurified with AAV2 virions. It was demonstrated that nucleolin
bound to intact AAV capsid but not to denatured viral proteins and colocalization of nucleolin was observed both in cytoplasm as well as in the nucleus (Qiu and Brown, 1999).

**AAV DNA Replication**

When cells are coinfected with AAV and helper virus, AAV DNA replication occurs via a single stranded displacement mechanism (Hauswirth and Berns, 1977). There is no evidence of RNA primers or Okazaki fragments being provided for the initiation of AAV DNA replication. Upon uncoating the incoming AAV genome is single stranded and forms identical hairpins at either end due to the inverted palindromic repeats. The 3’-OH end from these hairpins serve as a primer for full length extension by cellular DNA polymerase (Muzyyczka, 2001). The product of this extension is a full length duplex genome with one end in a closed hairpin conformation. This replication intermediate is also referred to as replicative form monomer (RF$_m$). The closed hair pinned end has a single copy of the inverted terminal repeat whereas the open end has two copies, one on each strand. The closed end is then nicked by Rep78/68 endonuclease activity near the end of the terminal palindrome. Nicking is followed by synthesis from the newly created 3’ terminus so that the inverted terminal repeat is copied. This process is called “terminal resolution” and the strand specific nicking site is called the “terminal resolution site”. The end result of this process is conversion of closed hairpin to a duplex open end with one copy of the inverted terminal repeat on each strand. Each end of the duplex genome can fold back upon itself generating a new free 3’-OH end that initiates leading strand displacement.
synthesis to generate a single-stranded genome (which is packaged) and a
duplex genome that is closed at one end. The process of terminal resolution,
strand displacement and synthesis is repeated again. Duplex dimers are created
if extension from a 3'-OH terminus on a genome with a closed hairpin at its other
end, reaches that closed hairpin before it has been terminally resolved.
Extension will continue through the hairpin, resulting in a dimer of two inverted
copies of the AAV genome with one duplex copy of the inverted terminal repeat
at the junction. This intermediate form is called as replicative form dimer (RF$_d$
)(Figure 3). This model of AAV DNA replication was originally proposed by Straus
and Ward in two different studies (Straus et al., 1976; Tattersall and Ward, 1976).

A large amount of experimental evidence supports this model of AAV DNA
replication. All of the predicted replicative form intermediates have been found in vivo and can be generated with in vitro replication systems. Using baculovirus-
expressed Rep78, 68 and 52 in vitro replication of AAV DNA has been
demonstrated (Ni et al., 1998; Ni et al., 1994). It was observed that with AAV
DNA as the starting substrate, the replication reaction required an adenovirus-
infected cell extract and the presence of either Rep78 or Rep68. Rep52,
however, did not support DNA replication. A mutation of the AAV terminal
resolution site was defective for DNA replication in the in vitro assay. Uninfected
HeLa extracts were less efficient in supporting AAV DNA replication than
adenovirus-infected extracts. The reduced ability of uninfected HeLa extracts to
support complete DNA replication was not due to a defect in terminal resolution
rather due to a defect in the reinitiation reaction or in elongation.
In vitro experiments demonstrated that in the extracts prepared from human cells that have been infected with both adenovirus and AAV, covalently joined ends of linear AAV DNA can be resolved (Snyder et al., 1990). The reaction is initiated by a site-specific and strand-specific endonucleolytic cut at a terminal resolution site near the end of the AAV terminal palindrome. During resolution, the orientation of the terminal palindrome is inverted, and the 3’ viral strand is extended by DNA synthesis. These experiments thereby provided direct
evidence of the terminal resolution step which is an essential step in the model for AAV DNA replication. It has also been demonstrated that purified Rep68 protein contains a site-specific and strand-specific endonuclease activity that specifically cuts the AAV origin at the terminal resolution site (TRS) (Im and Muzyczka, 1990). The TRS endonuclease activity requires the presence of ATP for activity and becomes covalently attached to the 5’ end at the cut site. In addition to the specific endonuclease activity, Rep68 also contains a DNA helicase activity. These studies have provided direct evidence that larger AAV Rep proteins are directly involved in AAV DNA replication. Furthermore it was demonstrated that a purified maltose binding protein/Rep68 (MBP-Rep68) fusion protein has DNA-DNA helicase activity similar to purified Rep68 protein (Wonderling et al., 1995). The fusion protein was also shown to have DNA-RNA helicase property. The helicase activity requires the hydrolysis of ATP which is carried out by Rep68 itself; mutation of the putative nucleotide binding domain inhibited both helicase and ATPase activity of MBP–Rep68.

Mutational analysis of the AAV terminal repeat has demonstrated that it contains at least three specific sequences that direct the Rep mediated terminal resolution endonuclease reaction (McCarty et al., 1994). Rep68 binds to a linear DNA sequence that is contained within a 25-bp sequence of the stem of the adeno-associated virus (AAV) terminal repeat T structure. The binding was further confirmed by demonstrating that Rep68 could specifically bind to a synthetic oligonucleotide containing the 25-bp region in the absence of the other sequences within the terminal repeat. This sequence, the Rep binding element
(RBE) within the stem of the T structure is sufficient to bind Rep78/68 complex that contains 2 to 6 molecules of Rep protein (Dignam et al., 2007). Most of the RBE consists of a tandem (GAGC)$_4$ repeat. One of the functions of the RBE is to assemble the Rep complex in proper orientation (Brister and Muzyczka, 2000) that is subsequently required for terminal resolution site endonuclease activity. The RBE orientation appears to align Rep on the AAV terminal repeat, allowing specific nucleotide contacts and directing nicking to the terminal resolution site. Alterations in the polarity or position of the RBE relative to the terminal resolution site significantly inhibit Rep nicking. Rep contacts with the RBE appear necessary for both the DNA helicase and terminal resolution site cleavage steps of the endonuclease reaction.

Once bound to the terminal repeat, Rep carries out two sequential reactions. Firstly the DNA helicase activity of Rep unwinds the stem of the terminal repeat in a reaction that requires ATP hydrolysis and generates a single stranded terminal resolution site. In the second step of the reaction, the endonuclease active site carries out a transesterification reaction, in which the tyrosine residue at amino acid position 156 of Rep is covalently linked to the 5' phosphate end of the nick at terminal resolution site (Davis et al., 2000). The minimal terminal resolution sequence necessary for Rep cleavage is 5'-GT/TGGCC-3', and this 7-base core sequence is required only on the nicked strand (Brister and Muzyczka, 1999). The confirmation that ATP is not required for endonuclease activity came from studies (Snyder et al., 1993), demonstrating that Rep nicks a variety of substrates in an ATP independent manner provided
that the RBE is double stranded and the terminal resolution site is single stranded. The Rep protein remains bound to the 5' end of the nick through subsequent steps of AAV DNA replication and remains attached during packaging of the progeny strand. By immunoprecipitation analyses, it was observed that Rep78 protein is covalently attached to viral DNA in a preformed virion (Prasad and Trempe, 1995). The positioning of Rep78, outside the virion was confirmed by the observation that Rep78 was susceptible to antibody binding and protease digestion, and the DNA linkage was susceptible to nuclease digestion.

The enzymatic requirement of Rep78/68 protein has been confirmed by the mutational analysis of Rep proteins. The C-terminal domain of Rep78 that is also present in Rep52/40 contains the Rep protein associated DNA helicase activity (Smith and Kotin, 1998). It was observed that purified MBP-Rep52 possesses 3'-to-5' DNA helicase activity that is strictly dependent upon the presence of nucleoside triphosphate and divalent cation cofactors. Furthermore the fusion protein was also observed to have a constitutive ATPase activity that was active in the absence of DNA effector molecules. Furthermore it was demonstrated that after deletion of C-terminal, Rep78 protein lacked the helicase and ATPase activity but retained the ability to bind RBE and to nick single stranded terminal resolution (Davis et al., 2000).
Host Cellular Factors Involved in AAV DNA Replication

Evidence from in vitro reconstitution replication assays showed that DNA polymerase alpha was not required for AAV DNA replication (Ni et al., 1998). However, the presence of replication protein A (RPA), proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) was required for replication. A requirement for PCNA indicated that DNA polymerase delta or epsilon is involved in the elongation of the leading strand during replication of AAV DNA. Reconstitution of an in vitro reaction with one or both of these polymerases did not restore AAV DNA replication indicating a requirement of additional cellular proteins (Muzyczka, 2001).

Fractionation of adenovirus infected crude extracts to detect the enzymes involved in AAV DNA replication revealed that fractions that contained replication factor C (RFC) and proliferating cell nuclear antigen (PCNA) were essential for reconstituting AAV DNA replication (Nash et al., 2007). These could be replaced by purified PCNA and RFC to retain full activity. Fractions containing polymerase delta, but not polymerase epsilon or alpha, were capable of replicating AAV DNA in vitro. Requirement of DNA polymerase delta was further confirmed by the use of purified polymerase delta complex from baculovirus expression clones. Further characterization of factors required to reconstitute efficient AAV DNA replication in vitro also revealed the requirement of minichromosome maintenance (MCM) complex, a cellular helicase complex that is believed to be the replicative helicase for eukaryotic chromosomes (Nash et al., 2008a). Interfering RNAs targeted to MCM and polymerase delta inhibited AAV DNA replication in vivo,
suggesting that one or more components of the MCM complex and polymerase delta play an essential role in AAV DNA replication *in vivo* as well as *in vitro*.

One of the cellular proteins required for AAV DNA replication is high mobility group 1 protein (HMG1) (Costello et al., 1997). HMG1 physically interacts with Rep protein, promotes the formation of Rep-DNA complexes and stimulates the activity of Rep protein in site- and strand-specific cleavage of DNA and the hydrolysis of ATP, functions essential for AAV DNA replication.

Interaction of AAV Rep68 and two members of 14-3-3 proteins (14-3-3 epsilon and gamma) were identified while determining for Rep68 interacting partners, by one step affinity purification (Han et al., 2004). Phosphorylation of 535Ser at the carboxy terminus of Rep68 was critical for its association with 14-3-3. The association of 14-3-3 proteins to Rep68 resulted in a reduction of the affinity of Rep68 for DNA. Furthermore, DNA replication of a recombinant mutant virus carrying a phosphorylation-deficient Rep68 (Ser535Ala) was more efficient than that of the wild-type virus suggesting that Rep’s interaction with 14-3-3 may affect AAV DNA replication.

Another cellular factor involved in an interaction with AAV DNA is a protein that specifically binds to one strand of the D sequence within the AAV terminal repeats; hence it is referred to as single-stranded DNA binding protein (ssD-BP). This protein is phosphorylated on a tyrosine residue in response to signaling pathway from epidermal growth factor receptor (EGFR) (Mah et al., 1998; Qing et al., 1997; Smith et al., 2003). When bound by the phosphorylated form, the input single stranded AAV genome is unable to synthesize the second strand to form
the first replicative intermediate. The protein, which appears to be 52-kDa FK506 binding protein, FKBP52, binds to the 10 bases of the D sequence proximal to the terminal resolution site and thus prevents DNA replication. Inhibition of the phosphorylation of FKBP52 using EGFR inhibitors dramatically increases the transduction of cells by recombinant AAV (rAAV) vectors in the absence of the helper virus. This is because the input vector single stranded DNA must synthesize the second strand to produce a substrate for RNA polymerase II transcription.

In further efforts to identify Rep interacting partners, affinity tagged Rep protein was used to purify cellular protein complexes that were associated with Rep protein in cells that had been infected with Ad and AAV (Nash et al., 2008b). In all, 188 cellular proteins from 16 functional categories, including 14 transcription factors, 6 translation factors, 15 potential splicing proteins, 5 proteins involved in protein degradation, and 13 proteins involved in DNA replication or repair were identified. Computer analysis indicated that 45 of these proteins could be placed in a pathway of interacting proteins involved in DNA replication. Furthermore, the interaction of Rep protein with Ku70/80 helicase was confirmed. In vitro DNA synthesis assays demonstrated that although Ku helicase activity could substitute for MCM to promote strand displacement synthesis, its presence was not essential.

**AAV Gene Expression**

During a productive infection in the presence of a helper virus, AAV genes are transcribed and six transcripts are discernible by northern blotting. These
transcripts are responsible for both structural and non-structural proteins and are under the control of three conventional RNA polymerase II promoters. These promoters are located at map units 5, 19 and 40 (p5, p19 and p40) (Muzyczka, 2001). These promoters control synthesis of six capped polyadenylated mRNA transcripts of size 4.2kb, 3.9kb, 3.6kb, 3.3kb, 2.6kb and 2.3kb. The p5 promoter synthesizes 4.2kb and 3.9 kb mRNAs which are translated into Rep78 and Rep68 proteins for the unspliced and spliced transcript respectively. Two transcripts under the control of p19 are of size 3.6kb (unspliced) and 3.3kb (spliced) and translate Rep52 and Rep40 proteins, respectively. The transcription map of AAV is depicted in figure 4.

**AAV genome and transcriptional map**

![Diagram of AAV genome and transcriptional map](image)

Inverted Terminal Repeats are represented at the either end of genome. Closed circles p5, p19 and p40 represent promoters at map unit 5, 19 and 40 respectively. rep represents the nonstructural rep gene while cap represents structural cap gene. Open boxes represent transcripts for non-structural proteins (Rep). Hatched boxes represent transcripts for structural proteins (Cap).
The smallest 2.3kb transcript is most abundant and under the control of the p40 promoter. The 2.3 kb species consists of two alternatively spliced mRNAs. The major spliced mRNA translates to either VP2 or VP3 capsid protein depending upon the start codon used. It directs the synthesis of the major capsid protein VP3, from a conventional AUG codon at nucleotide position 2809 and the minor capsid protein, VP2, from a 195 nucleotide upstream unconventional in-frame ACG start codon. The minor spliced 2.3kb message includes an upstream AUG codon that allows the translation of the entire Cap open reading frame to produce the minor capsid protein VP1 (Trempe and Carter, 1988a). The role of unspliced 2.6kb transcript is not known.

Kinetic studies have demonstrated that there is a temporal order in accumulation of AAV transcripts during infection (Mendelson et al., 1986; Mouw and Pintel, 2000; Trempe and Carter, 1988b). First, unspliced p5-generated transcripts, which encode Rep78, are detectable prior to the significant accumulation of other AAV RNAs. Ultimately, p19-generated products accumulate to levels greater than those generated from p5, and p40-generated transcripts predominate in the total RNA pool. In addition, the percentage of each class of AAV RNA that is spliced increased during infection, and the degree of this increase is different for the p5/p19 products than for those generated by p40. At late times post coinfection, approximately 90% of p40 products, but only approximately 50% of RNAs generated by p5 and p19, are spliced. It appears that each of the AAV RNAs are quite stable; the majority of each RNA species persists 6 hours after treatment with actinomycin D. Quantification of the
accumulation of individual AAV RNAs, over intervals during which degradation is negligible, demonstrates that at late times during infection the relative strength of p5, p19, and p40 is approximately 1:3:18, respectively, consistent with the steady-state accumulated levels of the RNAs generated by these promoters.

AAV RNAs generated from all three viral promoters (p5, p19 and p40), contain the same intron, which when excised, utilizes a single splice donor site and either one of two splice acceptor sites. Coinfection of cells with a helper virus stimulates splicing of mRNA (Mouw and Pintel, 2000) but this occurs only in the presence of AAV Rep protein. In the absence of helper virus, splicing of both p19 and p40 transcripts is very poor. AAV2 Rep78/68 proteins can act to increase the ratio of spliced to unspliced AAV RNA when they are targeted to the transcription template via a Rep binding element (RBE) (Qiu and Pintel, 2002). The requirement for RBE is both location and orientation independent; however, Rep enhancement of splicing decreases as the distance between the promoter and the intron of the affected transcription unit increases. The RBE can be provided either by the terminal repeats or the p5 promoter. The increase in splicing however requires the presence of adenovirus gene products.

**Sequence Elements and Transcription Factors in AAV Gene Expression**

The sequences responsible for regulation of AAV gene expression have been extensively characterized. It was observed that the activity of the AAV p5 promoter was induced by adenovirus E1A gene products. A pair of adjacent sequence elements was found to mediate both basal and E1A-induced p5 activity (Chang et al., 1989). The first element is a binding site for the major late
transcription factor (MLTF) while the second element is a tandemly repeated 10-base-pair sequence that binds the cellular transcription factor YY1. Computer analysis demonstrated that sequences allowing binding of Rep78/68 referred to as Rep binding element (RBE) are present within the all three AAV promoter regions. Furthermore RBE within the p5 promoter is located between the YY1 initiator sequence and the TATA binding site (McCarty et al., 1994). Identification of this element suggested that Rep78 could act as repressor or transactivator of the p5 promoter by interaction with YY1 or TBP. The two YY1 binding sites are located upstream of the TATA box and overlapping with p5 message start site. The Rep binding element was also observed in p19 promoter. The presence of Rep binding elements upstream of both promoters suggested that these sites may be involved in coordinated regulation of AAV transcription.

In a bid to identify transcription factors that bind the p19 promoter, interaction between with the cellular factor Sp1 at positions -50 and -130 relative to p19 transcription start site was observed (Pereira and Muzyczka, 1997b). Additionally, an unknown cellular protein referred to as cellular AAV activating protein (cAAP) with an approximate molecular mass of 34 kDa was found to interact with an element at position -140. Mutational analysis of the p19 promoter suggested that the Sp1 site at -50 and the cAAP site at -140 were necessary to mediate Rep78 mediated activation of the p19 promoter. On the basis of interaction of the Rep78 with Sp1 and with p19 promoter, Rep’s ability to transactivate the p19 promoter likely involves Sp1-Rep protein contacts facilitating Rep interaction with the p19 DNA sequence.
Mutations of the p40 promoter elements showed that two Sp1 sites at approximately -50 and -70 base pairs upstream and the TATA box at -30 of the start of the p40 messages are necessary for maximal promoter activity (Pereira and Muzyckza, 1997a). Sequences for the binding of transcription factors ATF-80 and AP1-40 were identified. Mutation of these elements resulted in a modest decrease in p40 transcription, but DNA binding experiments did not clearly demonstrate binding of transcription factors to these sites. Thus, in the p40 promoter the Sp1 sites and the TATA box are essential for transcriptional regulation. Furthermore, ATF and AP1 may also play a role in regulation of p40 promoter.

During AAV infection in the absence of helper virus coinfection only a very small amount of Rep78 is synthesized from the p5 promoter. This is sufficient to bind to the p5 RBE and completely repress further p5 transcription (Beaton et al., 1989). The negative regulation of p5 promoter by Rep78 was discovered when the p5 and p19 promoters of the AAV2 genome were fused to reporter genes and the constructs were used to transfect HeLa cells. These promoters functioned constitutively but were repressed in trans by the AAV rep gene product. This repression was relieved by adenovirus infection. It was also observed that transfection of increasing amounts of the wild-type rep gene in trans from a heterologous promoter inhibited p5 and p19 mRNA accumulation from a plasmid containing the entire AAV genome with an amber mutation in the rep gene (Kyostio et al., 1994). Cotransfections with plasmids producing individual wild-type Rep proteins in trans demonstrated that p5 and p19 mRNA accumulation
was inhibited 5- to 10-fold by Rep78. It was also observed that mutation of the RBE in the p5 promoter within a full length AAV genome abolished Rep binding and resulted in a 40-50% reduction in ability of the Rep68 to inhibit p5 transcripts \textit{in vivo} (Kyostio et al., 1995). It has been reported that mutations of the p5 RBE can lead to higher expression of p5 transcripts and thereby make some cell lines partially permissive for AAV DNA replication (Wang and Srivastava, 1998). Although there is no strong RBE within the p19 promoter, Rep52 is capable of repressing p19 transcription (Kyostio et al., 1994). This suggests that binding to the RBE at the p5 promoter is not the only mechanism by which Rep proteins can repress transcription. The evidence for transcriptional repression by Rep proteins using a mechanism other than binding to RBE also comes from the study wherein effects of the four Rep proteins on the accumulation of mRNA transcribed from the human papillomavirus type 18 upstream regulatory region (HPV18 URR), the human immunodeficiency virus long terminal repeat, and the AAV2 p5 and p19 promoters by transient transfection experiments in HeLa cells were analyzed (Horer et al., 1995). It was observed that Rep78 showed a greater than 10-fold inhibition of the four promoters studied, transcriptional repression mediated by Rep68 and Rep52 was reduced and nearly completely abolished for Rep40. These promoters do not contain any RBE and mutational analysis demonstrated that several different \textit{cis}-acting elements were needed for inhibition by Rep78 proteins. These findings suggested that transcriptional repression is mediated by protein-protein interactions of the Rep proteins with multiple transcription factors. Such interactions of Rep proteins have been
reported with proteins such as Sp1 (Hermonat et al., 1996), TATA binding protein (TBP) (Hermonat et al., 1998) and PC4 (Weger et al., 1999). Cytomegalovirus (CMV) promoter driven overexpression of PC4 led to the transient accumulation of non-phosphorylated PC4 with concomitant downregulation of all three AAV2 promoters in the absence of helper virus (Weger et al., 1999). However, in the presence of adenovirus, this effect was relieved implying an involvement of the PC4 in the regulation of AAV2 gene expression in the absence of a helper virus. Although there is an active repression of p5 and p19 promoter activity by the Rep proteins, repression of the p40 promoter has not been demonstrated. Yet, there is little accumulation of p40 transcripts or capsid protein in the absence of adenovirus infection.

**Regulation of AAV Gene Expression during Coinfection with Adenovirus**

In the presence of adenovirus coinfection a cascade of transcriptional regulation rapidly induces synthesis of all AAV genes. Firstly the adenovirus E1a transactivator protein induces transcription from the p5 promoter (Chang et al., 1989). The transcriptional activation of the p5 promoter is brought about by displacing Rep78 protein from p5 RBE by adenovirus E1a or YY1 protein. Once displaced from p5 RBE, Rep78/68 now acts like a transcriptional activator rather than a repressor and induces transcription from the p5 and p19 promoters. Frameshift mutations within the p5 or p19 gene severely inhibit synthesis and accumulation of all AAV transcripts. Furthermore the defective accumulation of transcripts could be complemented in trans by transfection of an AAV plasmid lacking the capsid region (Labow et al., 1986). The dependence of p40
transcription on the Rep-responsive elements within the p5 and p19 regions could be overcome by the presence of the AAV terminal repeats (McCarty et al., 1991), suggesting that the terminal repeats contained redundant Rep-responsive elements. Also, in the presence of adenovirus the p19 Rep proteins that do not bind to the RBE can eliminate repression of the p5 promoter by Rep78 and Rep68 (Pereira et al., 1997). This possibly occurs by the association of Rep52 with Rep78 or Rep68 to produce a Rep78/68-Rep52 protein complex which can be detected in vivo by immunoprecipitation.

In the presence of adenovirus, the p5 RBE represses p5 transcription while the RBE in the terminal repeat activates p5 (Pereira et al., 1997). However, both the terminal repeat RBE and the p5 RBE transactivate the p19 and p40 promoters. Thus, to act as a transactivator, Rep apparently needs to be bound to the RBE in the terminal repeats as the absence of the RBE from the terminal repeats reduces transactivation of p19 and p40 promoters (Muzyczka, 2001). Binding of Rep at the RBE in the terminal repeats is also required to derepress transcription from the p5 promoter. Thus presence of Rep at RBE in terminal repeats transactivates p5 as well as p19 and p40 promoter.

The p19 transcription requires p5 or terminal repeat RBE, as well as the p19 Sp1 site at position -50 and the cAAP site upstream of p19 promoter (Pereira and Muzyczka, 1997b). Transcription of p40 requires the elements similar to that for p19 transactivation and the proximal -50 site upstream of the p40 transcription start site (Pereira and Muzyczka, 1997a).
Unspliced AAV RNAs from all three promoters can accumulate in the cytoplasm at high levels. During adenovirus infection, the adenovirus E1B55k-E4ORF6 complex inhibits the transport of cellular mRNA from nucleus to cytoplasm and promotes the transport of viral mRNA (Pilder et al., 1986). The same complex is also responsible for the timely cytoplasmic accumulation of AAV RNA during coinfection (Samulski and Shenk, 1988).

**AAV Genome Encapsidation**

The assembly of empty AAV capsids is relatively rapid, while insertion of the single stranded genome is much slower. Empty capsids are the predominant intracellular particles during infection. In the absence of capsid accumulation, single stranded AAV genomes do not accumulate (Myers and Carter, 1980). Immunofluorescence experiments have demonstrated that during late stages of coinfection with AAV and adenovirus all three capsid proteins colocalized with the Rep proteins and AAV2 DNA in nuclear replication foci (Wistuba et al., 1997). This suggests that AAV DNA replication and encapsidation may occur concurrently in the nucleoplasm. As mentioned earlier, the AAV Rep protein is covalently attached to the AAV DNA and remains attached even during packaging. Rep domains responsible for Rep-Cap interaction involve amino-acid 322-482 (Dubielzig et al., 1999) and it was observed that Rep52 and, to a lesser extent, Rep78 are most abundantly recovered with capsids. These complexes are thought to represent intermediates in the DNA packaging pathway. It was further demonstrated that Rep52 helicase activity was required for AAV DNA insertion into the capsid (King et al., 2001). Insertion occurred from 3’ end of the
genome and the authors proposed that the capsid-immobilized helicase functions as a molecular motor to pump single stranded DNA into the capsid (King et al., 2001).

It has been observed that by using AAV replicative-form DNA as the substrate, it is possible to synthesize an infectious AAV particle \textit{in vitro} (Zhou and Muzyczka, 1998). The packaging procedure required the presence of both the AAV Rep and capsid proteins. Two kinds of \textit{in vitro} products were formed which facilitated DNA transfer. Both were resistant to heat and had a density in cesium chloride gradients that was indistinguishable from that of the in vivo-synthesized wild-type virus. This indicated that the particles formed had the appropriate protein-to-DNA ratio and a structure that shared the heat resistance of mature AAV particles.

\textbf{AAV Latent Infection and Rescue}

AAV needs a helper for its DNA replication and productive infection. In the absence of a helper virus, AAV undergoes a latent infection. The latent infection gives AAV an advantage whereby it persists for a long time in the host and awaits for the arrival of a helper virus to carry out its replication. There appear to be at least three mechanisms by which the AAV genome can persist in host cells. The first is site specific integration into the host DNA at AAVS1 on chromosome 19. The second mode of persistence is non-specific integration. The third mechanism involves the persistence of AAV DNA as episomal concatamers in the nucleus.
Initially it was thought that AAV integration was random with respect to cellular DNA. It is now clear that integration occurs at a specific site on human chromosome 19q13.2-qter. Integration occurs by non-homologous recombination, although there are 4-5 base homologies at the site of recombination and integration is often associated with rearrangements and inversions of both the viral DNA and the cellular sequences (Kotin et al., 1992; Kotin et al., 1991; Kotin et al., 1990). Although integration is site specific, it is not specific at an individual nucleotide but occurs within a range of several hundred nucleotides (Samulski et al., 1991). The pre-integration site and the region of integration is referred to as adeno-associated virus integration site 1 (AAVS1). A gene proximal to the integration site codes for the slow skeletal muscle troponin T gene (TNNT1) (Dutheil et al., 2000).

It has been observed that the pre-integration site contains a Rep binding element (RBE) and an abbreviated version of the terminal resolution sequence. Mutation of either the RBE or the putative terminal resolution site dramatically reduces site-specific integration (Linden et al., 1996). It was observed that transfected plasmids containing the rep gene and a selectable marker flanked by the AAV inverted terminal repeats integrated site specifically at AAVS1 (Kearns et al., 1996; Ponnazhagan et al., 1997) whereas those that did not have the rep gene did not integrate in chromosome 19. This study indicated a role of Rep proteins in site-specific integration. Rep68 can bind to a plasmid containing a fragment of the pre-integration site (Weitzman et al., 1994) and can also serve as a bridge between the AAV terminal repeat and the chromosome 19 RBE. Thus, in
in vitro, Rep can bring the chromosome 19 target site and AAV genome together through Rep-Rep contacts. Rep can both nick a terminal resolution sequence and ligate the nicked DNA to another sequence in vitro (Smith and Kotin, 2000). In vitro nicking and joining ability of Rep has been demonstrated in multiple studies, further providing biochemical evidence for the role of Rep in site-specific integration.

Evidence suggests that the N-terminal domain of Rep78/68 is required for targeted integration (Cathomen et al., 2000; Yoon-Robarts and Linden, 2003; Yoon et al., 2001). Firstly the Rep52/40 proteins, which are missing the N-terminal 224 residues of Rep78/68, are unable to mediate targeted integration into the AAVS1. Secondly, the DNA binding and endonuclease domains are both located in the N-terminal region of Rep78/68 protein and this is the region that interacts with viral RBE both in vitro and in vivo. Thirdly, charged-to-alanine mutations of the N-terminal half of the Rep78/68 demonstrated that all the mutations affecting DNA binding and/or nicking resulted in a complete loss of targeted integration (Urabe et al., 1999). It is thought that cellular enzymes are responsible for the recombination and Rep is essential for site specificity (McLaughlin et al., 1988). The evidence in favor of this argument comes from the observation that efficiency of Rep-minus integration can be as high as 80% and appears to depend on the cell.

The elements required in cis for integration are not well characterized. Interestingly it has been demonstrated that the viral terminal resolution sequence within the inverted terminal repeat is not required for AAV site specific integration
An AAV vector plasmid consisting of a reporter gene flanked by the viral inverted terminal repeat with a mutated terminal repeat sequence was able to mediate targeted integration at the same frequency as plasmids carrying a wild type inverted terminal repeat in presence of the Rep78 protein. It has been demonstrated that a 138-bp cis element, the p5 integration efficiency element (p5IEE), mediates efficient integration (Philpott et al., 2002a; Philpott et al., 2002b). It was concluded that the p5IEE is not only required for efficient site-specific integration, it is also sufficient because integration mediated by the p5IEE occurred in the absence of the AAV inverted terminal-repeat elements as well. A better understanding between different functions of the p5 promoter revealed that the elements of p5IEE that define its role as a promoter also define its function as a highly optimized substrate for Rep mediated site specific integration (RMSSI) and replication (Murphy et al., 2007). The p5 RBE is essential for RMSSI; however replacement of RBE with the AAV inverted terminal repeats did not enhance or compromise RMSSI. On the other hand manipulations of the p5 TATA/RBE/YY1 compromised RMSSI. Thus it has been proposed that, depending on Rep expression and the presence or absence of the helper virus, the p5IEE operates as a transcription or integration switch sequence element.

The targeted integration of AAV is directed by AAVS1 and the critical sequences are located within 500 base pairs (Giraud et al., 1994). This region of AAVS1 contains sequences that are similar to the viral terminal resolution sequence and RBE (Weitzman et al., 1994). It has been demonstrated that a 33
nucleotide sequence containing the terminal resolution sequence and RBE at AAVS1 is necessary and sufficient for integration of AAV at AAVS1 (Linden et al., 1996). Furthermore, for integration to occur, AAVS1 DNA should have the proper topology and, along with the presence of AAVS1 terminal resolution sequence and RBE, proper sequence and position of spacer elements separating them (Lamartina et al., 2000; Meneses et al., 2000).

Rescue of AAV can be defined as the induction and packaging of wild type AAV by supplying helper functions to a cell containing AAV genome in either the chromosomally integrated or episomal state. After rescue, encapsidated AAV genomes and infectious AAV particles are produced from latently infected cells. It was demonstrated that infection of Detroit 6 cells with adenovirus, 67 passages after AAV infection resulted in production of infectious AAV2 particles (Berns et al., 1975).

AAV rescue can be triggered by either helper virus super infection, a wide variety of DNA damaging agents or by transient inhibition of DNA replication. Studies with a cloned duplex form of AAV genome in a pBR322 vector have helped elucidate the mechanism involved in rescue of integrated genome (Laughlin et al., 1983). Expression of Rep proteins is necessary during rescue. Mutations and deletions in different regions of the inverted terminal repeats has demonstrated that the D sequences (Wang et al., 1995; Wang et al., 1996) increased the efficiency of rescue and replication of the AAV genome. Furthermore, if the inverted terminal repeat closest to the p5 promoter is deleted, the RBE in p5 promoter acts as the excision site leading to AAV rescue.
Helper virus and AAV interactions

Adenovirus, herpes simplex virus (HSV), human papilloma virus etc. all serve as complete helpers for AAV replication (Atchison et al., 1965; Buller et al., 1981; Ogston et al., 2000). However, as mentioned earlier the requirement of helper virus is not an absolute requirement; under certain stress conditions infected cells become partially permissive for productive replication. The helper activities provided by adenovirus are most extensively studied. Initial studies involving micro injection of mRNA molecules indicated that among Ad genes, only those required for the early functions are required for the synthesis of infectious AAV particles (Richardson et al., 1980). Two adenovirus E1a proteins with 289 and 243 amino acids have been identified as transcriptional regulators (Jones, 1995). The 289 amino acid E1a protein is responsible for transactivation of AAV gene expression (Chang et al., 1989). The relief of the AAV p5 promoter repression by E1a requires both the major late transcription factor (MLTF) and YY1 binding sites in the p5 promoter. Adenovirus E1a proteins induce cells to enter S phase and to synthesize proteins for DNA replication which are required for AAV DNA replication (Wang et al., 1993). Schematic of adenovirus genome is shown in figure 5.
Adenovirus transcriptional map

The adenovirus E2a gene encodes a 72 Kd single stranded DNA binding protein that is required for Ad DNA replication but it does not appear to be absolutely required for AAV DNA replication. However mutations in the E2a gene greatly inhibits AAV particle formation (Carter et al., 1992; Jay et al., 1981) (Carter et al., 1992). The E2a protein also transactivates the p5 promoter (Chang and Shenk, 1990) but the exact mechanism of this transactivation is not known. E2a is also reported to be involved in AAV mRNA transport and stability (Janik et al., 1989) . In vitro AAV DNA replication assays demonstrated that E2a enhanced AAV DNA replication but it could be replaced by human single stranded DNA binding protein, RPA (Ward et al., 1998). Furthermore, it has been demonstrated in vitro that the AAV Rep78/68 protein interacts with single stranded DNA binding
proteins (Stracker et al., 2004) of adenovirus (E2a), HSV-1 (ICP8), and the cell (RPA) and these interactions enhance binding and nicking of Rep proteins at the terminal resolution sequence.

The E1b55 Kd and E4ORF6 34 Kd proteins form a complex that regulates AAV gene expression post-transcriptionally by stabilizing AAV mRNA transcripts and transporting them to the cytoplasm (Bridge and Ketner, 1989; Samulski and Shenk, 1988). The E4ORF6 was observed increasing the transduction of rAAV vectors by 100-1000 fold (Ferrari et al., 1996; Fisher et al., 1996) and this increase in transduction was because E4ORF6 promoted conversion of single stranded input genome of AAV to transcriptionally active double stranded form. Expression of E4ORF6 in 293 cells leads to the degradation of cyclin A and p53 (Grifman et al., 1999) and the inhibition of kinase activity of cdc2 causing an inhibition of cell cycle progression and an accumulation of cells in the S phase. Since AAV DNA replication takes place during the S phase, these effects of E4ORF6 suggest a mechanism by which it can promote AAV DNA replication.

The adenovirus E2b gene encodes the Ad terminal protein and Ad DNA polymerase, both of which are directly involved in the process of adenovirus DNA replication but are not required for AAV DNA replication. Adenovirus VA1 RNA stimulates AAV protein synthesis by preventing the host induced cell shut-off of translation (Janik et al., 1989). Cotransfection of plasmids expressing the VA1 RNA and a modified rAAV genome that expresses a reporter gene demonstrated increased levels of transgene expression as compared to reporter gene plasmid alone. It is believed that VA1 RNA, in addition to increasing translation also alters
cytoplasmic accumulation of AAV RNA (West et al., 1987). Recently, it has been demonstrated that adenovirus coinfection also facilitated the nuclear translocation of AAV capsids (Xiao et al., 2002). This effect was also observed using empty adenoviral capsids suggesting that one or more components of the adenovirus capsid affect AAV trafficking.

It has been reported that coinfection of cells with AAV2 and HSV-1 or -2 results in AAV titers similar to those observed in the presence of adenovirus but the AAV life cycle progressed more rapidly than with adenovirus (Buller et al., 1981; Handa and Carter, 1979). Studies conducted using mutated HSV strains or plasmids expressing individual HSV genes (Stracker et al., 2004; Weindler and Heilbronn, 1991) indicated that the HSV genes encoding for the helicase/primase complex (UL5, 8 and 52) and for the ssDBP are needed to provide helper functions.

**Effects of AAV on Helper Virus**

The AAV also has profound effects on the life cycle of its helper viruses. AAV inhibits helper virus DNA replication, gene expression, helper virus mediated oncogenic transformation and tumorigenicity. Inhibition of adenovirus DNA replication requires AAV gene expression or AAV DNA replication (de la Maza and Carter, 1981) because AAV defective interfering particles, which contain large internal deletions, had no effect on the helper virus. The effects of AAV on adenovirus DNA replication have been attributed to AAV Rep gene expression (Weitzman et al., 1996). It has been demonstrated that during coinfection of cells with AAV and adenovirus, there is a 50-fold decrease in
infectious adenovirus production (Timpe et al., 2006) and a 10- to 40-fold reduction in adenovirus DNA replication. With the exception of the E3 gene, AAV decreases all steady state adenovirus mRNA levels. Wild type AAV but not UV-inactivated AAV or rAAV vector, delayed Ad-induced cytotoxicity, inhibited Ad E2a gene expression, and inhibited Ad DNA replication (Jing et al., 2001). Furthermore it was demonstrated that AAV Rep protein repressed expression from adenovirus E1a, E2a, and E4 promoters but trans-activated the E1b and E3 promoters. However, when an E1a expressing plasmid was cotransfected Rep78 repressed expression from all five promoters. It has been demonstrated that all four Rep proteins repress E2a expression at the protein level, with Rep40 showing the weakest repression (Nada and Trempe, 2002). The apparent decrease in E2a expression is due to Rep mediated inhibition of transcription of adenovirus E2a. Using in vitro transcription assays it was demonstrated that Rep68 blocked transcription of a template containing the Ad major late promoter. A 38 base pair sequence has been identified in the adenovirus E2a promoter that contains a weak TATA box and has a canonical Rep binding element (Casper et al., 2005) indicating that Rep binding to the E2a promoter contributes to the inhibition of transcription from the adenovirus E2a promoter and subsequent adenovirus replication. Studies involving Rep protein mediated inhibition of transcription of the adenovirus major late transcription promoter (Needham et al., 2006) in vitro demonstrated that Rep78/68 can suppress transcription from this promoter. The purine nucleotide binding site of Rep is required for complete repression and since Rep proteins can bind to the promoter suggesting that
protein-DNA interactions are required for inhibition. There is further evidence that decreased early gene expression is probably not the sole mechanism responsible for suppression of adenovirus DNA replication by AAV, and this suppression occurs prior to, and independently of, the inhibition of early gene expression (Timpe et al., 2006).

The exact mechanism by which AAV inhibits adenovirus DNA replication is not yet known. This inhibition could occur by binding to the adenovirus origin of replication and thereby preventing the binding of the DNA replication machinery. Interactions of the AAV Rep protein with multiple cellular and viral proteins raise the possibility that interaction of Rep with proteins required for adenovirus DNA replication can prevent DNA replication. AAV also inhibits replication of herpes simplex virus (HSV) during coinfection (Heilbronn et al., 1990; Kleinschmidt et al., 1995). AAV Rep protein inhibits HSV DNA replication and the N-terminal DNA binding region is sufficient for this inhibition.

Coinfection of AAV with oncogenic adenovirus reduces the frequency of and delays the occurrence of tumors in Syrian hamsters (Mayor et al., 1973). The decrease in adenovirus oncogenesis seems to be due to the effect of AAV on the E1b expression levels in transformed cells. Furthermore, defective interfering AAV particles can inhibit adenovirus induction of tumors in hamsters (de la Maza and Carter, 1981). AAV has also been reported to inhibit the oncogenicity of HSV-2 transformed cells after infection (Muzychka, 2001). AAV has also been shown to inhibit transformation by human pappiloma virus 16 (HPV16) (Hermonat et al., 2000; Walz et al., 1997) and this inhibition is attributed to the
AAV Rep protein. Rep mediated inhibition appears to involve binding of Rep to an HPV-16 promoter and direct interaction with HPV E7 oncoprotein. It has been demonstrated that the complete AAV rep gene inhibits SV40 DNA and E1a/ras gene-mediated transformation of mouse fibroblasts (Khleif et al., 1991). The Rep68/40 or Rep52/40 proteins alone do not suppress transformation.

**AAV and Host Cell Interactions**

AAV is generally believed to have antiproliferative effects on cells and, since it interferes with tumor formation in animal models, it has been proposed to have oncosuppressive activities (Schlehofer, 1994). Studies into the effects of AAV on host cells have revealed that infection of non-permissive cells with AAV or ultra violet (UV) light inactivated AAV inhibits their division in culture and interfered with their transit through the cell cycle (Winocour et al., 1988). The perturbation of the cell cycle leads to accumulation of cells in the late S or G phase of the cell cycle. AAV has also been reported to induce differentiation associated antigens in a human leukemia cell line (Klein-Bauernschmitt et al., 1992) as well as in two different immortalized human keratinocytes. Simultaneously, it was observed that expression of c-myc and c-myb oncogenes was down regulated whereas c-fos expression increased in AAV infected cells.

AAV has also been reported to sensitize cells towards anti cancer agents. Analysis of integration of AAV DNA in Hela cells has revealed that these cells had (Walz and Schlehofer, 1992) (a) a reduced growth rate, (b) an increased serum requirement, (c) a reduced capacity for colony formation in soft agar, (d) a reduced cloning efficiency on plastic, (e) an elevated sensitivity to genotoxic
agents (N-methyl-N'-nitro-N-nitrosoguanidine, 7,12-dimethylbenz[a] anthracene, human tumor necrosis factor alpha, UV irradiation [256 nm], and heat [42°C]), and (f) an enhanced sensitivity to gamma irradiation were also observed in vivo when tumors from these cells were transplanted into nude mice. The infection of Hela cells with AAV also increased cell killing and reduced plating efficiency after irradiation compared to uninfected cells (Walz et al., 1992). Similarly, HeLa cell tumors in nude mice displayed a reduced growth rate and were more sensitive to gamma irradiation when the animals were infected with AAV type 2 prior to or after tumor cell inoculation. Studies on the effects of AAV in sensitizing other tumor cells towards chemotherapy revealed that AAV infection significantly increased the cytotoxicity of cisplatin on a variety of cancer cell lines and freshly explanted tumor biopsies (Klein-Bauernschmitt et al., 1996). Sensitization of the cell lines resistant to chemotherapeutic agents, such as cisplatin and etoposide, has also been reported by concomitant AAV infection (Hillgenberg et al., 1999).

Furthermore it has been reported that stable integration of the Rep gene in SV40 transformed Chinese hamster embryo cells (OD4) did not interfere with cell proliferation (Winocour et al., 1992). However, it did make cells more sensitive to UV light and these cells had lost the ability to promote SV40 origin containing DNA amplification in vitro, in contrast to the parental OD4 cells. Cells carrying the stably integrated AAV constructs with deletion in the Rep gene did not display the above mentioned changes. These studies suggest that stable integration of rep gene interferes with cellular processes connected with DNA repair and gene amplification. Cell lines which express AAV Rep proteins under the control of an
inducible mouse metallothionein transcription promoter (Yang et al., 1994) grew at a significantly slower rate than uninduced cells. The presence of the Rep proteins increased the population of cells in the S phase of the cell cycle. SV40 DNA replication was inhibited by the presence of the rep gene in human 293 cells, and the inhibition was not linked to suppression of SV40 early gene expression (Yang et al., 1995). Expression of the Rep78 and Rep68 proteins correlated with an inhibition of cellular DNA synthesis in NIH3T3 cells. It was suggested that rep gene ensures its anti-proliferative effects either by direct inhibition of DNA replication or by a cell cycle block.

It has been demonstrated that infection with AAV has an effect on several factors involved in cell cycle regulation. In response to AAV infection, p21WAF1 mRNA and protein was quickly upregulated (Hermanns et al., 1997) and cyclin E and cyclin A dependent kinase activities declined to low levels and thus arrested cell cycle progression. Investigations into whether the Rep protein interferes with cell division cycle revealed that Rep78/68 inhibited the growth of primary, immortalized and transformed cells while Rep52/40 did not. Rep78 expressing cells were stalled in the S-phase and almost exclusively contained hypophosphorylated retinoblastoma protein (Saudan et al., 2000). It was also observed that primary mouse embryonic fibroblasts with homozygously deleted Rb gene displayed a strongly reduced S phase arrest when challenged with Rep78 as compared to cells with wild type Rb gene. Further investigations into Rep mediated S-phase block of cells revealed that Rep78 inhibits Cdc25A activity by a novel method (Berthet et al., 2005) in which binding between the two
proteins stabilizes Cdc25A, thus increasing its abundance, while at the same time preventing access to its substrates, cyclin-dependent kinase (Cdk) 2 and Cdk1. In addition, Rep78, as well as Rep68, produce nicks in the cellular chromatin, inducing a DNA damage response mediated by the ataxia telangiectasia mutated (ATM) pathway leading to G(1) and G(2) blocks.

There is also evidence that AAV selectively induces apoptosis in cells that lack an active p53 (Raj et al., 2001). Cells with intact p53 are not killed but instead undergo arrest in the G2 phase of the cell cycle. The arrest and the cell killing did not depend upon AAV encoded proteins. Rather it was suggested that AAV DNA elicits a DNA damage response in infected cells, which in the absence of p53 leads to cell death.

AAV and adenovirus coinfection induces a DNA damage response (Collaco et al., 2008) involving ATM and DNA-PK mediated phosphorylation of CHK1/2 kinases, RPA2, NBS1 and H2AX. Inhibition of these kinases did not alter the AAV protein expression dramatically; however, AAV replicative genome copies produced per cell were decreased by ATM knock-down.

**Interactions of Rep Protein with Host Cellular Factors**

AAV Rep proteins interact with a variety of cellular proteins. Interaction of AAV Rep78 has been reported with cellular Sp1 (Hermonat et al., 1996), High mobility group chromosomal protein 1 (HMG1) (Costello et al., 1997), PrKX (Di Pasquale and Stacey, 1998), cellular TATA Binding Protein (TBP) (Hermonat et al., 1998), transcriptional coactivator PC4 (Weger et al., 1999), p53 (Batchu et al., 1999), E2F-1 (Batchu et al., 2001), Topors (Weger et al., 2002).
Binding of the Rep78 protein to Sp1 has been shown in vitro (Hermonat et al., 1996) and this interaction has been proposed to be responsible for inhibition of heterologous promoters having Sp1 binding sites during an AAV infection. Rep protein mediated activation of p19 and p40 promoters require Sp1 (Pereira and Muzychka, 1997a; Pereira and Muzychka, 1997b). Lack of a strong RBE in these promoters suggests that the Rep mediated activation of these promoters occur through Rep-Sp1 interaction. HMG1 interaction enhances the binding of Rep at the inverted terminal repeats of the viral genome, ATPase and endonuclease activity in vitro (Costello et al., 1997). In vivo HMG1 interaction enhances Rep mediated repression of the p5 promoter. Interaction of the Rep78 protein with TBP has been reported by a variety of different assays and is thought to be involved in modulating transcriptional regulation by the Rep78 protein at different proteins (Hermonat et al., 1998). Another general transcription factor interacting with Rep78 protein is positive coactivator 4 (PC4) (Weger et al., 1999). Stable in vivo interaction of Rep and PC4 requires ATP as a cofactor. Overexpression of PC4 in the absence of adenovirus coinfection suppresses all 3 AAV promoters, and this suppression was relieved when cells were coinfected with adenovirus. The Rep78 protein is also reported to interact with E2F-1 (Batchu et al., 2001) and thereby stabilizes the pRb-E2F-1 complex. Rep78 also binds to a putative site at E2F-1 promoter and down regulates the adenovirus induced E2F-1 transcription. This dual level regulation of E2F-1 by Rep78 is proposed to be one of the mechanisms of AAV’s oncosuppressive activity. It has been suggested that the interaction of Rep and Topors is involved in regulation of AAV gene
expression in the absence of helper virus (Weger et al., 2002). Furthermore the Rep domains necessary for interaction with Topors are similar to the domains required for Rep mediated inhibition of cellular transformation by E1a/ras suggesting a role of Rep-Topors interaction in Rep mediated inhibition of cellular transformation.

Rep78 protein also interacts with the protein kinase A catalytic subunit (PKAc) homologue, PrKX and autophosphorylation of PrKX is believed to be inhibited by this interaction (Chiorini et al., 1998; Di Pasquale and Chiorini, 2003; Di Pasquale and Stacey, 1998). PrKX mediated activation of cAMP responsive element binding (CREB) protein is suppressed by Rep78. Rep52 also inhibits trans and autophosphorylation of PKA and PrKX leading to reduction in CREB and cyclin A protein levels. Furthermore it is suggested that the interaction of Rep78 with PrKX during coinfection with adenovirus is not necessary for AAV2 replication and packaging, this interaction helps in inhibiting the replication of adenovirus and gives AAV a selective advantage to use the cellular machinery for its own replication.

**AAV as Vector for Gene Therapy**

A number of characteristics have made AAV a vector of choice for gene therapy. Some of these properties include (a) efficient transduction of dividing and quiescent cells (b) non-pathogenicity (c) low immunogenicity (d) long term gene expression. Hermonat et al. proposed the use of AAV as a mammalian transduction vector after replacing the capsid gene with a transgene (Hermonat and Muzychka, 1984). In a recombinant AAV vector (rAAV) genome, the only
elements required in cis are the viral inverted terminal repeats. Hence, both rep and cap genes can be replaced by the transgene and promoter of interest (McLaughlin et al., 1988). A map of recombinant AAV (rAAV) vector is depicted in figure 5. This type of AAV vector is in various phases of clinical trials for a variety of genetic diseases (Kay et al., 2000; Wagner et al., 1998).

Wild type and rAAV vectors infect a wide variety of tissue types. Some of the tissues which have shown transgene expression are lungs, neurons, retina, cochlea, liver, muscle and heart (Ali et al., 2000; Flotte et al., 1993; Kaplitt et al., 1994; Kaplitt et al., 1996; Snyder et al., 1997). The tissues that have shown low permissiveness to rAAV transduction include airway epithelial cells, fast muscle fibers, neural progenitor cells, ovarian cancer cells and hematopoietic cells (Duan et al., 2000; Handa et al., 2000; Hughes et al., 2002; Pruchnic et al., 2000; Vermeij et al., 2001). Efficacy of transduction of different cell types by rAAV is

Map of rAAV vector genome

Solid arrow represents promoter of interest in this case CMV promoter. Empty box represents transgene of interest in this case luciferase transgene. Inverted terminal repeats at either end are the only elements required in cis.
impeded by similar characteristics as discussed earlier for a successful infection (Ding et al., 2005) i.e. (a) availability of receptors, (b) efficient nuclear transport (c) conversion of single stranded DNA genome into a transcriptionally active double stranded form (d) persistence of expression of the transgene. Mechanisms by which some of these impediments are overcome have been discussed in the AAV life cycle section described above.

The different serotypes of AAV show variations in the capsid regions. This difference between capsids is responsible for alteration in host ranges and tissue tropism among serotypes (Chiorini et al., 1999; Chiorini et al., 1997; Rutledge et al., 1998). Taking advantage of these differences in serotypes AAV2 inverted terminal repeats were packaged into different capsid serotypes (Rabinowitz et al., 2002). These pseudotyped vectors had a higher transduction efficacy in different tissues as compared to vectors having capsid from AAV2, in different tissues. The use of different serotypes have an added advantage in that they might help the vector elude the host humoral immune responses (Halbert et al., 2000).

Transgene expression mediated by rAAV vectors persists for long durations. Initially, it was thought that the persistent expression of rAAV transgenes is because of random integration of rAAV genomes in cells (Nakai et al., 1999; Wu et al., 1998). However, now it is known that recombinant vector genomes exist as double stranded circular monomers and high molecular weight concatemers which are responsible for stable rAAV transduction in the liver and muscle (Malik et al., 2000; Nakai et al., 2001).
Limitation of rAAV Vectors

One of the challenges in gene therapy using viral vectors is modulating the host immune response against the vector. AAV vectors are associated with low immunogenicity and toxicity resulting in long term gene expression and vector persistence. The inability of AAV vector to efficiently transduce or activate antigen presenting cells may account for their decreased immunogenicity (Jooss et al., 1998). It has been reported that use of rAAV vector generates both humoral and cellular immune response against the gene product (Favre et al., 2002; Fields et al., 2000).

Eighty percent of the US population is considered to be seropositive to AAV2. 50% of the population has neutralizing antibodies. AAV mediated gene therapy leads to development of antibodies against the vector capsid (Chirmule et al., 2000; Erles et al., 1999). Anti-AAV antibodies have neutralizing effects that decrease the efficacy of in vivo gene therapy and can prevent readministration of the vector. Neutralizing antibodies to AAV limit rAAV transduction in liver (Murphy et al., 2008) and lung (Halbert et al., 1998); however, no such effect was seen in muscle, brain or retina (Daya and Berns, 2008). The problem of neutralizing antibodies can be overcome by using a different serotype for readministration. New cell mediated response to rAAV vectors may be dependent upon the route of delivery and serotype of AAV used (Brockstedt et al., 1999; Wang et al., 2007). For example, it was observed that in DBA/2 mice intravenously administered AAVlacZ rapidly induced the expression of a number of chemokines including TNF-alpha, RANTES, IP-10, MCP-1 and MIP-2. However,
the chemokine mRNA level returned to baseline within 6 hours (Zaiss et al., 2002).

One of main disadvantages in the use of rAAV vectors is their limited genome packaging ability. The maximum size that rAAV vectors can package is 5.2kb (Dong et al., 1996). Different strategies that are being developed to overcome this problem using a hybrid viral capsid structure (Ponnazhagan et al., 1998) or the use of split genes in separate rAAV vectors (Nakai et al., 2000; Sun et al., 2000). In split gene technology, one vector carries the transcriptional promoter, 5’ part of the gene and splicing signals flanked by the inverted terminal repeats, while another vector carries the 3’ part of the gene and splice acceptor signal flanked by the inverted terminal repeats. Cotransduction of these dual vectors into the host results in head to tail concatenemerization and splicing of the intervening inverted terminal repeats. This strategy doubles the size of the gene that can be introduced into the host.

In order to limit the tissue tropism and increase the specificity of rAAV vectors, methods have been developed to alter the capsid structure. To this end, it was demonstrated that incubation of AAV2 vectors with a bispecific antibody that recognized AAV capsid and a surface receptor resulted in efficient transduction of non-permissive megakaryoblast cells (Bartlett et al., 1999). Other approaches to genetically modify the AAV capsid are also being pursued wherein the goal is to directly insert a ligand into the capsid sequence. For example, it has been observed that VP2 is non-essential for making the viral particles and different peptides such as fractalkine (76 amino acid), leptin (146 amino acid)
and GFP (238 amino acids) could be inserted exclusively at the VP2 N-terminal (Warrington et al., 2004). Similarly a His tag has been inserted at the C-terminal of VP3 (Zhang et al., 2002).

Given the advantages of rAAV vectors and the development of methods to produce vectors without the infection of adenovirus, rAAV vectors are becoming the vector of choice for gene therapy. Currently AAV vectors are in different phases of gene therapy clinical trials for diseases such as cystic fibrosis, Canavan’s disease, Parkinson’s disease, prostate cancer, hemophilia B, alpha-1-antitrypsin deficiency, Alzheimer’s disease, and arthritis.

**Adenoviridae**

The *Adenoviridae* family of viruses is divided into two genera, *Mastadenovirus* and *Aviaadenovirus*. The *Mastadenovirus* genus includes human, simian, murine, bovine, equine, porcine, ovine, canine and opossum viruses. Adenoviruses were first isolated and characterized in 1953 from human adenoids and were named adenovirus in 1956.

Adenovirus is a nonenveloped DNA virus with a regular icosahedral structure with a diameter of 70-90 nm. The viral genome is linear and double stranded with approximately 36,000 nucleotides (Chroboczek et al., 1992). The genome contains a virally encoded terminal protein covalently linked to 5’ end of the genome and inverted terminal repeats at both ends which also have the viral origins of replication (Rekosh et al., 1977). The viral genome encapsidation signal is present close to the left inverted terminal repeat (Hearing et al., 1987). The genome has five early transcription units (E1a, E1b, E2a, E2b, E3 and E4)
(Berk, 2007), and one late transcription unit that generates five precursor mRNAs (L1-L5) (Figure 4) transcribed by RNA polymerase II. The genome also encodes for virus associated (VA) genes transcribed by RNA polymerase III.

The adenovirus replication cycle is divided into two phases that are separated by the onset of DNA replication. The early phase begins as soon as virus interacts with the cell surface receptor. Adenovirus enters the host cell by attachment of its fiber to a specific cell surface receptor (Bergelson et al., 1997) and is internalized rapidly via receptor mediated endocytosis. The best studied adenovirus receptor is CAR (coxsackievirus and adenovirus receptor), a 46Kd protein that also mediates infection by group B coxsackieviruses. The virus is later released from the endosomes and is transported to the nucleus (via a nuclear targeting signal in the capsid protein) where the various early transcriptional regions are expressed. These gene products modulate the cellular environment by inducing cell cycle progression, blocking apoptosis and antagonizing host antiviral responses to support viral replication (Berk, 2007).

**Adenovirus Proteins and Their Role**

Human adenovirus E1a encodes two closely related proteins, of 289 amino acid (aa) residues (13S) and of 243 residues (12S). The E1a (13S) is one of the most extensively studied viral transcriptional regulators. The E1a gene products activate transcription of other viral early genes and are important for modulation of the cell cycle to facilitate viral replication (Harlow et al., 1986; Spindler et al., 1985). These functions are brought about by both direct and indirect interactions with cellular proteins such as retinoblastoma protein (pRb),
the Rb family proteins p107 and p130 (Harlow et al., 1986; Yee and Branton, 1985), p300/CBP, CtBP (Schaeper et al., 1995), Sur2 (Boyer et al., 1999b), p400 (Fuchs et al., 2001) and TRRAP/GCN5 (Deleu et al., 2001). The adenovirus E1a (12S) protein is also involved in down regulating transcription of interferon alpha and interferon gamma induced genes (Leonard and Sen, 1996; Reich et al., 1988).

The products of the E4 gene mediate transcriptional regulation, mRNA transport, modulate DNA replication and apoptosis. E4ORF1 and E4ORF4 pathways cooperate to activate mTOR leading to high rate of protein synthesis (O'Shea et al., 2005). Furthermore, E4ORF6 interacts and inhibits p53 and p73 independently of E1b55 Kd (Steegenga et al., 1999). These interactions prevent the transcriptional activities of p53 and p73 and thus E4ORF6 blocks their potential inhibitory effects on viral replication.

E1b encodes two proteins that block apoptosis. E1b55 Kd complexes with E4ORF6, binds to p53 and targets it for proteosomal degradation (Querido et al., 1997). In the absence of E4ORF6, E1b55 Kd binds to p53 and tethers to it a strong repression domain thereby converting p53 from a transcriptional activator to a repressor (Teodoro and Branton, 1997). Viral E1b19 Kd protein binds to the proapoptotic BCL-2 family members, BAK and BAX (Cuconati and White, 2002). Sequestration of these proteins prevents the cell from undergoing premature apoptosis and thus allows the virus to undergo a complete lytic cycle.

The E1b and E4 proteins are also involved in neutralizing the cellular DNA damage pathways in response to viral infection by directing the MRE11 and
RAD50 subunits of MRN complex for proteosomal degradation during the late phase of infection (Stracker et al., 2002). The adenoviral E4ORF3 protein mislocalizes DNA repair proteins such as Mre11 complex, and the E1B55Kd/E4ORF6 complex targets them for proteosome mediated degradation (Carson et al., 2003; Evans and Hearing, 2005; Stracker et al., 2002). During adenovirus infection E4ORF6 and E4ORF3 proteins individually prevent the formation of concatemers of the linear viral genome in infected cells (Boyer et al., 1999a). It has been suggested that the E4 products block the formation of concatemers of the viral genome by inhibiting DNA PK-dependent double strand break repair and they act by forming a physical complex with DNA PK.

Shortly after the infection the E2 proteins begin to accumulate as a result of the transcriptional activation by E1a. E2 encodes three proteins [pTP, DNA polymerase (Ad pol) and the DNA binding protein (DBP)] that play a role in DNA replication (Berk, 2007). pTP protein interacts with the Ad pol, binds to single stranded DNA and stabilizes Ad pol on the unwound viral origin. Ad pol an eukaryotic DNA polymerase-α, has both 5’ to 3’ DNA polymerase and 3’ to 5’ exonuclease activities and is involved in both initiation and elongation. The DBP protects the single stranded DNA from nucleases and helps in stabilizing and properly orienting the pTP-Ad pol complex at the origin during the initiation of DNA replication.

The E3 region was termed as “nonessential” since adenovirus mutants with almost entire E3 region deleted replicated to wild type adenovirus levels in cultured cells (Kelly and Lewis, 1973). However later role of a critical role of E3 in
adenovirus pathogenesis was established (Ginsberg et al., 1989). Various proteins encoded in the adenovirus E3 transcription unit protect cells from killing mediated by cytotoxic T cells and death-inducing cytokines such as TNF-α, Fas ligand and TRAIL (Lichtenstein et al., 2004). The viral protein E3-gp19Kd blocks the MHC class-I-restricted antigen presentation, E3-RID and E3-6.7Kd stimulates clearance from the cell surface and subsequent degradation of the receptors for Fas ligand and TRAIL. Adenoviruses lyse the cell to allow efficient virus release and dissemination. Finally adenoviruses synthesize a protein late in infection named Adenovirus Death Protein (ADP) (formerly E3-11.6 Kd) that is required for efficient virus release.

Adenovirus late gene expression begins at the onset of DNA replication. The single large late transcript is processed by differential poly(A) site utilization and alternative splicing to generate multiple mRNAs. Expression of this family of genes is under the control of the major late promoter (MLP) (Shaw and Ziff, 1980). This promoter has low activity during early infection but its activity increases several hundred fold during the late stage of infection. It is activated by E1a (Parks and Shenk, 1997), a cis-activating change in viral the chromosome and at least one virus encoded transactivating factor (Thomas and Mathews, 1980). Proteins encoded by the late transcriptional unit are responsible for the production and assembly of capsid proteins.

Once the adenovirus DNA replication begins and all the late mRNAs are synthesized, the cytoplasmic accumulation of cellular RNA is blocked (Beltz and Flint, 1979). This inhibition of cellular mRNA transport is mediated by the E1b-
55Kd protein (Pilder et al., 1986) and the E4ORF6 gene product (Halbert et al., 1985). In addition to the block once in the cytoplasm the viral mRNA is preferentially translated over cellular mRNA. This facilitated translation of viral mRNA is facilitated by a protein encoded by L4 called 100K (Cuesta et al., 2004).

The genome of mouse adenovirus (MAV-1) is similar to human adenovirus (Cauthen et al., 2007); there is a set of genes transcribed early after infection, and a set of late genes transcribed after the onset of viral DNA replication. A terminal protein is covalently attached to the 5’ end of each strand. The E1a region of MAV-1 encodes only one mRNA and one protein, which has a 40% similarity to the 289-amino acid E1a protein of human adenovirus-5 (Ad5). E1b, E2a and the late structural genes are predicted to encode proteins similar to the human adenoviruses, whereas E3 and E4 proteins have little or no similarity. MAV-1 E1a, protein similar to human adenovirus E1a, interacts with pRb, p107 (Smith et al., 1996), p130 and mSur2 (Fang et al., 2004). Furthermore just like human adenovirus E1a, MAV-1 E1a also plays a major role in antagonizing the antiviral effects of interferons (Kajon and Spindler, 2000). Further, the MAV-1 E1a gene is not required for viral replication in cell culture (Ying et al., 1998) but is a virulence gene in both outbred and inbred mice (Smith et al., 1998; Spindler et al., 2001). The similarities between MAV-1 and human adenoviruses make it an excellent system to study adenovirus pathogenesis in a mouse model.

Considering the similarities between MAV-1 and Ad-5 we hypothesized that MAV-1 should be able to provide helper functions to AAV2 for a productive infection in murine cells. To confirm our hypothesis we studied the replication of
AAV2 in murine cells with MAV-1 provided helper functions. These studies will be a prelude to study the replication of AAV2 in a mouse model with MAV-1 provided helper functions.
MATERIALS AND METHODS

Cells and Viruses

Hela cells and mouse NIH 3T3 cells were grown in MEM and DMEM respectively supplemented with 10% FBS, Gultamine and antibiotics. All cells were maintained as monolayer cultures at 37°C in a 5% CO₂ atmosphere.

Mouse adenovirus (MAV1) was kindly provided by Dr. K.R. Spindler (University of Michigan). MAV1 was used to infect fifteen 10 cm dishes of NIH 3T3 cells. 96 hours later cells were harvested by scraping in the medium. Cells were lysed by 3 freeze-thaw cycles. The crude lysate was cleared of cell debris by centrifugation it at 10,000 rpm for 10 minutes at 4°C and the supernatant containing virus was saved. Physical particle and infectious titer of virus was determined by real time PCR and western blotting for MAV E1A protein after limiting dilution infection.

AAV was prepared by transfecting 293 cells with pNTC244 plasmid and infecting them with Ad-5. 40 hours later cells were harvested, subjected to three freeze-thaw cycles, and treated with DNase. Extracts were then fractionated by heparin-agarose column chromatography as described previously and infectious titer of AAV was determined by dot blot analysis (Casper et al., 2005).

Infections and Transfections

Ad-5 or MAV were used at multiplicity of infection (MOI) 5 and AAV was used at the MOI indicated in experiments. Cells were infected when they reached 80-90% confluency. Infections were performed in serum free medium for 2 hours after which the medium was replaced with complete medium. Cells were plasmid
transfected at 90% confluency using Lipofectamine 2000 (Invitrogen) in serum free medium for 4 hours according to manufacturer’s recommendation. The DNA to Lipofectamine 2000 ratio used was 1μg:2.5μl, respectively and 4μg was transfected in 6 well plates. Four hours after transfection, medium was replaced with complete medium.

**Real Time PCR**

Replicative genome copy numbers for AAV, Ad-5 and MAV were determined by quantitative real time PCR. The forward primer for AAV was 5’ AAC TGG TTC GCG GTC ACA A 3’ (AAV nt 708) and the reverse primer was ACC CGA CCA GCT CCA TGT AC (AAV nt 1008). These primers amplify a 301 base pair fragment. The forward primer for MAV was 5’ ATG TCG CGG CTC CTA CG 3’ and the reverse primer was 5’ CAA CGA ACC ATA AAA AGA CAT CAT 3’. These primers amplify a 512 base pair fragment of the MAV E1A gene. Primers were used at a final concentration of 0.3 μM. Applied Biosystems Power Sybr-Green master mix was used to perform PCR.

Amplification was performed at 95°C for 10min to activate the polymerase followed by 40 cycles at 95°C for 30 sec, 54°C for 45 sec and 72°C for 45 sec. Genome copy number was determined by comparison to a standard curve plotted after amplification of the same fragment from plasmid pNTC244 or a MAV E1A fragment cloned into pGEM-T (Promega) at 10 fold serial dilutions starting from 1x10^{10} copies to 1x10^{2} copies. All experimental and serial dilution templates were run in triplicate. Data was analyzed using 7500 system SDS software (Applied Biosystems).
Southern Blot

Low molecular weight viral DNA was extracted from Hela or NIH 3T3 cells 24 or 48 hours post infection and/or transfection, respectively, using Qiagen QIAmp DNA mini kit. Samples were subjected to RNase treatment and extracted DNA was electrophoresed on a 1% agarose gel. DNA extracted after plasmid transfection was subjected to DpnI digestion before electrophoresis. After electrophoresis, the gel was treated with 0.2N HCl for 20 minutes to depurinate DNA. The gel was then washed several times to remove traces of acid and then placed in denaturation solution (.5M NaOH, 1.5M NaCl) for 30 minutes. The gel was then kept in neutralization solution (1M (NH₄)₂ acetate). The DNA was transferred to nytran membrane by capillary transfer. DNA was cross-linked to the membrane by exposing it to 1200J/m² of UV light in a Stratalinker (Stratagene). The membrane was prehybridized in hybridization buffer [5X SSC, 5X Denhardt solution (100X Denhardt solution: 10g Ficoll 400, 10g polyvinylpyrolidone and 10g BSA in 500 ml), 1% SDS and 100μg/ml denatured salmon sperm] at 42°C. Four hours later 5x10⁶ cpn of α³²P-dATP labeled DNA probes for AAV, Ad-5 or MAV DNA were added for hybridization overnight at 42°C. The probe was removed and the membrane was washed 3 times with 1X SSC and 0.1% SDS and then two times with 0.25X SSC and 0.1% SDS at 42°C. The membrane was then exposed to HyBlot Cl autoradiography film. For quantification of data the membrane was exposed to phosphor screen and the
signal was quantified using a Typhoon Phosphor-imager and Image Quant Software.

**DNA Probe preparation**

The AAV2 DNA fragment used for radiolabeling was obtained by digesting pNTC244 with HindIII enzyme and purifying an AAV fragment, for Ad5 by digesting an adenoviral plasmid with SacII and purifying a 1.6 kb fragment, for MAV by amplifying a 512 bp fragment of MAV E1A gene by PCR. These DNA fragments were gel purified (Qiagen). Purified DNA was heated at 95°C for 10 minutes and then immediately transferred to ice and kept for 5 minutes. The denatured DNA was mixed with .02 mM each of dCTP, dGTP, dTTP, 1μl Klenow fragment, 5μl of α 32P-dATP and 5μl of 5X labeling buffer (Random Primed DNA labeling Kit) (Roche). The reaction was allowed to continue for ~4hours at 37°C and was stopped by adding 2.5μl of 0.2M EDTA. Total volume was brought to 50μl by adding TE (10mM Tris, 1mM EDTA pH8.0).

In a 1cc syringe barrel a pinch of silanized glass wool was added to form a plug. The syringe was filled with G-25 sephadex beads resuspended in TE pH 8.0. The syringe was placed in a 15ml centrifuge tube and spun at 4500 rpm for 5 minutes to pack the column. The labeling reaction mixture was passed over this column to purify radio labeled DNA away from unincorporated nucleotides.

**Immunofluoroscence**

1.5 x 10^4 Hela or 3T3 cells were plated in each well of a 8 well chamber slide, 24 hours before infection. Cells were infected and/or transfected (1μg per well) as described above. 24 or 48 hours post infection or transfection cells were
fixed and permeabilized with 100% cold methanol. Cells were then washed and blocked with 2% Bovine Serum Albumin (BSA) in Phosphate Buffer Saline (PBS) (BSA-PBS). After blocking the cells were treated with RIPA buffer for 10 minutes (50mMTris ph 8, 150mM NaCl, .5% DOC, .1% SDS, 1% NP40) to lyse the cells. Fixed cells were incubated with primary antibody used at a dilution of 1:50 in BSA-PBS (affinity purified rabbit anti-Cap, and 1: 50 mouse anti-BrdU) for 1 hour. Primary antibody was removed and cells were washed with BSA-PBS. Cells were incubated with secondary antibody diluted in BSA-PBS [1:200 donkey anti-rabbit (Molecular Probes) and anti-mouse conjugated AlexaFlour488 (Molecular probes)] for 1 hour followed by washes with BSA-PBS. 5 minute incubation with 150 nM DAPI (4’, 6-diamidino-2-phenylindole) was done to stain nuclei. Cells were washed with BSA-PBS and then PBS alone to remove traces of DAPI. The gasket was removed and the slide was covered with a layer of DAKO Fluorescent Medium and a coverslip. A Nikon eclipse E 800 fluorescent microscope was used for analysis of the stained cells.

**Immunoblotting**

Hela or 3T3 cells were harvested at 24 or 48 hours post infection, respectively. For detecting protein expression AAV Rep, Cap and MAV E1A proteins nuclear extracts were prepared. Infected cells were scraped in medium, pelleted and washed with PBS + 5mM MgCl₂. Cell pellets were resuspended in STM-NP buffer [10mM Tris (pH8.0), 0.25mM sucrose, 10mM MgCl₂, 0.5% NP40, 1mM PMSF and 0.1mM DTT]. Cells were kept on ice for 15 minutes with intermittent vortexing. Nuclei were pelleted by spinning at 2000 rpm for 5
minutes. The supernatant (cytoplasmic fraction) was removed carefully and pellet was resuspended in IPP buffer [50mM Tris, 150mM NaCl, 20mM EDTA and 0.5% NP40 and 1mM DTT supplemented with protease inhibitors (1mM PMSF, 1μM leupeptin, 1μg/ml pepstatin and 1mM benzamidine)]. Extracts were kept on ice for 1 hour with intermittent vortexing. After 1 hour, extracts were spun at high speed (13000 rpm) for 5 minutes and the supernatant was saved. These nuclear extracts were separated on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA in Tris Buffered saline containing 0.5% Tween 20 (TBST). Rabbit anti-E1A serum (provided by KR Spindler) diluted in 1% BSA-TBST (1:3000) was used to probe for MAV E1A protein. AAV Rep and Cap proteins were probed using affinity purified rabbit anti-Rep and anti-Cap antibody diluted in 0.5% BSA-TBST (1:3000). After 1° antibody treatment the membrane was washed 3 times with TBST and the membrane was then incubated with horse radish peroxidase (HRP) conjugated anti-rabbit 2° antibody diluted in 0.5% BSA-TBST (1:10000). Proteins were then detected by ECL reaction and exposure of membrane to HyBlot Cl autoradiography film.
RESULTS

AAV DNAse I resistant particle production from 3T3 cells versus Hela cells

There are few studies to analyze replication of AAV in murine cells with helper functions provided by mouse adenovirus. We determined if DNAse I resistant particle (DRP) are produced after coinfection of murine fibroblasts with AAV-2 and MAV-1. We chose NIH-3T3 cells as a model murine cell line and compared DRPs produced from these cells to those produced by Hela cells. To this end, equivalent numbers of 3T3 and Hela cells were coinfected with 500 MOI of AAV and 5 MOI of MAV-1 or Ad-5, respectively, in serum free MEM. Hela cells were harvested 48 hours post infection whereas 3T3 cells were harvested after 48, 72 and 96 hours post infection. Crude viral extracts were prepared from harvested cells by 3 freeze-thaw cycles to lyse cells, DNAse I treatment for 30 minutes to remove any free viral DNA followed by inhibition of DNAse I by heating extracts at 65°C. Viral DNA was extracted from these extracts and real time PCR was performed to amplify AAV DNA, which would result in the number of DNAse resistant AAV genomes produced per cell from both cell lines.

As seen in fig. 7 at 48 hours post infection a negligible number of AAV DRPs are produced from 3T3 cells as compared to Hela cells at 48 hours. After 72 hours the number of AAV particles produced from 3T3 cells is approximately 40 fold less than the particles produced from Hela cells after 48 hours. Even after 96 hours, the number of particles produced from 3T3 is approximately 10 fold less than those produced by Hela cells. Hela cells showed extensive cytopathic effect at MOI 5 of Ad-5 after 48 hours, hence, these infections were not allowed
to progress further than 48 hours to determine the AAV DRP produced. The cytopathic effect in 3T3 cells due to MAV-1 at MOI 5 was comparable to Hela after 96 hours. These experiments allowed us to determine the infection conditions and timings at which highest yield of AAV is produced. Similar conditions were used in further experiments unless noted otherwise.

**AAV DNAse Resistant Particles Produced From NIH 3T3 and Hela cells.**

Equivalent number of 3T3 and Hela cells were coinfected with 500 MOI of AAV and 5 MOI of MAV or Ad-5 respectively. Crude AAV extracts were prepared at indicated time and real time PCR was performed to determine DNAse I resistant AAV particle number produced per cell.
Replication of AAV DNA in NIH-3T3 and Hela Cells

The dramatic difference between the AAV particle numbers produced from 3T3 cells as compared to that of AAV particles produced from Hela cells (Figure 7) prompted us to determine if AAV DNA replication in 3T3 cells with help provided by MAV-1 is equivalent to that observed in Hela cells with help provided by Ad-5. Equivalent numbers of 3T3 and Hela cells were coinfected with increasing MOI of AAV (0.1, 1, 10, 100, and 500) and 5 MOI of MAV1 or Ad-5, respectively. 3T3 or Hela cells were harvested 48 or 24 hours later, respectively, and low molecular weight AAV DNA was extracted. The DNA was electrophoresed on a 1% agarose gel and then analyzed by Southern hybridization with a radio-labeled AAV DNA fragment to detect replicative forms of AAV DNA. This experiment revealed that mouse adenovirus was able to provide helper functions for AAV DNA replication in 3T3 cells, however replicative forms of AAV DNA were observed only at AAV MOI of 10, 100 and 500 (fig. 8a). In comparison, replicative forms were detected from Hela cells even at the MOI of 0.1 (fig. 8b). Depending on the MOI of AAV used the replication signal was approximately 2-200 fold lower in 3T3 cells as compared to Hela cells as determined by phosphorimager analysis (fig. 8c). This data demonstrates that AAV DNA replicated in murine cells with MAV-1 provided helper functions but the level of replication is lower than that observed in Hela cells which are highly permissive for AAV infection.
Southern Blot to detect replication of AAV DNA in 3T3 and Hela cells

![Southern Blot](image)

AAV DNA replicative forms were detected after coinfecting (a) 3T3 and (b) Hela cells with 0.1, 1, 10, 100 and 500 MOI of AAV (lanes 3, 4, 5, 6 and 7 respectively) and 5 MOI MAV or Ad-5 respectively. AAV DNA was extracted and Southern blot was performed. (c) Replication signal was quantified by exposing membrane to phosphorimager.
To obtain a more quantitative assessment of the difference in replication of AAV between 3T3 and Hela cells we determined AAV genome copy numbers per cell after coinfection with AAV and MAV-1 or Ad-5. To this end, we coinfected 3T3 or Hela cells with increasing MOI of AAV (0.1, 1, 10, 100 and 500) and 5 MOI of MAV-1 or Ad-5. 3T3 or Hela cells were harvested 48 hours or 24 hours later, respectively. Low molecular weight AAV DNA was extracted and real time PCR was performed to amplify AAV DNA. As shown in fig. 8(d) and (e) the number of AAV genome copies per cell at the highest MOI in 3T3 cells is approximately 30 fold less than the AAV genome copies in Hela cells. This difference in DNA replication and genome copy number corresponds to difference noted in AAV DNAse resistant particles produced from 3T3 and Hela cells.

**Replicative Genome copy number of AAV per 3T3 cell**

![Graph showing AAV Genome copy number per 3T3 cell](Fig. 8(d))
Real time PCR was performed to detect the replicative genome copy number of AAV DNA per (d) 3T3 and (e) Hela cell after coinfecting cells with indicated MOI of Adenovirus and AAV.

**Adenovirus Replication in 3T3 and Hela cells**

Previous observations prompted us to question if the MAV-1 replicates efficiently in 3T3 cells compared to the replication of Ad-5 in Hela cells.

To determine the replication levels of MAV-1 in 3T3 cells and the replication of Ad-5 in Hela cells, equivalent numbers of 3T3 or Hela cells were infected with 5 MOI of MAV-1 or Ad-5 respectively with or without coinfection of AAV. 3T3 or Hela cells were harvested 48 or 24 hours later. Low molecular weight adenovirus DNA was extracted and analyzed by Southern hybridization.
As shown in fig. 9a we observed a robust replication of MAV-1 in 3T3 cells in the absence of AAV. At the highest MOI (500) of AAV in coinfected 3T3 cells the levels of MAV-1 DNA replication decreased by a modest 2 fold as determined by phosphorimager analysis (Fig. 9c).

As expected Ad-5 DNA replication in Hela cells was robust and the levels of Ad-5 DNA replication decreases with increasing MOI of AAV (fig. 9b). Quantification by phosphorimager analysis (fig. 9c) revealed that Ad-5 DNA decreased by approximately 15 fold at the highest MOI of AAV. This inhibition of Ad-5 DNA in presence of has been reported previously (Timpe et al., 2006) and is a well documented phenomenon.

Southern Blot to detect Replication of MAV and Ad-5 DNA in 3T3 and Hela cells respectively

<table>
<thead>
<tr>
<th>Fig. 9(a) 3T3</th>
<th>(b) Hela</th>
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<tbody>
<tr>
<td>Negative MAV AAV</td>
<td>Negative Ad-5 AAV</td>
</tr>
<tr>
<td>1 2 3 4 5 6 7</td>
<td>1 2 3 4 5 6 7</td>
</tr>
</tbody>
</table>
Phosphorimager analysis of Southern

(a) MAV-1 and (b) Ad-5 DNA replication was detected after coinfecting 3T3 or Hela cells with MAV-1 or Ad-5 at MOI 5 respectively and AAV at MOI 0, 0.1, 1, 10, 100 and 500 (lanes 2, 3, 4, 5, 6 and 7). Adenovirus DNA was extracted and Southern blot was performed. (c) Replication signal was quantified by exposing membrane to phosphorimager.

To directly compare the replication of MAV-1 in 3T3 and Ad-5 in Hela cells and to determine the effect of AAV on adenovirus DNA replication we determined the replicative genome copy number of adenovirus DNA by real time PCR. 3T3 or Hela cells were infected with 5 MOI of MAV-1 or Ad-5, respectively, and increasing MOI of AAV. Cells were harvested 48 or 24 hours later respectively, low molecular weight DNA was extracted and real time PCR was performed. As shown in fig. 9d there is only a slight difference in genome copy number of MAV-
1 and Ad-5 per cell in the absence of AAV (1.25 fold). Further, with the increasing MOI of AAV, Ad-5 genome copy number decreased. At the highest MOI of AAV there was an approximately 16 fold lower level of Ad-5 DNA replication than in the absence of AAV. However, the effect of AAV on MAV-1 replication was not as pronounced and demonstrated a decrease of only 5.5 fold at the highest MOI of AAV.

**Replication of MAV-1 and Ad-5**

Real time PCR was performed to detect the replicative genome copy number of MAV-1 DNA per 3T3 cells when infected with or without AAV.
These results demonstrate that MAV-1 replicates in 3T3 cells robustly and there were modest differences in the replication of MAV-1 in 3T3 cells as compared to that of Ad-5 in Hela cells. Ad-5 DNA replication decreases in the presence of AAV however the decrease in MAV-1 replication is less pronounced. This might be due to the fact that as observed earlier in (Fig. 8a, 8c and 8d) replication of AAV is much less in 3T3 cells as compared to that of Hela cells.

Next we sought to determine if the number of 3T3 cells supporting MAV-1 replication is comparable to the number of Hela cells supporting Ad-5 replication. To this end equivalent numbers of 3T3 or Hela cells were plated in an 8 well chamber slide. These cells were infected with 5 MOI of MAV-1 or Ad-5 respectively. Two hours post infection cells were treated with 5mM of Bromodeoxyuridine (BrdU). 3T3 or Hela cells were fixed 48 or 24 hours later, respectively, and stained using a primary antibody against BrdU. As shown in fig. 10 (a) and (c), we did not see any signal from uninfected cells however BrdU stained cells are observed in Ad-5 or MAV infected Hela or 3T3 cells, respectively (fig. 10b and d). As shown in table 1, we observed that the number of 3T3 or Hela cells supporting replication of MAV-1 or Ad-5 respectively was comparable at approximately 8%. 

85
Table 1 BrdU positive Hela and 3T3 cells

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<thead>
<tr>
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<th>Hela Cells</th>
<th>3T3 Cells</th>
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<tbody>
<tr>
<td>DAPI stained nuclei</td>
<td>1715</td>
<td>1475</td>
</tr>
<tr>
<td>BrdU positive cells</td>
<td>142</td>
<td>127</td>
</tr>
</tbody>
</table>

DAPI and BrdU stained cell were counted from 10 different fields to determine the percentage of cells showing BrdU staining.

BrdU staining to detect cells supporting MAV-1 and Ad-5 DNA replication

MAV-1 or Ad-5 (a, c) uninfected or (b, d) infected 3T3 or Hela cells, respectively cells were treated with BrdU. BrdU incorporation in viral DNA was detected by immunofluoroscence.
These results demonstrating comparable levels MAV1 or Ad-5 DNA replication suggest that the difference in the number of cells supporting replication of adenovirus in 3T3 versus Hela cells is probably not the reason for fewer DNase resistant particles produced or decreased levels of AAV DNA replication in 3T3 cells compared to that of Hela cells.

**Availability of receptors, receptor avidity and internalization of AAV in 3T3 cells and Hela cells**

Our results suggest that lower levels of AAV replication in 3T3 cells as compared to Hela cells is probably due to lower efficacy of AAV to infect 3T3 cells. The lower efficacy of infection can be attributed to any of the following stages of infection:

1. Availability and binding of AAV to its receptor on the cell surface.
2. Internalization of AAV.
3. Transport of AAV from cell membrane to nucleus.
4. AAV protein expression and DNA replication.

We analyzed each of these steps during the infection of AAV in 3T3 and Hela cell lines.

To determine the difference in binding of AAV to its receptors on 3T3 cells and Hela cells, an equivalent numbers of cells were infected with $10^5$ MOI of AAV. High MOI was used to saturate the receptors available on the cell surface with AAV. Infections were performed at 4°C for 1 hour to prevent internalization of virus. One hour later, cells were washed extensively with cold PBS on ice to remove any free virus. Cells were harvested and viral DNA was extracted as
described earlier. Real time PCR of AAV DNA was performed to determine the number of AAV genomes bound per cell. As shown in fig.11 the number of AAV genomes bound per cell was comparable in both 3T3 and Hela cells. This result indicated that the number of receptors available for binding of AAV on 3T3 cells and Hela cells are comparable.

**Number of AAV genomes bound per cell**

![Bar Chart](image)

Equivalent numbers of 3T3 and Hela cells were $10^5$ MOI of AAV at 4°C. Cells were washed and real time PCR was performed to detect to number of AAV genomes bound per cell.
Number of AAV genome bound per cell

![Graph showing AAV genome bound per cell](image)

Equivalent numbers of 3T3 and Hela cells were co-infected with 1, 10, 100 and 500 MOI of AAV and 5 MOI of adenovirus at 4°C. Two hours post infection cells were harvested and real time PCR was performed to determine the AAV genome bound per cell.

Next, we sought to determine if receptor binding of AAV in 3T3 cells is comparable to that of Hela cells. 3T3 and Hela cells were coinfected with increasing MOI of AAV (1, 10, 100, and 500) and 5 MOI of MAV-1 or Ad-5, respectively. In this experiment, low MOI for AAV infection were used to mimic the conditions that were used for analyzing AAV DNA replication. Infections were carried out at 4°C to prevent internalization of virus. One hour later cells were harvested. Viral DNA was extracted and real time PCR of AAV DNA was performed to determine the number of AAV genomes bound per cell. As shown
in fig.12, the number of AAV genomes bound per cell is comparable for both 3T3 and Hela cells. This indicated that binding avidity of AAV for its receptor in 3T3 and Hela cells was comparable.

**AAV genome internalized per 3T3 or Hela cell**

3T3 and Hela cells were coinfected with 5 MOI of adenovirus and 1, 10, 100, 500 MOI of AAV at 37°C for two hours. Cells were lysed and real time PCR was performed to detect the number of AAV genomes internalized per cell.

Next we analyzed if the internalization of virus is different in both cell lines. To this end 3T3 and Hela cells were coinfected with increasing MOI of AAV (1,
10, 100 and 500) and 5 MOI of MAV-1 and Ad-5, respectively at 37°C for 2 hours following which cells were washed with PBS three times and trypsinized to remove any virus bound to the receptors on cell surface and viral DNA was extracted. Real time PCR was performed to detect the number of AAV genome copies internalized. Fig. 13 shows comparable numbers of genome copies internalized per cell for both 3T3 and Hela cells.

These results (figure 11, 12, and 13) show that the number of genomes binding to the cell surface receptors, receptor binding of AAV and the AAV genomes internalized are comparable in both 3T3 and Hela cells. Thus AAV can indeed gain entry into both the cell lines with equal efficacy. Thus, the lower number of AAV DNAse resistant particles produced from 3T3 cells as compared to Hela cells is probably caused by the differences in intra cellular stages of viral infection in either cell line.

**AAV protein expression in 3T3 and Hela cells**

Observing a comparable number of AAV genomes bound to the receptors and internalized in both 3T3 and Hela cells we wanted to confirm if AAV Rep and Cap proteins were expressed in 3T3 cells to support AAV DNA replication and packaging. 3T3 cells were coinfectected with 10 or 100 MOI of AAV and 5 MOI of MAV-1. Cells were harvested 48 hours post infection and nuclear extracts were prepared, separated on 10% SDS-PAGE gel transferred onto a nitrocellulose membrane and probed for AAV Rep and Cap proteins. As shown in fig. 14a, all the Rep and Cap proteins of AAV were expressed in 3T3 cells.
AAV Rep and Cap expression in 3T3 cells

3T3 cells were coinfected with indicated MOI of AAV and MAV-1 nuclear extracts were prepared and separated by SDS-PAGE. AAV Rep and Cap proteins were detected by western blotting.

To determine if there was a difference in the number of cells supporting AAV protein expression between 3T3 and Hela cells, we plated equal number of cells in an 8 well chamber slide. Cells were then coinfected with increasing MOI of AAV and 5 MOI of MAV-1 or Ad-5, respectively. 3T3 and Hela cells were fixed 48 or 24 hours later, respectively, and stained for Cap proteins. As shown in figures 14b and 14c and table 2, at the highest MOI, the number of cells showing Cap protein expression is approximately 20 fold lesser in 3T3 cells than in Hela cells.
Immunofluorescence to detect cells expressing AAV cap protein

(b) Hela and (c) 3T3 cells were co-infected with 5 MOI of Adenovirus and 500 MOI of AAV. Cells were stained for AAV Cap proteins.

Table 2

<table>
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<tr>
<th></th>
<th>Hela cells</th>
<th>3T3 cells</th>
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<tbody>
<tr>
<td>DAPI stained nuclei</td>
<td>970</td>
<td>1015</td>
</tr>
<tr>
<td>Cap positive nuclei</td>
<td>417</td>
<td>37</td>
</tr>
</tbody>
</table>

Localization of Cap proteins in 3T3 and Hela cells

Having observed comparable binding, and internalization of AAV genomes in 3T3 and Hela cells we next determined if a difference in trafficking of AAV in
either cell line accounted for different levels of DNA replication and protein expression of AAV in 3T3 and Hela cells.

Equivalent numbers of 3T3 or Hela cells in 8 well chamber slides. Cells were co-infected with AAV MOI (1, 10, 100, and 500) and 5 MOI of MAV-1 or Ad-5. 3T3 and Hela cells were fixed 48 and 24 hour later respectively, and stained for Cap proteins in both cell lines. As seen in fig. 15a, we observed foci of Cap proteins in the nucleus of Hela cells; a hallmark of Cap expression. However, in 3T3 cells we observed the signal for Cap proteins in the peri-nuclear space (fig. 15b) (results shown only at the highest MOI of AAV). This signal increased with increasing MOI of AAV.

Cap localization in Hela versus 3T3 cells

<table>
<thead>
<tr>
<th>Fig.15(a) Hela</th>
<th>(b) 3T3</th>
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<tr>
<td><img src="image1.png" alt="Hela Image" /></td>
<td><img src="image2.png" alt="3T3 Image" /></td>
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</table>

(a) Hela or (b) 3T3 cells were co-infected with 500 MOI of AAV and 5 MOI of MAV-1 or Ad-5, respectively. Cells were then stained for AAV Cap proteins.
The presence of AAV Cap protein in the peri-nuclear space indicates that it is input virus rather than newly synthesized for two reasons; one, we see this signal only at the higher MOI and two that newly synthesized Cap expression is localized exclusively in nucleus.

To further confirm the localization of AAV DNA, we infected an equivalent number of 3T3 and Hela cells with 10 or 100 MOI of AAV with or without adenovirus. Cells were incubated for 4 hours at 37°C for virus to be internalized. Cells were then harvested and their nuclear and cytoplasmic fractions were separated. AAV DNA was amplified by PCR from both the fractions and separated on 2% agarose gel (figure 16).

**AAV DNA localization Hela versus 3T3 cells**

Hela and 3T3 cells were infected with indicated MOI of AAV and adenovirus for 4 hours at 37°C. Nuclear and cytosolic extracts were separated and AAV DNA was amplified.
As observed in fig. 16 it is clear that AAV DNA in Hela cells was present exclusively in the nucleus both with and without Ad-5. However, in 3T3 cells the majority of AAV DNA was present in the cytoplasmic fraction. Even in the presence of MAV-1, AAV DNA was detected in the cytoplasm even though it is known that adenovirus aids in the trafficking of AAV from cell membrane to the nucleus (Lux et al., 2005).

These experiments suggest that there is a defect in cell membrane to nucleus trafficking in 3T3 cells as compared to Hela cells for AAV2. This defect keeps AAV in the cytosol in 3T3 cells thereby causing decreased replication of AAV in 3T3 cells as compared to Hela cells even in the presence of adenovirus.

**AAV protein expression after plasmid transfection**

The genome of wtAAV was cloned into a plasmid backbone of pBR322 and is referred as pNTC244. AAV proteins are expressed and infectious DNAse resistant AAV particles can be generated by transfecting cells with this plasmid followed by adenovirus infection.

After observing a defect in AAV trafficking in 3T3 cells as compared to Hela cells we decided to analyze if the protein expression and DNA replication of AAV was comparable in 3T3 and Hela cells when transfected with pNTC244 and infected with adenovirus. This approach bypasses the intracellular trafficking of AAV and allows us to directly compare the levels of AAV replication.

First we decided to analyze if there was a difference in expression levels of Cap proteins in either cell line after transfection with pNTC244 and infection with adenovirus. Equivalent numbers of 3T3 or Hela cells were plated in 8 well
chamber slides. Cells were then transfected with pNTC244 and infected with MAV-1 or Ad-5, respectively. 3T3 and Hela cells were fixed and stained for Cap proteins. As expected we observed Cap protein expression in cells transfected and infected, however the number of Cap expressing 3T3 cells, as shown in fig. 17a and 17b, was much lower than Cap expressing Hela cells. This result demonstrated that even without the need for virus transport to the nucleus the number of cells expressing AAV proteins remain lower as compared to Hela cells.

**AAV Cap expression in Hela versus 3T3 cells after pNTC244 transfection**

<table>
<thead>
<tr>
<th>Fig. 17(a) Hela cells</th>
<th>(b) 3T3 cells</th>
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<tbody>
<tr>
<td><img src="image1" alt="Hela Cells" /></td>
<td><img src="image2" alt="3T3 Cells" /></td>
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</table>

(a) Hela or (b)3T3 cells were transfected with pNTC244 and infected with Ad-5 or MAV-1 respectively. Cells were stained for AAV Cap proteins.
AAV DNA replication after plasmid transfection

To analyze if there was a difference in DNA replication between 3T3 and Hela cells, cells were transfected with pNTC244 into the cell lines in presence of adenovirus and AAV DNA replication was determined by Southern blot.

Comparison of transfection efficacy of Hela and 3T3 cells.

Hela or 3T3 cells were transfected with pNTC244 and were harvested after 6 hours and southern blot was performed using AAV fragment as probe.* is the DpnI digested pNTC244 fragment as detected by southern hybridization.

To compare the transfection efficacy, equal numbers of 3T3 and Hela cells were transfected with pNTC244. 6 hours post transfection cells, were harvested and low molecular weight DNA was extracted. Extracted DNA was subjected to
DpnI treatment to remove the input plasmid DNA. DNA was electrophoresed on a 1% agarose gel, and analyzed by Southern hybridization. This experiment revealed comparable levels of AAV DNA in both cell lines as shown in fig. 18a thereby demonstrating that the transfection efficacy of both cell lines was comparable.

To analyze the replication efficacy of AAV DNA, equivalent numbers of 3T3 and Hela cells were transfected with pNTE244. Three hours after plasmid transfection, cells were infected with 5 MOI of MAV-1 or Ad-5. 3T3 or Hela cells were harvested 48 or 24 hours respectively post infection. Low molecular weight viral DNA was extracted, subjected to DpnI digestion to remove any free plasmid DNA, electrophoresed on a 1% agarose gel and analyzed by Southern hybridization to detect replicative forms of AAV DNA using a radio-labeled AAV DNA fragment. As seen in fig. 18b and c we observed AAV DNA replication in both Hela and 3T3 cell lines respectively. However quantification of the signal revealed that in presence of adenovirus, replication of AAV DNA in 3T3 cells is approximately 5 fold less than AAV replication in Hela cells. This result suggests that along with having a defect in trafficking there is also a defect in replication of AAV DNA in 3T3 cells as compared to Hela cells.
AAV DNA replication after plasmid transfection

(b) Hela or (c) 3T3 were transfected with pNTC244, infected with MAV-1 or Ad-5, harvested 48 or 24 hours, respectively post transfection and southern blot was performed to detect AAV DNA replication.

Results from these experiments confirm that along with trafficking defect, AAV also has a replication defect in 3T3 nucleus as compared to the Hela nucleus. These two defects acting together lead to lower levels of AAV transduction and decreased numbers of AAV DNAse resistant particles produced from 3T3 cells as compared to Hela cells.
DISCUSSION

Given the nature of the effects of AAV on host cells and helper virus, and due to the interest in the use of AAV as vector for gene therapy because of its non-pathogenicity and low immunogenic effects, it is essential that effects of AAV on host and helper virus in animal models be studied in detail. We studied the dynamics of coinfection of AAV and MAV-1 in murine fibroblasts as a prelude to studying the coinfection of AAV and adenovirus in mice. MAV-1 is an excellent model for studying adenovirus infection in vivo in mice. The first experiment we performed was to determine if murine fibroblasts supported the production of AAV DRP when coinfected with MAV-1. We compared AAV/MAV coinfections of NIH 3T3 cells to coinfection of AAV and Ad-5 in Hela cells. We observed that 48 hours post infection the number of AAV particles produced from 3T3 cells was negligible as compared to Hela cells. However the number of AAV particles produced from 3T3 cells at 96 hours post infection, when 3T3 cells start showing extensive cytopathic effect is approximately 10 fold less than the number of particles produced from Hela cells (Fig. 7). To follow up on this observation we analyzed AAV DNA replication in 3T3 cells compared to Hela cells. To detect AAV DNA replication in 3T3 an MOI of 10 or higher was required (Fig. 8a). However in Hela cells AAV DNA replication was observed at an MOI as low as 0.1 of AAV (Figure 8b). Real time PCR analysis indicated that at the highest MOI of AAV, there were approximately 30 fold fewer AAV genome copies per 3T3 cell as compared to Hela cells (Fig. 8c and 8d).
These observations led us to question whether the number of 3T3 cells supporting MAV-1 replication is lower than the number of Hela cells supporting Ad-5 replication thereby leading to decreased AAV replication. Southern analysis for replication of MAV-1 in 3T3 cells showed robust replication of MAV-1 DNA. BrdU staining of Hela and 3T3 cells after infection with Ad-5 and MAV-1, respectively, revealed comparable numbers of Hela and 3T3 cells supporting adenovirus replication.

It is well established that the transduction of rAAV vectors in different cell types can be limited at multiple stages. These stages can be lack of receptors or co-receptors on cell surface thereby preventing binding and internalization of virus, decreased trafficking from cell membrane to nucleus or inefficient conversion of single stranded AAV DNA to transcriptionally active double stranded form (Sanlioglu et al., 2001). We systematically analyzed if infection of wtAAV in 3T3 cells is limited at one or more of these stages and if coinfection of MAV-1 helps wtAAV to overcome these obstacles.

Infection of 3T3 and Hela cells with AAV at a very high MOI ($10^5$), to saturate the AAV receptors on the cell surface, revealed comparable numbers of AAV genomes bound to either cell line (Fig 11). Next, the coinfection of cells at 4°C for one hour with MOI of AAV and adenovirus identical to that used for analyzing DNA replication and production of DRP, revealed comparable numbers of AAV genomes bound per cell for both cell lines (Figure 12). These results indicated that the number of genomes binding to the receptors and the receptor avidity of AAV in 3T3 and Hela cells is comparable. However, the infection of
AAV in 3T3 cells is much poorer compared to Hela cells. This data is in agreement with previous studies (Qing et al., 1999) where it was observed that even though rAAV vector could bind to both Hela and 3T3 cells efficiently, the transduction in 3T3 cells was much lower than Hela cells.

Coinfection of Hela and 3T3 cells with AAV and MAV-1 for 2 hours at 37°C revealed that comparable levels of AAV were internalized in 3T3 and Hela cells (Fig. 13). These experiments helped us rule out the possibility of inefficient AAV receptor binding or internalization as a reason for the difference in the AAV DNA replication in 3T3 cells compared to Hela cells.

We confirmed expression of AAV Cap proteins in 3T3 and Hela cells by immunofluorescence and observed that the number of Cap positive 3T3 cells was approximately 15 fold lower than Hela cells (fig. 14b and 14c). Observing similar levels of AAV genomes bound and internalized in both Hela and 3T3 cells we questioned whether AAV can traffic from the cell membrane to the nucleus in 3T3 cells. As seen in fig. 15b, 3T3 cells coinfected with MAV-1 and AAV showed Cap staining in the perinuclear region at the highest MOI. However, this was not the case with Hela cells. PCR analysis for detecting AAV DNA in nuclear versus cytosolic fraction in each cell line showed that in 3T3 cells, AAV DNA was in the cytosolic fraction as compared to Hela cells. These results support earlier observation (Hansen et al., 2000) that even 48 hours after infection of 3T3 cells with rAAV, majority of vector DNA was in the cytoplasmic fraction as compared to 293 cells that showed nuclear localization as analyzed by southern hybridization. We observed AAV Cap signal in the cytosol upto 48 hours post infection, even
though we had coinfected cells with MAV-1. Typically, adenovirus is shown to help AAV traffic from cell membrane to nucleus (Xiao et al., 2002).

Next we questioned whether bypassing the trafficking stage of AAV infection in 3T3 cells allowed AAV DNA to replicate at levels similar to that of Hela cells. We transfected 3T3 or Hela cells with pNTC244 and infected them MAV-1 or Ad-5, respectively. Southern hybridization to detect AAV DNA replication demonstrated that even after transfection, the levels of AAV DNA replication in 3T3 cells is lower compared to Hela cells. This lower level of DNA replication in 3T3 cells could be due to differences in the support provided by host nuclear factors in 3T3 versus Hela cells. These cellular differences may cause inefficient single stranded to double stranded conversion of AAV genomes leading to decreased AAV DNA replication.

These results indicate that the intracellular milieu in Hela cells is more conducive for AAV replication than 3T3 cells. It would be interesting to follow up these studies with experiments to identify the cytosolic compartment in which AAV is obstructed in 3T3 cells. Further, since EGFR inhibitors can increase transduction by rAAV vectors in non-permissive cell lines by phosphorylating ssDBP (Mah et al., 1998), (Smith et al., 2003) it would be interesting to determine if treatment with these chemical agents increases AAV DNA replication in 3T3 cells.

For studying the replication of wtAAV in mice with MAV-1 provided helper functions, we will use Swiss outbred mice strain. This strain will serve as a good model as they are reported to support transduction by AAV2 for upto 1.5 years.
(Xiao et al., 1996). MAV DNA was detected (Kajon et al., 1998) in endothelial cells of the brain and spinal cord and also in lungs and kidney after intraperitoneal or intranasal delivery of virus in Swiss outbred mice. Infection of MAV-1 results in chemokine upregulation and cellular inflammation in the lung (Weinberg et al., 2005). We will coinfect the mice with MAV and AAV2 and then analyze if presence of AAV during infection alters the profile of chemokine expression. These studies will help us to determine if AAV changes the pathogenic effects of MAV for good or bad. We will also analyze tissues for the expression of AAV replication and determine the tissues in which AAV might establish a latent infection.
CONCLUSIONS

1. Coinfection with mouse adenovirus (MAV-1) provides the necessary helper functions for a productive infection of AAV in NIH 3T3 cells.

2. The number of AAV particles produced from NIH 3T3 cells after coinfection with MAV-1 and AAV2 is approximately 10 fold less than the number of AAV particles produced from Hela cells after coinfection with Ad-5 and AAV2.

3. AAV DNA can replicate in NIH 3T3 cells at higher MOI with help provided by MAV-1 however level of replication is approximately 30 fold less than level of replication in Hela cells at identical MOI.

4. Replication of MAV-1 in NIH 3T3 and Ad-5 in Hela cells is at comparable levels.

5. The decrease in MAV-1 replication after coinfection with AAV in 3T3 cells is approximately 5.5 fold at the highest MOI (500) whereas Ad-5 replication decreased by approximately 16 fold at an identical MOI.

6. Receptor binding and internalization of AAV is comparable in NIH 3T3 and Hela cells.

7. As compared to Hela cells AAV has a trafficking defect in NIH 3T3 cells that prevents efficient entry of AAV into the nucleus of infected NIH 3T3 cells.

8. Once into the nucleus replication of AAV DNA is slower in 3T3 cells as compared to Hela cells.
SUMMARY

In this dissertation, the replication of adeno-associated virus (AAV) with mouse adenovirus (MAV-1) provided helper function in murine fibroblasts was studied. We observed that upon coinfection of NIH 3T3 cells with MAV-1 and AAV, AAV DNAse resistant particles (DRP) are produced indicating a productive infection of AAV in these cells. However, the number of AAV particles produced from 3T3 cells was dramatically lower in comparison to the number of particles produced from Hela cells after coinfection with AAV and Ad-5. Furthermore, analysis of AAV DNA replication in 3T3 cells as compared to Hela cells demonstrated replication of AAV DNA only at higher MOI in 3T3 cells. Even at the highest MOI (500), replication of AAV was approximately 30 fold less in 3T3 cells as compared to Hela cells.

Comparison of MAV-1 and Ad-5 DNA replication in 3T3 and Hela cells, respectively, demonstrated a robust replication by Southern blot. Real time PCR data indicated the accumulation of comparable number of replicative forms MAV-1 and Ad-5 DNA. As expected, replication of Ad-5 DNA decreased with increasing MOI of AAV with a decrease of approximately 16 fold at highest MOI of AAV. Furthermore, the number of 3T3 and Hela cells supporting MAV-1 and Ad-5 DNA replication respectively was comparable as observed by BrdU staining of infected cells.

A systematic analysis of the various stages of AAV infection revealed that binding of AAV to its receptor on the cell surface of Hela or 3T3 and its internalization in both cell lines is comparable. However, once internalized the
3T3 cells have a trafficking defect that prevents efficient entry of AAV into the nucleus of 3T3 cells thereby leading to lower levels of AAV DNA replication. Additionally, even after abrogation of this trafficking defect by transfecting a proviral plasmid in 3T3 or Hela cells and infecting them with MAV-1 or Ad-5 respectively, replication of AAV DNA is lower in 3T3 cells.
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ABSTRACT

The adeno-associated virus (AAV) replicates to high titer when host cell are coinfected with a helper virus. Here we analyzed the coinfection of AAV and mouse adenovirus (MAV-1) in murine fibroblasts. We observed that MAV-1 coinfected 3T3 cells produced approximately 10-40 fold less AAV DNAse resistant particles (DRP) than Hela cells. Levels of AAV DNA replication were approximately 30 fold less in 3T3 cells as compared to Hela cells coinfected with human adenovirus (Ad-5). A study of these lower levels of infection in 3T3 cells compared to Hela cells revealed that receptor binding and internalization of AAV in 3T3 and Hela cells was comparable. However, AAV did not enter into the nucleus of 3T3 cells as efficiently as it does in Hela cells. Furthermore, viral DNA replication levels of AAV DNA were found to be lower in 3T3 cells than Hela cells even after transfection of proviral plasmid indicating a defect in support for the AAV DNA replication as well in 3T3 cells as compared to Hela cells.