PROTEIN PROCESSING STRATEGIES BY ADENO-ASSOCIATED VIRUS TYPE 5 (AAV5) AND THE EFFECTS OF THE ADENOVIRUS E4ORF6/E1B-55K/CULLIN 5 E3 UBIQUITIN LIGASE COMPLEX ON AAV PROTEIN STABILITY.

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by

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

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Presented by **Kerry David Farris**,

A candidate for the degree of DOCTOR OF PHILOSOPHY

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ABSTRACT

We report the initial identification of the ubiquitination of a parvovirus non-structural protein by the adenovirus E4orf6/E1b-55k/Cullin 5 E3 ubiquitin ligase complex. The small Rep and capsid proteins of adeno-associated virus type 5 (AAV5) were found to be specifically targeted for degradation in a proteasome-dependent manner by the adenovirus E3 ubiquitin ligase complex. This effect was at least partially dependent upon the levels of substrate protein within the cell, as significant overexpression of Rep during transient transfection prevented the degradation of the protein in 293 cells in the presence of E4orf6. This finding is in agreement with the observation that during adenovirus infection or transient co-transfection, adenovirus VA RNA is capable of overcoming this degradative effect by enhancing the overall levels of translated AAV protein. AAV5 small Rep proteins were found to associate with adenovirus E1b-55k in the absence or presence of E4orf6; however, Rep only co-immunoprecipitated with Cullin 5, the largest anchor protein portion of the complex, when E4orf6 was present. We demonstrate that the adenovirus E3 ligase functions to add ubiquitin moieties in a specific manner to AAV proteins and results in the targeting of these AAV proteins for destruction by the

proteasome. The AAV5 small Rep proteins were also found to be ubiquitinated in 293 cells during transient transfection in the absence of E4orf6. This is the first example of poly-ubiquitination of a parvovirus non-structural protein.

The AAV2 small Rep proteins Rep52 and Rep40 both display superfamily 3 (SF3) helicase activities and are both required for efficient unwinding of replicated AAV DNA and packaging into preformed capsids. In AAV2, these proteins differ in their C-termini due to alternative splicing of the pre-messenger RNA (pre-mRNA) transcript, and it has been suggested that the size of Rep40 allows for it to form a hexameric structure upon binding of its substrate; however, Rep52 remains a monomer in solution. The transcription profile of AAV5 has revealed that upstream Rep-encoding transcripts are predominantly poly-adenylated within the intron and are thus not spliced. This lack of splicing of Rep-encoding pre-mRNAs predicts that only Rep52 and not Rep40 would be made; however, significant expression of a Rep40-like protein in AAV5 was consistently observed during infection and transient transfection. We report that unlike AAV2, AAV5 likely utilizes an additional internal methionine translation start site within the Rep52 reading frame to encode Rep40, a start site that also is within AAV2, but is not used. Therefore AAV5, and the other animal AAVs, have likely evolved an alternate strategy from that of the AAV2-like AAVs to encode this potentially critical protein.

The splicing of small internal introns is often governed by the rules of intron definition, where all of the *cis* splicing signals required for splicing regulation are within the intron. During transient transfection of AAV RepCap in 293 cells, the levels of AAV1, AAV2, and AAV6 pre-mRNA splicing in the absence of adenovirus are low. However, in contrast to AAV2, whose levels of pre-mRNA splicing in the presence of

adenovirus are significantly enhanced, the levels of pre-mRNA splicing of AAV1 and AAV6 pre-mRNAs remains low in the presence of adenovirus. We report that this splicing difference is due to *cis*-acting sequences within the intron, and the improvement of the nonconsensus AAV donor to that closer or identical to that of the U1 snRNP splicing factor recognition site significantly enhances splicing of all AAV pre-mRNAs regardless of serotype. Enhancement of the AAV donor to that closer to the consensus donor also resulted in the improvement of recombinant AAV (rAAV) production, whether the Rep and capsid proteins were supplied on a single plasmid or on separate plasmids. In a split-vector system, levels of rAAV were further enhanced by the replacement of the cytolomegalovirus immediate early promoter (CMV-IE) with that of the AAV5 P41 promoter to drive expression of the capsid proteins. In 293 cells, transcripts from the constitutively-active AAV5 P41 promoter were more efficient at splicing and thus resulted in an additional increase in rAAV production such that levels of production were approximately 10-15-fold greater than those obtained through traditional methods. Statistically speaking, these rAAVs were equally as efficient at transducing HeLa cells as those made in the traditional manner, indicating that the enhancement of splicing has revealed to be a simple method by which to enhance the production of rAAV during transient transfection.

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I. INTRODUCTION

AAV and the other parvoviruses.

The adeno-associated viruses (AAVs) are small, non-enveloped, single-stranded DNA (ssDNA) viruses belonging to the family *Parvoviridae*. This family can be subdivided into two sub-families: the *Parvovirinae* and the *Densovirinae*, which infect animals and insects, respectively. The *Parvovirinae* can be even further divided into five distinct genera: the *Parvoviruses* (e.g., Minute Virus of Mice, MVM), *Erythroviruses* (B19), *Dependoviruses* (AAV), *Amdoviruses* (Aleutian Mink Disease Virus, AMDV), and *Bocaviruses* (Bovine Parvovirus) **(Figure 1-1)**. The *Parvoviridae* are among the smallest known viruses (usually between 4 and 6 kilobases (kb) in length), are universally consisted of ssDNA genomes, and can infect a variety of animal species. Some *Parvoviridae*, such as B19, are capable of causing serious diseases; however, others, such as AAV, are considered non-pathogenic (150). It is partially on this basis that AAV remains an attractive candidate as a human gene therapy vector, a topic that will be discussed in more detail later in this introduction.

As a member of the *Dependoviruses*, AAV is unique from the other *Parvovirinae* in that it is generally replication deficient in the absence of a helper virus, such as adenovirus or herpesvirus, although there are some instances in which low-level replication may occur in the absence of helper virus function (14, 163). In the absence of helper function, wild-type AAV integrates very efficiently into the long arm of human chromosome 19 (39) **(Figure 1-2)**. AAV remains in this latent form until it is rescued by co-infection with adenovirus or herpesvirus, or in some instances, DNA damage. During

co-infection with a helper virus, AAV will excise its integrated genome and begin its lytic phase through productive replication and by generating viral progeny for subsequent re-infection (102).

Figure 1-1. Members of the subfamily *Parvovirinae*.

Phylogenetic relationship as determined by sequence alignments between the non-structural proteins of members of the subfamily *Parvovirinae*. Numbers indicate percent amino acid identity among the non-structural proteins of the indicated parvoviruses. Adapted from (150).

Figure 1-1. Members of the subfamily *Parvoviridae*.

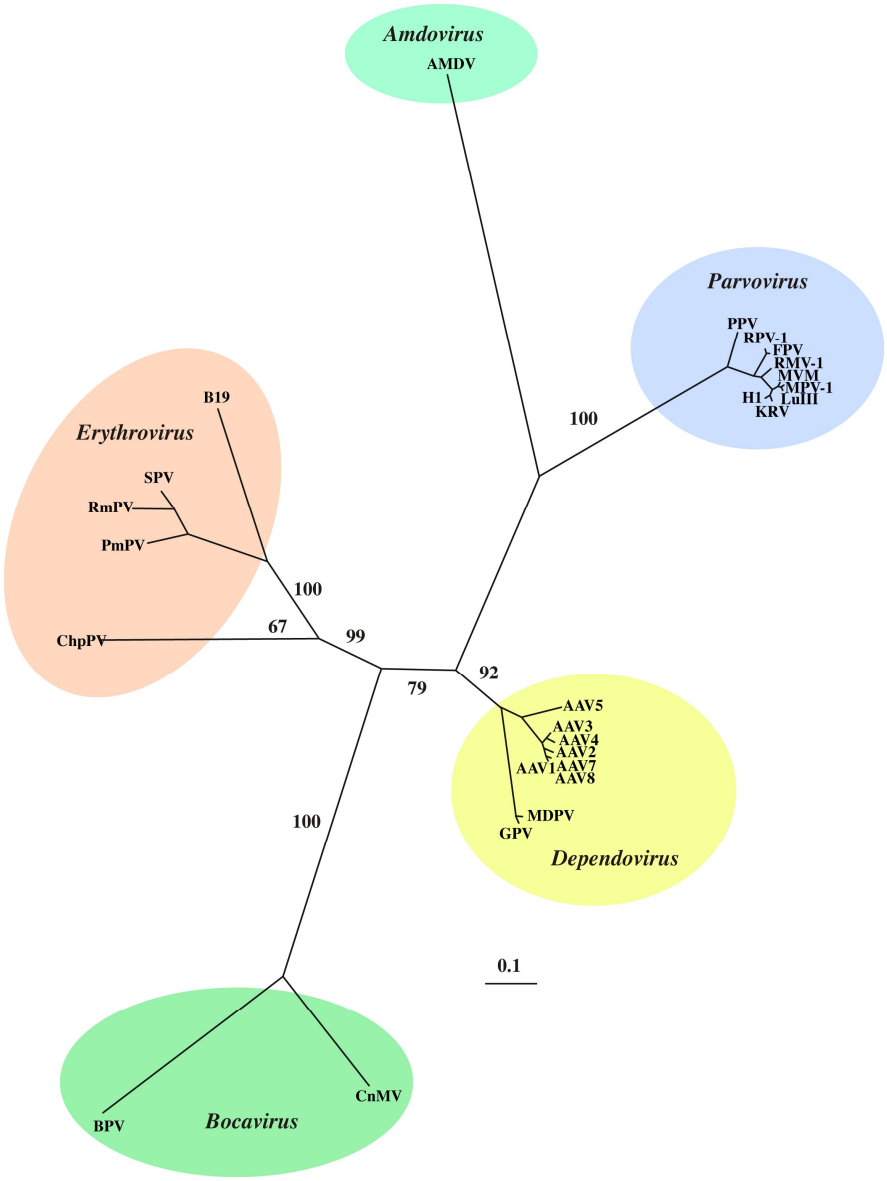
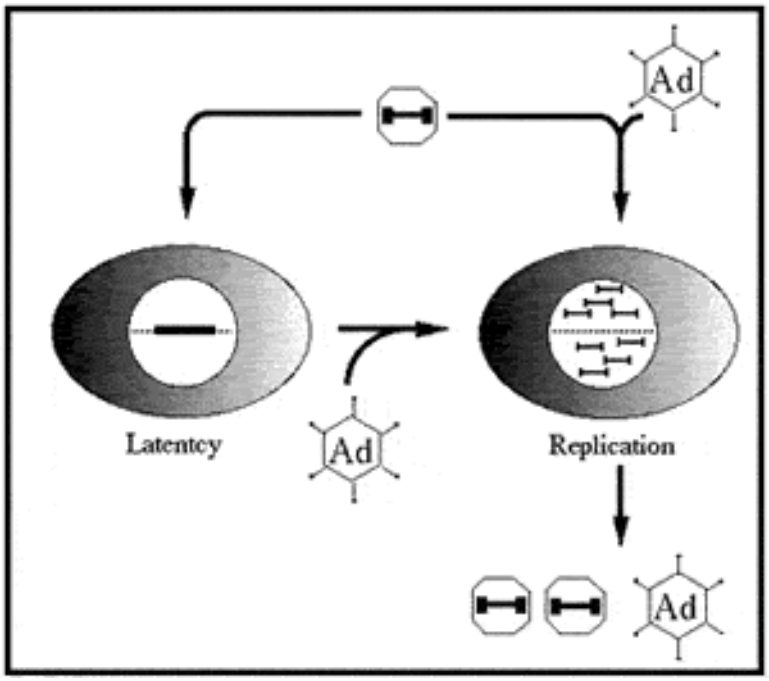


Figure 1-2. Diagram of the Life Cycle of AAV2.

In the absence of helper-virus co-infection, AAV2 integrates quite efficiently into the long arm of human chromosome 19 in a process that requires low-level Rep expression. During helper-virus co-infection, the AAV2 genome is excised from the chromosome and proceeds into its lytic phase. Sizes of AAV virions (with AAV genomes represented as "dumbbells") and adenovirus virions (Ad) are not to scale. Figure borrowed from **www.biology.ucsd.edu/faculty/weitzman.html**.

Figure 1-2. Diagram of the Life Cycle of AAV2.



Transcription profile of AAV2.

Members of the *Dependoviruses* share structurally similar, but slightly varied, transcription profiles (116-119). The general layout of an AAV genome involves two major open reading frames (ORFs) in the same orientation but in different reading frames. The first ORF encodes the large and the small non-structural proteins, called Rep, and the second ORF encodes the three structural proteins, or capsid proteins.

All adeno-associated viruses utilize three promoters to generate the pre-mRNAs that will encode all of the structural and non-structural proteins necessary for replication and productive infection in the presence of a co-infecting helper virus. Proteomic diversity, and stoichiometric balance, of the major structural and non-structural proteins made by AAV are the result of proper usage and alternative splicing of a single, small, internal intron of approximately 300 nucleotides (nt) in size. This internal intron consists of a single, non-consensus donor (D), and two acceptors (A1 and A2).

AAV2 **(Figure 1-3A)**, as well as most of the other human and non-human primate AAVs, have three promoters: P5, P19, and P40, each named for their corresponding map units, whereas AAV5 **(Figure 1-3B)** and the other animal AAVs utilize three promoters at map units P7, P19, and P41. Although these promoters vary slightly in their relative locations on the genome, their basic functions are identical. However, as our lab has demonstrated, and as will be described in more detail later, there are several key differences between AAV5 (and the other animal AAVs) and AAV2 (and the other human and non-human primate AAVs) that serve the basis and the motivation for much of our work.

Figure 1-3. Transcription profiles of AAV2 (and the AAV2-like AAVs) versus AAV5 (and the animal AAVs).

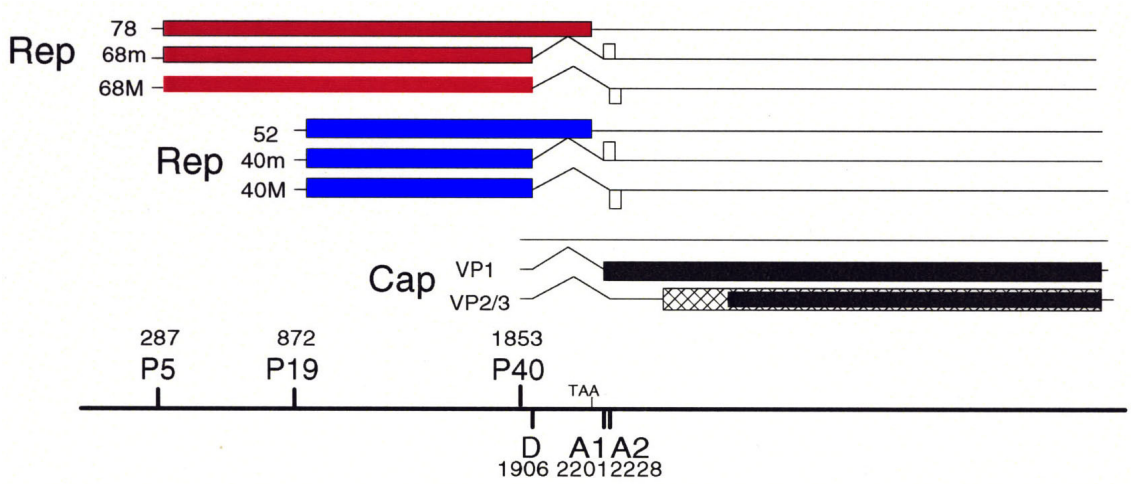
(A) The transcription profile of AAV2 and AAV2-like AAVs (inverted terminal repeats, ITRs, not shown) (from ref. 119). AAV2 utilizes three promoters, at map units P5, P19, and P40. These promoters, in addition to the alternative splicing of the small internal intron, provides all pre-mRNAs necessary for the coding of all AAV proteins. The numbers 78, 68m, 68M, 52, 40m, and 40M represent the kDa of the large and small Rep (non-structural) proteins. The major (M) and minor (m) forms of the Rep68 and Rep40 proteins differ in the utilization of either the first splice acceptor (A1 for the minor forms) or the second splice acceptor (A2 for the major forms). The capsid proteins VP1, VP2, and VP3 are generated from spliced P40 mRNAs, with VP1 originating from spliced messages utilizing the first acceptor (A1) and with VP2 and VP3 originating from spliced messages utilizing the second acceptor (A2). Both acceptors share the same donor (D). Nucleotide positions of the promoters and splice sites are indicated. Hatched bar in VP2/3 indicates region of VP2 (coded with a non-traditional ACG initiation codon) not shared by VP3. "TAA" refers to to the stop codon of Rep78 and Rep52.

(B) The transcription profile of AAV5 and the animal AAVs (from ref. 118). AAV5 also utilizes three promoters, at map units P7, P19, and P41. Unlike AAV2, AAV5 transcripts from the P7 and P19 promoters are preferentially polyadenylated within the intron. In addition, in cells expressing E1A, AAV5 promoters are constitutively active, even in the absence of AAV Rep proteins or any additional adenovirus gene products. Positions of the three promoters, the initiator sequence within the 5' ITR (Inr), as well as the splice donor and acceptors, are indicated by the numbers.

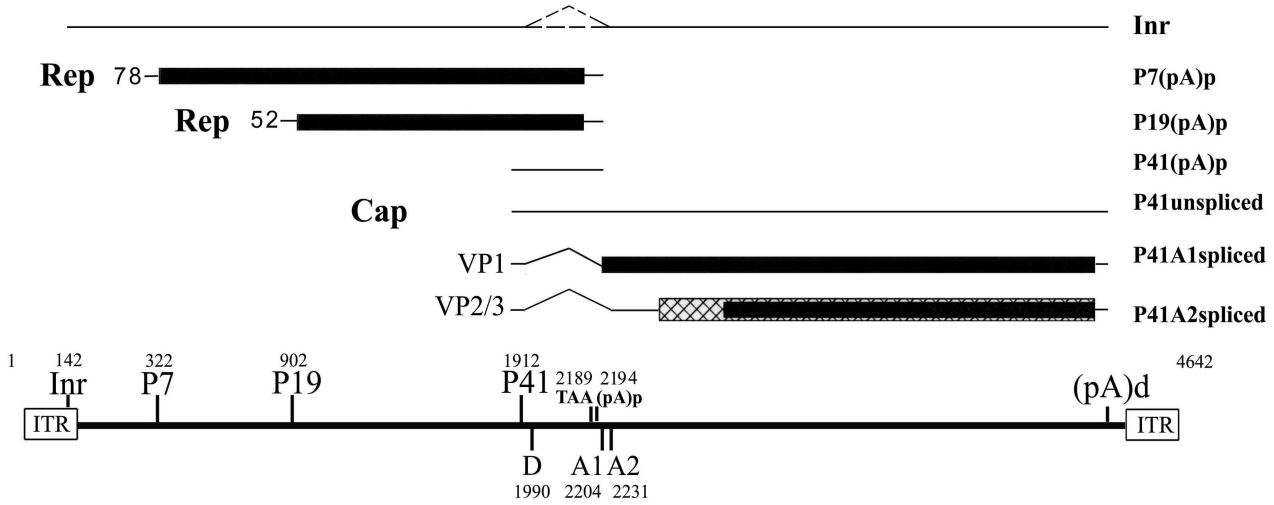
"TAA" refers to the stop codon of Rep78 and Rep52. (pA)p and (pA)d refer to the proximal and distal polyadenylation sites, respectively. On the right, Inr, P7(pA)p, P19(pA)p, P41(pA)p, P41unspliced, P41A1spliced, and P41A2spliced, are the names of the RNAs indicated by the lines. Lines without black bars represent mRNAs that are not hypothesized to be translated.

Figure 1-3. Transcription profiles of AAV2 (and the AAV2-like AAVs) versus AAV5 (and the animal AAVs).

(A)



(B)



In AAV2, transcripts from the P5 promoter are alternatively spliced to generate the large non-structural proteins Rep78 and Rep68. The large Rep proteins possess the vast majority of AAV-encoded protein functions necessary for AAV replication, including: DNA helicase activity, terminal resolution, replication, ATPase activity, site-specific integration, transcriptional activation, and splicing (14, 28, 99, 102, 121, 163, 164). Although alternative acceptor usage theoretically generates two spliced Rep68 proteins, the amino-acid differences between these two proteins is minute at best, and currently there are no known functional differences between the two spliced isoforms of the large Rep68 protein, called Rep68 major (68M) and Rep68 minor (68m). Several subtle differences in function have been noted previously, however, between Rep78 and Rep68; the primary differences involving large Rep protein interactions with cellular gene products (25, 32, 50, 140). Currently, there are no known functional differences between Rep78 and Rep68 regarding AAV itself, as in processes such as splicing, transcriptional activation, replication, or site-specific integration.

In AAV2, transcripts from the P19 promoter are alternatively spliced to generate the small non-structural proteins Rep52 and Rep40. Similarly to Rep68, alternative splice acceptor usage can theoretically generate two spliced isoforms of Rep40; however, there are currently no known functional differences between the two spliced forms of this protein. Until recently, little has been known about the functions of the small Rep proteins other than their well-characterized roles in the packaging of ssDNA genomes into pre-formed capsids (71). Recent studies have demonstrated significant functional differences between these two proteins. These important differences will be discussed in

greater detail later, and serve as part of the primary motivation for the experiments described in chapter 3.

Currently, the only known role of P40-generated pre-mRNAs is in the processing of the three capsid proteins: VP1, VP2, and VP3. It is well established that alternative splicing of the small internal AAV intron is required for capsid production (7, 22, 153). It is unknown whether unspliced messages from P40 generate a significant protein product, or if such a product would be functional. Splicing of the donor to the first acceptor yields mRNA that encodes the largest capsid protein, VP1. Splicing of the donor to the second acceptor yields mRNA that encodes the other two capsid proteins, VP2 and VP3. VP2 utilizes an atypical translation initiation site of ACG, which is predominantly read-through to the downstream AUG start codon for VP3. The relative ratios of these proteins that have been demonstrated previously to be most efficient at allowing the assembly of functional fully-assembled capsids is 1:1:10 (VP1:VP2:VP3). Disruption of this stoichiometric balance has significant adverse effects on packaging and infectivity.

Differences between AAV2 and AAV5.

Although AAV2 and AAV5 are similar in their overall design, their large Rep proteins share only approximately 60% identity, and our lab has found that there are several subtle but significant differences between these two viruses (118, 173). These differences have drastic impacts on the transcription profiles and protein processing strategies between the viruses. Many projects in our lab currently address these differences and how they affect the life cycle of AAV. We believe that studying these differences will give insight on the complex nature of these smallest of viruses, teach us how they interact with not only the host but other viruses, allow us greater understanding of their complex evolution, and may enable additional methods of improving the field of gene therapy.

Similarly to AAV2, AAV5 utilizes three promoters to generate the pre-mRNAs that encode the structural and non-structural proteins **(Figure 1-3B)**. Transcripts from the P7 promoter, like those from the P5 promoter in AAV2, encode the major non-structural protein, Rep78. The functions of AAV5 Rep78 are virtually identical to those of the AAV2 large Rep proteins. However, unlike AAV2, whose upstream pre-mRNAs are polyadenylated at the 3'-end of the genome, AAV5 P7 pre-mRNAs are preferentially polyadenylated within the intron (118). As a result, the splicing efficiencies of these upstream transcripts are extraordinarily low, and no detectible levels of Rep68 protein are generated by P7. Unlike the other human and non-human primate AAVs such as AAV1, AAV2, AAV6, and AAV8, whose large Rep proteins are virtually interchangeable, the large Rep78 protein from AAV5 is unable to perform many of its functions within the context of AAV2, and vice-versa (118).

Transcripts originating from the P19 promoter also encode the small Rep proteins of AAV5, which are required in AAV2 for packaging of the ssDNA genomes into preformed capsids (71). Surprisingly, AAV5 is able to generate significant levels of both Rep52 and Rep40 despite the low levels of splicing of the upstream transcripts. Investigations into the origins of the small Rep40 protein in AAV5 reveal that unlike AAV2, which uses alternative splicing to generate this critical gene product, AAV5 likely uses an alternate initiation codon within the P19 transcript to encode Rep40. Potential implications of this phenomenon will be discussed in chapter 3.

The adenovirus helper gene products E1b-55k and E4orf6, and their effects on host cell and AAV biology.

AAV lacks many protein functions necessary to autonomously replicate and requires helper function from viruses such as adenovirus or herpesvirus to establish a productive infection. Although wild-type adenovirus is quite efficient at allowing the replication of AAV, studies on the helper requirements of AAV from adenovirus have determined that only five early gene products are required for productive AAV replication and infection. These five adenovirus gene products are: E1a, E1b, E2a, E4orf6, and VA RNA. The primary focus of this section will regard the adenovirus gene products E4orf6 and E1b-55k.

The adenovirus type 5 (Ad5) early gene 1B (E1b) mRNA a single pre-mRNA that is alternatively spliced to generate three separate mature RNAs (74). Three proteins of molecular weights 14.4 kilodalton (kD), 21 kD, and 55 kD are encoded by these mature RNAs (85). Mutants in E1b-55k are 100-fold defective in virus production. E1b-55k is involved in post-transcriptional regulation of host cell as well as late-stage adenoviral gene products. Adenovirus mutants for E1b-55k are defective in their abilities to inhibit host cell protein synthesis. Furthermore, there is a significant reduction in the levels of late viral cytoplasmic mRNAs, which has subsequent negative effects on the expression of viral late proteins. Thus, the E1b-55k protein is involved in bringing about a change from the early to the late viral gene expression (45) and is also involved in viral and cellular mRNA nuclear export (75, 172).

In adenovirus-infected cells, the E1b-55k protein is found in a complex with the cell cycle regulation protein p53 (123). This complex also contains the adenovirus early

gene product E4orf6. During wild-type adenovirus infection, E4orf6 re-localizes E1b-55k to the nucleus; however, in the absence of E4orf6, E1b-55k remains almost exclusively in the cytoplasm (76, 101).

An adenovirus mutant null for the E1b-55k gene was defective in acting as a helper virus for AAV replication, and was defective for allowing the accumulation of AAV capsid proteins. Additional studies conducted with this same mutant suggested that E1b-55k was actively involved in the cytoplasmic export of AAV mRNA (129). In addition to its well-established role in the degradation of p53 (described in greater detail below), E1b-55k has the added ability to inhibit p53-dependent transcription by binding to its N-terminal activation domain (67, 131, 174) and can function as a general repressor of RNA polymerase II-mediated transcription (83, 174).

The adenovirus E4 mRNA, similarly to E1b, also consists of a single precursor pre-mRNA that is alternatively spliced to generate a number of different mature RNAs. In the case of E4, seven different spliced forms of the precursor pre-mRNAs are produced to encode seven different proteins. Most of these spliced mRNAs are identical at their 5' and 3' ends and carry a small 5' leader sequence (107, 161).

Initial experiments to determine the function of the different E4 gene products involved specific deletions of each of the reading frames. Interestingly, deletions in six of the seven reading frames had no effects on the growth of adenovirus; however, when a deletion was made in the E4orf6 coding region such that only the N-terminal region of the E4orf6 protein was produced, viral growth was significantly repressed (59, 60). An adenovirus mutant in which the entire E4 gene was completely deleted caused a further deficit in viral growth than the E4orf6 mutant alone, even though individual mutants had

no effect. This suggested that there was at least some degree of functional redundancy between the different gene products encoded by E4 pre-mRNAs. The Ad mutant for E4orf6 demonstrated similar defects in the accumulation of late mRNAs, the export of viral mRNAs, and the inhibition of host cell protein synthesis as the E1b-55k mutant (59, 60), indicating that these two gene products may work in concert to establish their effects.

The defect in viral growth of E4 mutant adenoviruses is partially due to the tendency of adenovirus DNA to form concatamers in the absence of E4orf6. These concatamers inhibit viral replication and are caused by an activation of the DNA damage response, otherwise inactive during wild-type adenoviral infection. The E4orf6 mutant induced a strong DNA damage response by a complex containing the DNA repair proteins Mre11 (meitotic recombination gene 11), Rad50 (radiation sensistive gene 1), and Nbs1 (nijmegen breakage syndrome gene 1). During wild-type infection, E4orf6 inhibits this response by mediating the degradation of this complex in a proteasome-dependent manner. This phenomenon also requires E1b-55k, and the two proteins together form an E3 ubiquitin ligase complex with a number of cellular proteins including elongin B, elongin C, Rbx1 (ring box protein 1), and cullin 5. Therefore, adenovirus employs an interesting strategy to overcome negative effects on the virus by the host antiviral innate immune response (144).

Two major E4 open reading frames (3 and 6) have been shown to possess redundant phenotypes with regards to the prevention of genome concatamer formation during adenovirus infection. The Ad5 E4orf6 accomplishes this task by mediating the ubiquitination and subsequent degradation of Mre11 (20, 144). E4orf3 functions to mislocalize Mre11 from its normally diffuse nuclear localization pattern to large nuclear

and cytoplasmic aggregates (2, 144). This altered localization pattern prevents concatamer formation by preventing the accumulation of the Mre11/Rad50/Nbs1 DNA repair complex within viral replication centers. Although the degradation of the Mre11 complex can occur in the presence of functional E4orf6 from all adenovirus serotypes (145), the E4orf3 proteins from adenovirus serotypes 4 and 12 are incapable of mediating the relocalization of Mre11 and its partners, indicating that there are significant functional differences between the adenovirus serotypes that warrant further study with respect to the functions of their E4orf6 and E4orf3 gene products. In addition, the process of adenovirus DNA concatamerization is also reliant upon the enzyme DNA ligase IV. DNA ligase IV has also been found to be a substrate of E4orf6/E1b-55k/Cullin 5-mediated ubiquitination and degradation by the proteasome (6).

The cell cycle regulator, p53, is the host cell's first line of defense against viral infection and other potentially devastating DNA inflictions. During adenovirus infection, p53 becomes activated and is briefly stabilized by adenovirus E1a. This brief stabilization is necessary for the initiation of adenovirus early gene transcription. However, prolonged activation of p53 causes a signaling cascade that induces the host cell to undergo apoptosis. Therefore, adenoviruses have developed an interesting strategy to overcome the inhibitory effects of p53 by bringing about the degradation of p53 by means of the E4orf6/E1b-55k/Cullin 5 E3 ubiquitin ligase complex in a manner identical to that utilized for the destruction of the Mre11 DNA damage complex (122-124).

During the late stages of adenoviral infection, the production of adenoviral proteins dominates the production of host cellular proteins. This block in host protein

accumulation is at the step of pre-mRNA export and is mediated by the adenovirus E3 ubiquitin ligase complex in a manner independent of p53 (12, 33, 167).

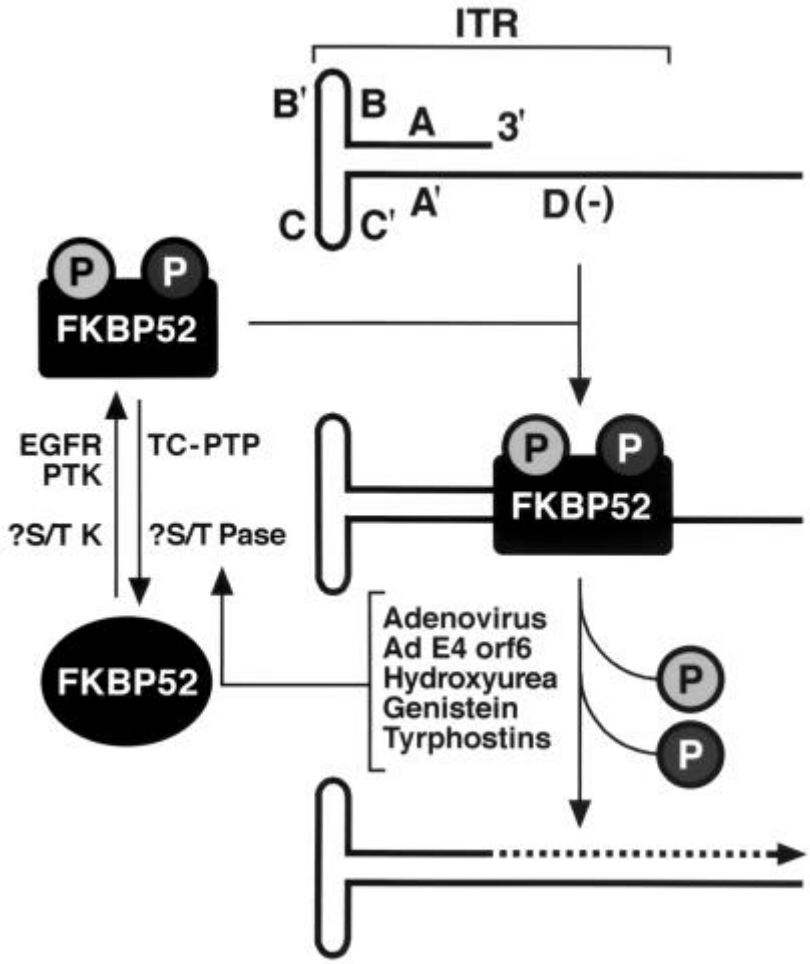
Much of the initial understanding of the functions of E4orf6 was the result of work on its role in the life cycle of AAV. One of the first discovered roles of E4orf6 in the biology of AAV regarded the requirement of E4orf6 in the second-strand synthesis of the nascent ssDNA genome for replication. Conversion of the ssDNA viral genome into a transcriptionally active double-stranded intermediate is currently believed to be one of the major rate-limiting steps in the transduction and expression of rAAV-encoded vector gene products (42, 43). During wild-type AAV and adenovirus co-infection, the E4orf6 protein is responsible for the head-to-tail concatamerization of AAV genomes, a process required for AAV replication (35, 36).

Phosphorylation of a 52 kilodalton (kD) cellular protein, FK506-binding protein (FKBP52), prevents the conversion of AAV ssDNA to the double-stranded replication intermediate **(Figure 1-4)**. Phosphorylated FKBP52 binds to the single-stranded D-sequence in the AAV2 inverted terminal repeat (ITR) (114). FKBP52 is phosphorylated at tyrosine residues by the epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK) and de-phosphorylated by T-cell protein tyrosine phosphatase (TC-PTP) (112, 113). During adenovirus co-infection, FKBP52 is de-phosphorylated and this allows for the conversion of AAV ssDNA to the double-stranded form. Preliminary data from our lab indicates that EGFR-PTK may be a substrate for the E4orf6/E1b-55k/Cullin 5 E3 ubiquitin ligase complex (Nayak, *et al.,* unpublished), suggesting multiple roles of E4orf6 and E1b-55k in the life cycle of AAV.

Figure 1-4. Role of FKBP52 in the life cycle of AAV.

When phosphorylated by EGFR-PTK, FKBP52 binds to the single-stranded D-sequence in the AAV2 inverted terminal repeat (ITR). This binding prevents conversion of the ssDNA genome into the double-stranded intermediate form. During adenovirus infection, FKBP52 becomes de-phosphorylated through a mechanism that is still not completely understood, and conversion of the ssDNA to dsDNA is allowed. Adapted from (111).

Figure 1-4. Role of FKBP52 in the life cycle of AAV.



In addition to their established roles in AAV replication, adenovirus E1b-55k and E4orf6 are required along with E1a, E1b, and VA RNA for transcription, splicing, and translation of AAV pre-mRNAs, although the precise requirements for these processes vary between AAV2 and AAV5 (96, 118, 119, 173). During transient transfection in 293 cells, the levels of AAV5 capsid proteins were also found to be negatively regulated in the presence of E4orf6, indicating a potential role for the adenovirus E3 ubiquitin ligase complex on the accumulation of AAV proteins (96).

The ubiquitin-conjugation and proteasomal pathways.

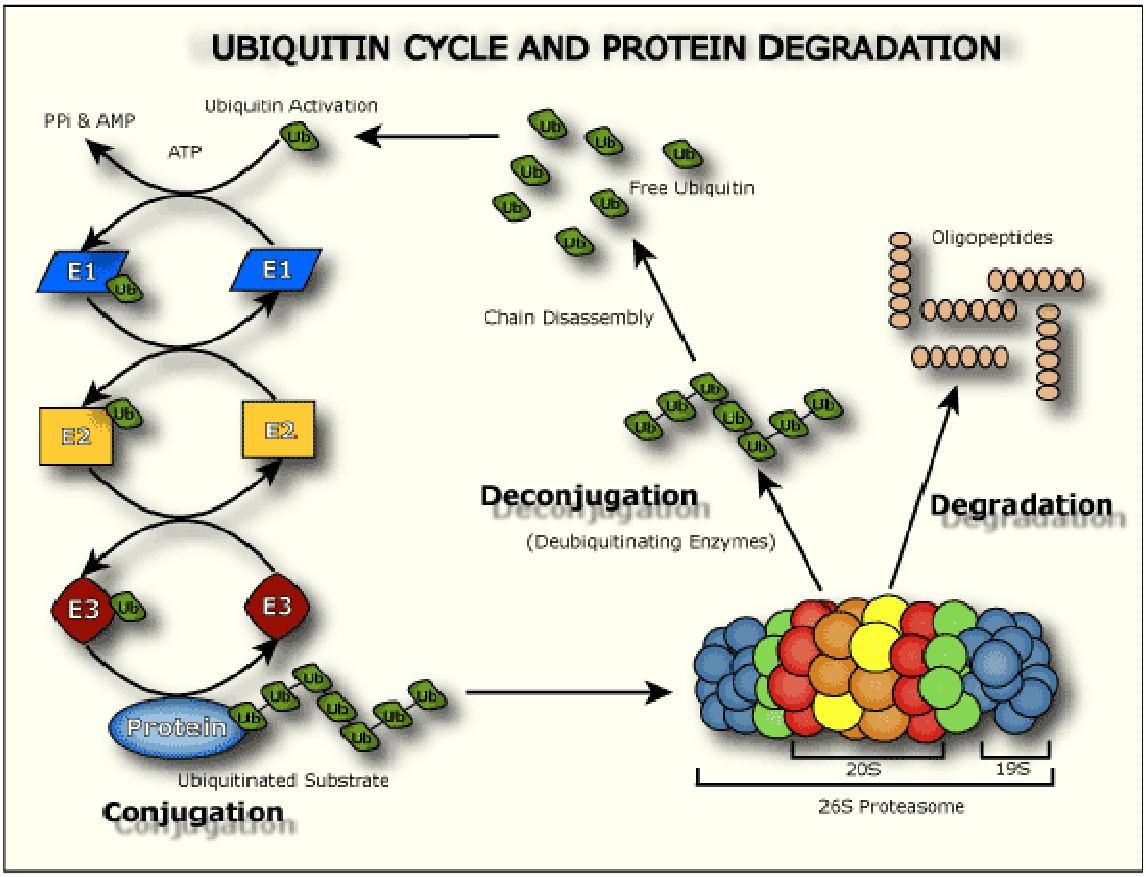
The stoichiometric balance and abundance of all proteins in the cell must be tightly regulated for the cell to survive. This can be accomplished through regulating the synthesis or turnover of the proteins. It is imperative that the process of protein turnover be extremely specific as to not cause the improper degradation of proteins needed by the cell at any given moment in the cell's life cycle. In addition, this specificity ensures that proteins not required for cellular function are eliminated from the cellular milleu, as improper protein expression can have adverse effects on the cellular environment. The processes of degradation by the proteasome and lysosomal degradation are the two methods by which the process of protein turnover is highly regulated. Prior to proteasomal degradation, substrate proteins are covalently tagged with poly-ubiquitin chains by ubiquitin ligases **(Figure 1-5)** (53, 110)

The protein ubiquitin is a small, "ubiquitous" protein of approximately 8 kD that is expressed in all cells. The process of ubiquitination typically involves three steps. In the first step, ubiquitin is activated at its C-terminus by an E1 activating enzyme to form an E1-ubiquitin thioester bond in an ATP-dependent reaction. Step two involves the transfer of the activated ubiquitin from the cysteine of E1 to the cysteine of one of many ubiquitin-conjugating enzymes, E2. From here, the E2-conjugated ubiquitin conjugating enzyme can either transfer the ubiquitin to the substrate itself or can transfer the ubiquitin to the substrate with the help of a third enzyme ubiquitin ligase, also called E3. Ubiquitin ligases are comprised of numerous families of protein complexes, each made up of a myriad of protein components. Each E3 ligase complex is generally specific for a particular protein substrate or set of substrates that have a common function, such as the

Figure 1-5: The ubiquitin ligase pathway.

The process of attaching ubiquitin moieties to a substrate protein involves a number of specific proteins. First, a ubiquitin-activating enzyme, termed "E1" binds and activates ubiquitin in a process that requires ATP. Next, a ubiquitin-conjugating enzyme, termed "E2" accepts the activated ubiquitin from E1 and can either transfer the ubiquitin to a substrate directly or facilitate this transfer through the use of a third ubiquitin ligase, termed "E3". This process can either be accomplished once or multiple times to result in either a mono- or poly-ubiquitinated substrate. The circumstance surrounding the type of ubiquitination that occurs determines the fate of the substrate protein, one of which is degradation by the proteasome. (Figure adapted from *www.bostonbiochem.com*).

Figure 1-5: The ubqiquitin ligase pathway.



case of the E4orf6/E1b-55k/Cullin 5 E3 ubiquitin ligase complex. E3 ligases are responsible for the transfer of the ubiquitin moiety from E2 to the substrate. After subsequent rounds of ubiquitin-chain elongation, the substrate is modified by a long chain of ubiquitin moieties. The function of the ubiquitin chain depends upon its size and its makeup and is largely dependent on the quaternary structure of the ubiquitin chain. In most cases, a poly-ubiquitin chain of four ubiquitins is generally the minimum length required to be recognized by the proteasome and targeted for degradation (70). The E3 ubiquitin ligase catalyzes the formation of a covalent isopeptide bond between the C-terminal glycine (G76) of ubiquitin to the epsilon amino group of a lysine on the substrate protein (53). The poly-ubiquitin chain serves as the recognition signal for targeting of the protein for degradation by the proteasome (31). The *cis* signals that determine whether a protein is targeted for poly-ubiquitination have been previously characterized (53).

In eukaryotes, the type of E3 ubiquitin ligases usually depends on the presence of either a RING (really interesting new gene) domain or a HECT (homologous to the E6-AP carboxyl terminus)-domain. The RING superfamily primarily consists of proteins that contain particular fold domains that bind zinc, although some do not (U-box). The types of RING superfamily E3 ubiquitin ligases most extensively studied are those of the Cullin-RING ligase (CRL) superfamily (106). The catalytic cores of these E3 ubiquitin ligases are comprised primarily of a RING protein bound to the C-terminal domain of a cullin protein (65, 100, 136, 149). This C-terminal region of cullin, bound to the RING protein, recruits an E2 ubiquitin-conjugating enzyme, whereas the N-terminal portion of cullin recruits specific co-factors. The type of co-factor recruited depends on the type of

CRL; for example, cullin 2 and cullin 5 recruit BC box proteins such as Elongin B and Elongin C. Cullin 1 and Cullin 7 CRLs recruit specific receptors that contain an F-box, and Cullin 3 recruits BTB domain proteins (53, 110).

Like the F-box proteins, the E4orf6 and E1b-55k proteins form a multi-enzyme complex involved in transferring ubiquitin moieties to the substrate molecule from the E2 complex. Cullin 5 acts as the major anchor protein to assemble the adapter proteins Elongin B and Elongin C, the Ring finger protein Rbx1, E4orf6, E1b-55k, and the substrate protein into an E3 ubiquitin ligase complex **(Figure 1-6)** (122).

The process of multiubiquitination is complex and is not a simple matter of poly-ubiquitin elongation. The manner in which a protein substrate is ubiquitinated has significant impact on its fate. Ubiquitin itself is also poly-ubiquitinated, and this can occur through the use of one of seven lysines on the molecule **(Figure 1-7)** (3, 5, 38, 44, 64, 84, 142, 143). It is believed that the mechanical forces exerted on a particular type of poly-ubiquitin chain determine the likelihood that it will serve as a marker for proteasome-mediated ubiquitination **(Figure 1-8)**. For example, poly-ubiquitin chains comprising of K48-linked moieties display moderate levels of mechanical resistance, such that the structure of the poly-ubiquitin chain can remain intact while still being flexible enough to allow changes in the substrate structure necessary for proper processing by the 19S subunit of the proteasome. However, poly-ubiquitin chains comprising of K63-linked moieties are predicted to be extremely rigid and lack the flexibility necessary for processing by the 19S subunit (41).

Figure 1-6: Diagram of Cullin E3 ubiquitin ligases.

The substrate specificity of a particular E3 ubiquitin ligase is primarily a result of the specific protein-protein interactions the make up the complex. Although all cullins recruit the ring finger protein Rbx1, many of the cullin ring ligases (CRLs) differ in the adapter proteins they recruit. For example, cullin 2 and cullin 5 recruit BC box proteins such as elongin B and elongin C. Cullin 1 recruits the F-box protein, Skp1. Figure adapted from (121).

Figure 1-6: Diagram of Cullin E3 ubiquitin ligases.

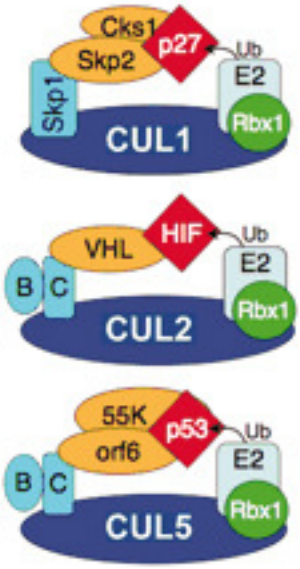


Figure 1-7: The type of ubiquitination determines the fate of the substrate protein.

The extention of the poly-ubiquitin chains through specific lysines on ubiquitin itself generates particular quaternary structures that likely play roles in the fate of the ubiquitinated protein. For example, K63-linked poly-ubiquitin chains have known roles in the activation of immune responses, target protein trafficking, and kinase activation. K48-linked, and possibly K29-linked, poly-ubiquitin chains are known to facilitate the recognition of the substrate by components of the 26S proteasome for degradation. (Figure adapted from *www.bostonbiochem.com*).

Figure 1-7: The type of ubiquitination determines the fate of the substrate protein.

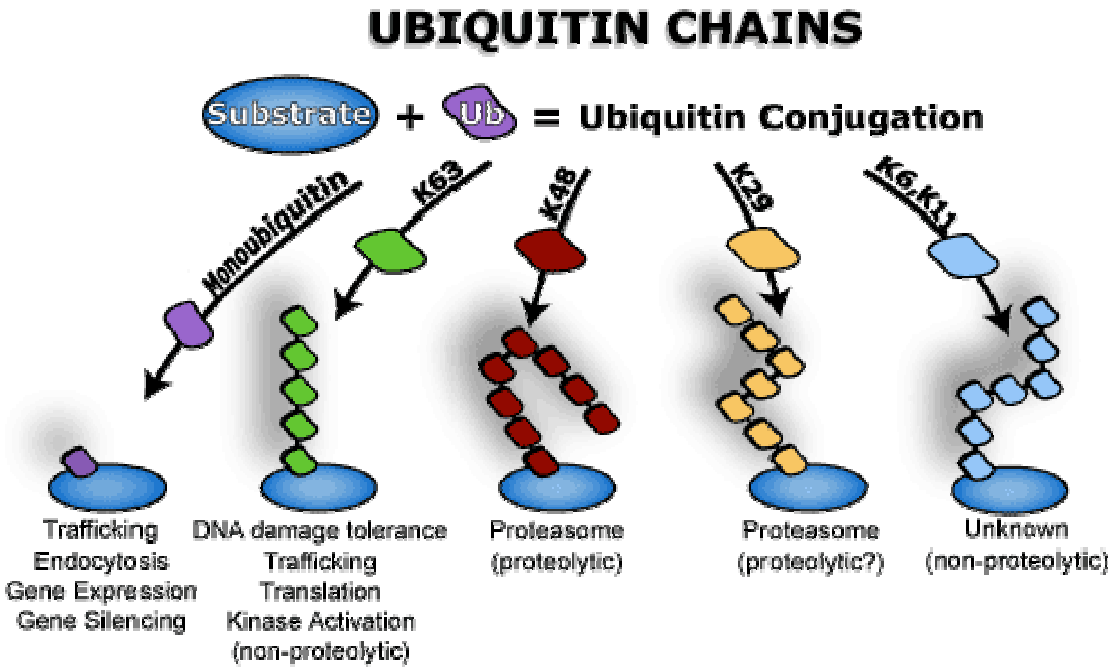


Figure 1-8: Different ubiquitin-ubiquitin linkages result in different poly-ubiquitin structures.

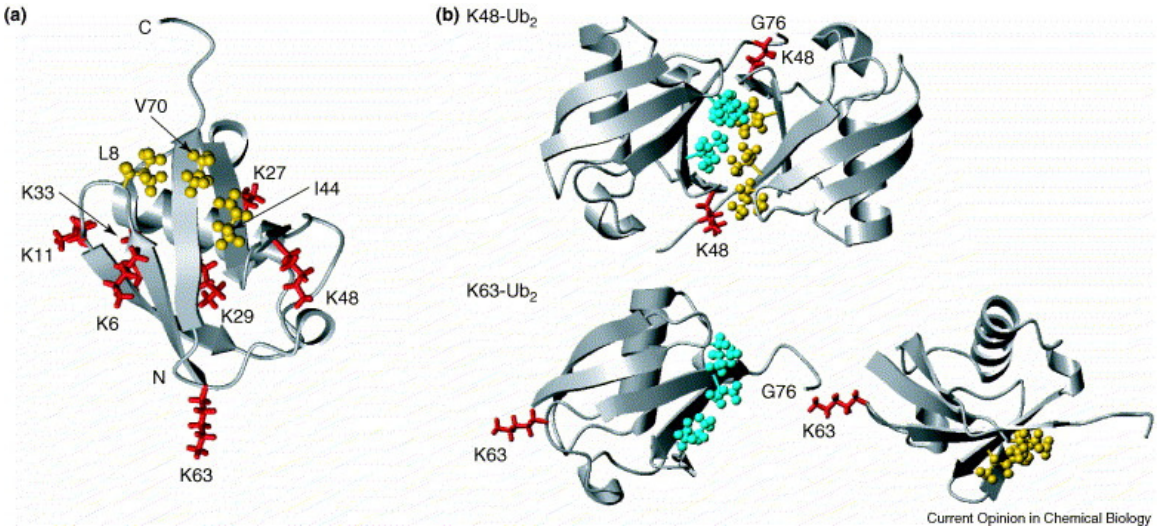
The preferential use of specific ubiquitin-ubiquitin linkages (shown: K63 vs. K48) results in varied quaternary structures of the poly-ubiquitin chain.

(A) The crystal structure of ubiquitin. The locations of lysine residues are indicated.

(B, top) Crystal structure of K48-linked di-ubiquitination.

(B, bottom) Crystal structure of K63-linked di-ubiquitination.

Figure 1-8: Different ubiquitin-ubiquitin linkages result in different poly-ubiquitin structures.



The vast majority of proteins that are known to be degraded by the proteasome are poly-ubiquitinated; however, a few exceptions to this phenomenon have been reported (90, 92, 137). Most notably, the parvovirus MVM nonstructural protein NS2 is degraded by the proteasome in a manner that is ubiquitin-chain independent (90). In that study, it was determined that the stability of NS2 could be enhanced upon use of proteasome inhibitors; however, the presence of high molecular-weight poly-ubiquitinated isoforms of the protein could not be detected *in vitro* or *in vivo*.

Much of the initial knowledge of ubiquitin and its function was determined in yeast. In *Saccharomyces cerevisiae*, K63-linked poly-ubiquitination of the large ribosomal subunit L28 is required for its function in translation *in vitro* and *in vivo* (141). In mammalian cells, the regulation of the nuclear factor kappa B (NF-κB) family of transcription factors is multifaceted and involves different mechanisms of poly-ubiquitination. The binding of inhibitor of kappa B (IκB) to NF-κB renders the transcription factor inactive; however, during DNA damage or in the presence of proinflammatory cytokines such as tumor necrosis factor α (TNFα), the proteasome-mediated degradation of IκB via K48-linked poly-ubiquitination releases NF-κB and activates the signaling cascade (68, 69). Specifically, TNFα stimulation leads to the modification of receptor-interacting protein (RIP) and the TNF receptor-associated factor 2 (TRAF2) through K63-linked poly-ubiquitination. This poly-ubiquitination ultimately leads to the activation of IκB kinase (IKK), which phosphorylates IκB. The phosphoylation of IκB results in its release from NF-κB, which activates the NF-κB family of transcription factors and renders IκB sensitive to K48-linked poly-ubiquitination and degradation (66, 147). Interestingly, the human gene product A20 is

known to act as a negative regulator of NF-κB signaling by acting as a deubiquitinating (DUB) enzyme, removing K63-linked poly-ubiquitin chains from ribosome inhibiting protein (RIP) and TNF receptor associated factor (TRAF2) (146). However, A20 was also discovered to stimulate K48-linked poly-ubiquitination of RIP (165). Therefore, A20 serves as an unusual example of an E3 ubiquitin ligase that also serves as a DUB enzyme. It is hypothesized that this dual function of A20 on RIP may occur sequentially in that it first removes K63-linked poly-ubiquitin chains from RIP and then subsequently adds K48-linked poly-ubiquitin chains (potentially on the same lysines) (165).

Parkin, an E3 ubiquitin ligase known to be a key player in the pathogenesis of Parkinson's Disease (PD), has also recently been found to possess dual poly-ubiquitin ligation activity (80, 81). In this report, the authors found that under normal conditions, parkin mediates the K48-linked poly-ubiquitination of protein factors such as α-synuclein and synphilin-1. This poly-ubiquitination targets these proteins for degradation by the proteasome. However, in patients with PD, there may be mutations in one or more of these proteins such that parkin no longer targets them for K48-linked poly-ubiquitination; rather, parkin serves to modify these proteins through K63-linked poly-ubiquitination. This specific type of poly-ubiquitination is thought to be primarily responsible for the formation of Lewy Bodies (LB), large protein aggregates found in the neuronal cells of patients with PD. Interestingly, this the same phenotypic effect could be observed when α-synuclein and synphilin-1 were overexpressed in cultured cells, indicating that aberrant accumulation of toxic levels of these proteins *in vivo* may cause a shift in K48-linked to K63-linked poly-ubiquitination by parkin. It is unknown if other E3 ligases, such as the

E4orf6/E1b-55k/Cullin 5 ubiquitin ligase, are also capable of this dual function possessed by A20 and parkin.

The ubiquitination of AAV capsid proteins plays an important role in the transduction efficiency of the virus during infection (37, 170). In those reports, the authors discovered that the transduction efficiency of rAAV could be enhanced by the use of proteasome inhibitors. As the proteasome was known to play a critical role in the internal processing of other viruses, such as human immunodeficiency virus (HIV) (134), it was hypothesized that internalized capsid proteins could be ubiquitinated and processed by the proteasome. Indeed, the addition of proteasome inhibitors enhanced viral capsid stability and trafficking to the nucleus; in addition, it was suggested that capsid proteins that had been endosomally processed were the preferential substrates for ubiquitination. Therefore, the poly-ubiquitination of AAV capsid proteins serves as an intrinsic innate immune response against AAV infection. It has not yet been previously established whether AAV proteins are ubiquitinated *de novo*; however, we demonstrate in chapter 2 that the AAV5 small Rep proteins are poly-ubiquitinated in 293 cells, even in the absence of additional adenovirus gene products.

Ubiquitin can also target cell surface proteins for degradation by lysosomes (54). Generally, this occurs through the continued attachment of additional ubiquitin proteins to the lysine 48 (K48) of the previous ubiquitin (24, 109, 151); however, there are also rare examples of K29-linked and K11-linked ubiquitination preceding proteasome-mediated degradation (111). Generally, a K48-linked poly-ubiquitin chain requires at least four ubiquitin moieties to be recognized by the proteasome (151); however, there are examples in which a single K48-linked poly-ubiquitin chain is adequate for

degradation by the proteasome (24). The crystal structure of K48-linked tetraubiquitin has been recently reported (40). K63-linked poly-ubiquitination has well-established roles in the inflammatory response, DNA-damage tolerance, the protein trafficking, and protein synthesis (55, 132, 141, 146, 155-157).

The elongation of some poly-ubiquitin chains can also be assisted by an additional ubiquitin conjugation factor, E4 (72). In yeast, the E1, E2, and E3 enzymes were not sufficient to mediate the poly-ubiquitination of protein substrates to the lengths required for recognition by the proteasome. E4 (also known as UFD2) functions in conjunction with these enzymes to assist in the addition of previously-assembled poly-ubiquitin chains to the mono-ubiquitinated substrate. E4 mutants did not display defects in protein degradation during normal growth conditions; however, they demonstrated a marked sensitivity to stress-inducing agents, implicating the E4 ubiquitin conjugation factor in the protection of yeast cells against environmental stress by eliminating unnecessary proteins (72, 154).

The 26S proteasome is comprised of a 20S proteolytic core and a 19S cap, which may bind to one or both ends of the proteasomal complex. The 26S proteasome is predominantly located in the cytoplasm of mammalian cells; however, in certain cases there is evidence that the proteasome will traffic into the nucleus, presumably to serve in the degradation of nuclear proteins (126).

The 19S cap directs poly-ubiquitinated proteins to the proteolytic core of the proteasome. 19S then acts as a deubiquitinating (DUB) enzyme to remove the poly-ubiquitin chain from the substrate in an ATP-dependent fashion. Finally, the protein substrate is unfolded and trafficked through the narrow pore of the 20S core complex (30).

These processes can be assisted by the activity of additional subunits known as the 11S regulators, which also bind to the 20S core complex to bring a conformational change that allows for enhanced access of substrate proteins to the catalytic core of the proteasome (56, 82).

The 20S subunit of the proteasome is comprised of 4 rings made up of seven alpha-type subunits forming the two outer rings and seven beta-type subunits forming the two inner rings. The alpha subunits form a protective barrier around the enzymatic portion of the proteasome, preventing the entry of non-selected proteins into the inner chamber, which is composed of the beta subunits that serve as the catalytic site (48). The 20S proteasome displays trypsin-like activity where proteolytic cleavage takes place after basic amino acids, chymotrypsin-like activity where proteolytic cleavage takes place after hydrophobic amino acids and caspase-like activity where cleavage takes place after acidic amino acids (30). Individually, the alpha and beta subunits of the 20S core proteasome structure form ring structures; in conjunction, they form a cylinder structure comprised of two internal beta rings surrounded by two external alpha rings (29, 48). The chymotrypsin-like, trypsin-like, and postglutamyl hydrolyzing activities of the core structure are associated with subunits beta-1, beta-2, and beta-5, respectively (29). In certain conditions, however, these subunits may be replaced with homologues that replace the normal subunits and alter the catalytic characteristics of the proteasome (176).

The proteasome inhibitor MG132, a proteasome subunit analog that competitively inhibits the chymotrypsin-like and peptidylglutamyl-peptide-hydrolyzing (PGPH) activities of the proteasome (127), has been utilized extensively in studies examining the proteolytic characteristics of the proteasome on protein substrates. The levels of poly-

ubiquitin conjugates are typically enhanced in the presence of proteasome inhibitors such as MG132 (26). Transient transfection of a lysine-less, dominant-negative form of ubiquitin, known as UbR7, blocks the degradation of proteins targeted for degradation by the proteasome by poly-ubiquitination (137).

The type of poly-ubiquitination determines the fate of the protein, resulting in alterations in protein trafficking (55), protein stability (110), function (146), as well as effects on signaling and protein-protein interactions (16, 57). Unfortunately, although there are antibodies that recognize mono- versus poly-ubiquitinated substrate proteins, to date there are no linkage-specific antibodies available to individually study endogenous poly-ubiquitin linkages.

In some cases, the poly-ubiquitination of a particular substrate can be eliminated by the specific mutation of targeted lysines (128). However, in other cases, non-specific ubiquitination of otherwise non-utilized lysines (or even non-lysine ubiquitination) can occur when the preferred site is unavailable (98). Mass spectrometry has also been used to determine the sites of ubiquitination in a protein (104); however, the extent to which a particular lysine is ubiquitinated can be difficult to assess as a pool of ubiquitinated proteins usually contains a myriad of substrates that are ubiquitinated at different levels. Clearly, the methods currently in use to examine ubiquitination *in vitro* and *in vivo* remain in the early stages of development; however, the understanding of the mechanisms driving the choice in ubiquitin-chain usage will be of significant benefit to our knowledge of many human diseases.

In chapter 2, we demonstrate that the small Rep proteins from AAV5 and AAV2 are subject to the same patterns of protein downregulation in 293 cells during transient

transfection in the presence of E4orf6 and that this downregulation is the result of specific poly-ubiquitination of these protein substrates by the adenovirus E4orf6/E1b-55k/Cullin 5 E3 ubiquitin ligase complex.

The Rep40 protein in AAV2.

In AAV2, the P19 promoter generates pre-mRNAs that are alternatively spliced to yield the small non-structural proteins Rep52 and Rep40. Although the functions of the large non-structural proteins Rep78 and Rep68 are well-characterized, the roles of the small Rep proteins in the life cycle of AAV2 remain largely unknown, with the exception of their well-known roles in the packaging of the AAV ssDNA genome into preformed capsids (71), although recent evidence also attributes a role of the small Rep proteins in the modulation of cell proliferation rates (78).

Until recently, the mechanisms of Rep52- and Rep40-mediated DNA packaging were not well understood. Indeed, there is still much to be learned about these critical proteins. Interestingly, all four Rep proteins in AAV2 share a common domain, known as the Walker domain **(Figure 1-9)**, which is a conserved motif common to the superfamily 3 (SF3)-type helicases (49, 62). In AAV2, both Rep52 and Rep40 possess 3'- to 5'-helicase activity, and are required for unwinding of the double-stranded replicative forms of AAV for packaging into preformed capsids (71).

Although both Rep52 and Rep40 possess some functional redundancy in their helicase activity, ATPase activity, and DNA binding activity, their precise mechanisms of action are quite different. Analyses of the crystal structure of these non-structural proteins revealed that upon binding its DNA substrate, Rep52 remains monomeric in solution (138). However, upon binding to DNA, Rep40 assumes a hexameric ring-like structure (27, 62) **(Figure 1-10)**. This structure is required for its function. Studies examining the individual activities of these proteins suggest that both Rep52 and Rep40 are required for efficient packaging. Although Rep52 and Rep40 both are capable of

Figure 1-9: Structure of the Walker motif domains within the AAV2 Rep SF3 helicases.

(Top) A diagram of the AAV2 Rep proteins, with N-terminal origin interaction domain (red), helicase domain (gold), and zinc finger domain (green). Dashed rectangles indicate the Walker motif region required for helicase and ATP-ase activity. From (61).

(Bottom) Sequence comparision of the helicase domain from AAV2 Rep and the helicase domains of other SF3-type helicases. Walker A, B, and B' residues, which are required for ATP-ase and helicase activity, are boxed. Identical amino acids are in orange; similar amino acids are in yellow.

Figure 1-9: Structure of the Walker motif domains within the AAV2 Rep SF3 helicases.

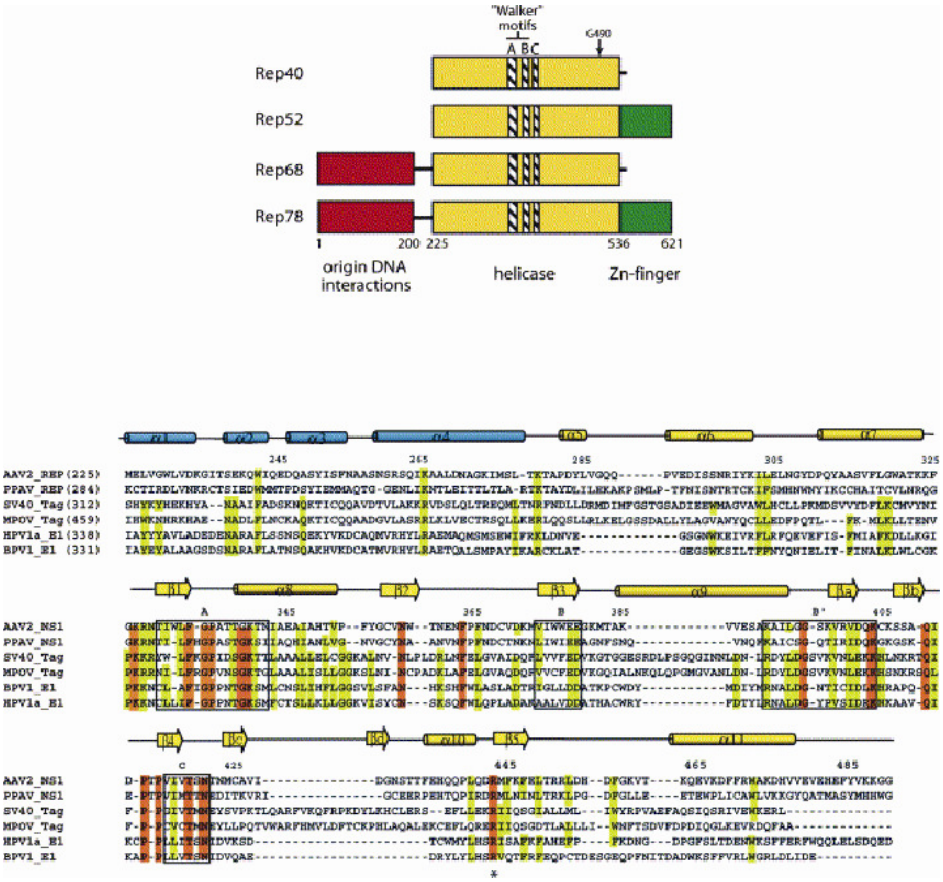
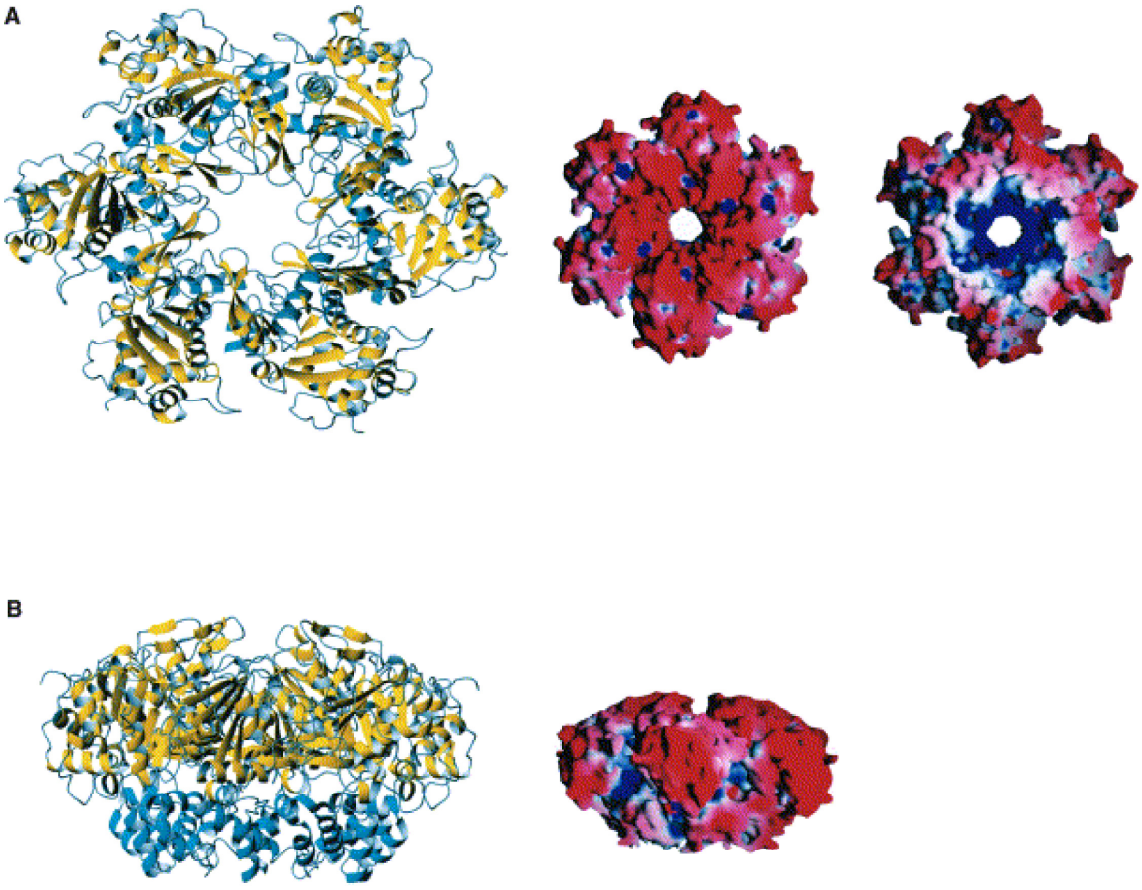


Figure 1-10: Proposed diagram of the AAV2 Rep40 hexameric ring structure.

(A) Top view of a ribbon model of the AAV2 Rep40 hexameric SF3 helicase. Note the pore complex in the center, through which the AAV DNA is proposed to be "pumped" into pre-formed capsids.

(B) Side view of same ribbon model. From (61).

Figure 1-10: Proposed diagram of the AAV2 Rep40 hexameric ring structure.



unwinding DNA substrates with ssDNA ends in a 3' to 5' fashion, Rep40 possesses the unique ability to unwind double-stranded DNA (27). Therefore, in the context of AAV replication, the Rep40 hexamer is necessary for the efficient initial unwinding of the first few bases of the dsDNA intermediate. Although Rep52 is capable of this phenomenon, it is much less efficient at doing so. Further unwinding may then be accomplished by Rep52 alone or by Rep52 in conjuction with Rep40 and the other Rep proteins (27).

In the context of AAV2, both small Rep proteins are required for the efficient packaging of AAV genomes. Studies of the transcription profile of AAV5 have revealed that unlike AAV2, pre-mRNA transcripts derived from the P19 promoter are preferentially poly-adenylated in the intron and not spliced (118). Therefore, significant quantities of Rep40 would not be expected to be encoded by P19-generated pre-mRNA. However, as demonstrated in chapter 3, AAV5 is capable of generating levels of a P40-like protein product that are comparable to those observed in AAV2. This finding, combined with the previously established importance of Rep40 in AAV2, is the basis for the work documented in chapter 3.

Recombinant AAV (rAAV) as a gene-delivery vector.

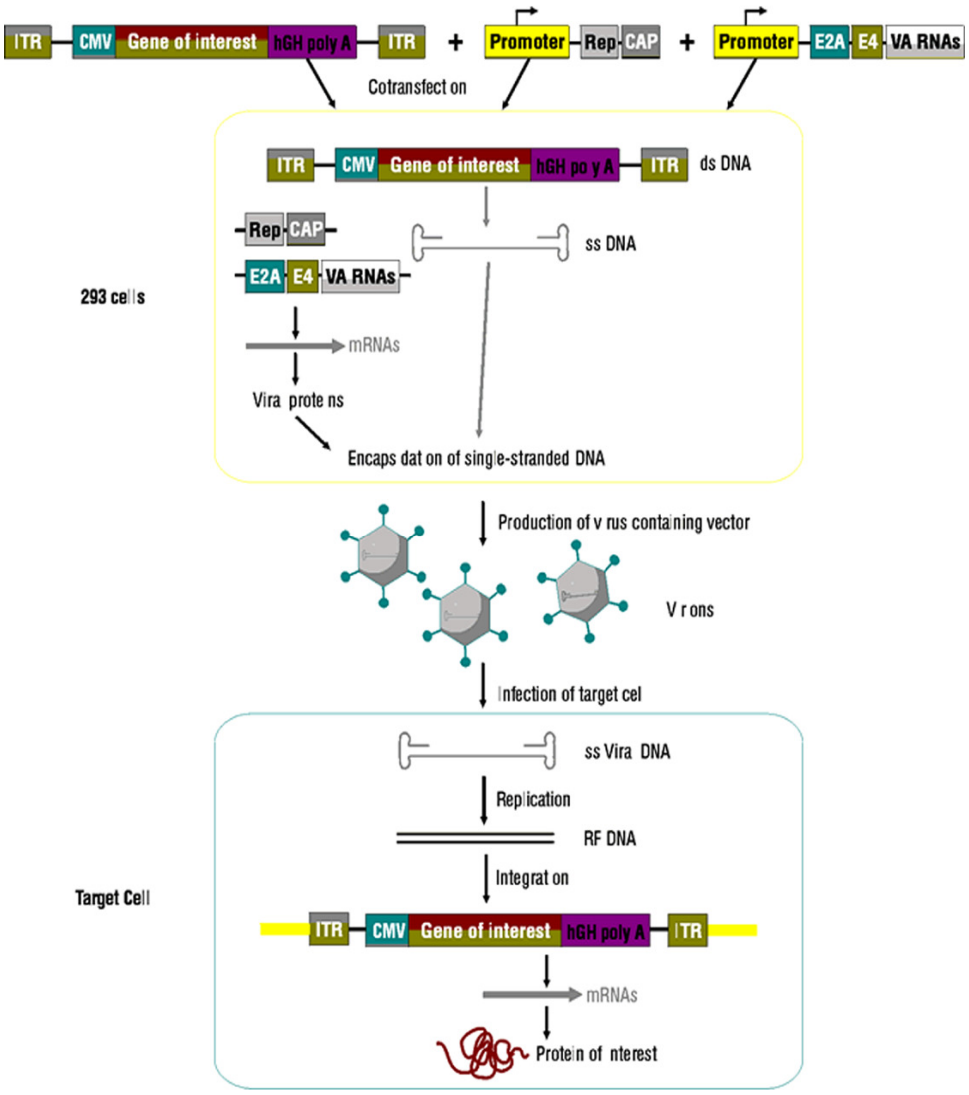
Wild-type AAV possesses several characteristics that make it a very attractive vector for gene therapy and other gene delivery applications. In the absence of helper virus, wtAAV integrates very efficiently into the long arm of human chromosome 19. AAV is capable of infecting a wide variety of cell types, can infect both dividing and non-dividing cells, is not species-specific (that is, it efficiently infects all tested animal species), is not known to cause any disease, and is only mildly immunogenic (although this phenomenon is under intense debate) (150). In addition, when the *rep* and *cap* genes of AAV are replaced by a promoter and a gene of interest flanked by the inverted terminal repeats (ITRs – the only *cis* sequences required in AAV for replication) to generate recombinant AAV (rAAV), these recombinant viruses are capable of stable, long-term expression in the infected cell type **(Figure 1-11)**. In the absence of Rep, rAAV genomes have been shown to persist episomally in the nucleus of many cell types (169). These characteristics make rAAV a highly sought-after gene therapy tool, having been successfully utilized in a plethora of early- and late-stage human clinical trials that are too numerous to mention (21).

Early attempts at producing rAAV involved a double-transfection method, in which the *rep* and *cap* genes of AAV between the ITRs were replaced with a promoter and gene of interest. This vector was co-transfected with another vector that contained the *rep* and *cap* genes in *trans*. Upon subsequent infection with adenovirus, the ssDNA genome containing the gene of interest was packaged into the AAV capsids. Following cell lysis, the adenovirus was typically heat-inactivated and the rAAV purified on a cesium gradient. However, this method would be questionable as a means of generating

Figure 1-11: Diagram of the production of rAAV via transient transfection.

Production of rAAV via transient transfection is typically accomplished by a triple or quadruple transfection method. The triple-transfection method (shown) requires a plasmid in which a gene of interest, driven by an endogenous promoter, has replaced the wild-type AAV sequence between the two AAV inverted terminal repeats (ITRs). The ITRs are the only *cis*-acting signals required for AAV replication. It also requires an AAV helper plasmid in which the Rep and capsid genes are supplied in *trans*-. This can be accomplished by the use of one or two separate expression vectors. Finally, a final plasmid, that in 293 cells, supplies the minimal adenovirus gene products required for AAV replication. Following transfection, the cell line packages recombinant ssDNA genomes into AAV capsids for subsequent harvest and re-infection of the target cell. Figure adapted from (*http://www.biocompare.com/technicalarticle/215/Exceptionally-Safe-AAV-Helper-Free-Gene-Delivery-And-Expression-System-from-Stratagene.html*)

Figure 1-11: Diagram of the production of rAAV via transient transfection.



therapeutic virions for human clinical trials due to potential adenovirus contamination; in addition, in all models there was a consistent presence of wild-type, replication competent AAV that arose from low-level recombination events between the *rep/cap* vectors and the ITR-containing plasmids (21).

As the potential benefits for rAAV as a gene therapy tool became apparent and its use became more mainstream, a number of researchers devoted much of their studies in the attempt at making a better vector. One of the first steps in making a safer vector for human clinical trials came with the design of a third plasmid vector that supplied the minimal adenovirus gene products required for AAV replication: E2a, E4orf6, and VA RNA. In 293 cells, which are transformed with adenovirus type 5 (Ad5) E1a and E1b, the use of the adenoviral vector eliminated the need for fully infectious adenovirus and significantly increased the safety of the recombinant vector (168).

However, one of the major limitations associated with using rAAV as a gene delivery vehicle involved the generally low levels of vector production that were generated using early methods, and the production of rAAV to the levels needed for human clinical trials remains costly and labor-intensive. Even under optimized conditions, traditional transfection methods can only generate approximately 50-150 transducing units (TU) per cell. In comparison, during wtAAV infection, the virus can generate as much as 5000 TU per cell (46).

The finding that large levels of the AAV non-structural protein Rep78 are capable of inhibiting transcription from the P40 promoter, which subsequently has a negative effect on capsid production and viral titers, led to the use of AAV mutants with reduced levels of Rep78 expression (79, 160). A mutation in the AAV2 Rep78/Rep68 AUG start

codon to the weaker ACG initiator codon resulted in a marked reduction in the amounts of the large Rep proteins, without affecting the levels of the small Rep proteins. Interestingly, levels of P40 pre-mRNA and capsid protein expression were increased in the presence of the ACG mutant, which subsequently resulted in a 10-fold increase in rAAV production. Alternate methods to reduce the levels of large Rep expression include the use of alternate, weaker promoters such as the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter (47).

A number of other strategies have been utilized to enhance the large-scale production of rAAV necessary for human clinical trials without increasing the levels of contaminating wtAAV that arise from low-level recombination events. One such strategy involves the splitting of the *rep* and *cap* genes into separate plasmids (166). Although this strategy requires the co-transfection of four plasmids at once as opposed to three, the replacement of the native P40 promoter with that of the cytomegalovirus immediate early (CMV-IE) promoter eliminates the downregulatory effects of Rep78 on the transcription of the capsid-encoding mRNA. In addition, the undesired production of wtAAV is also effectively eliminated, as two separate, equally inefficient recombination events would have to occur in the cell as opposed to one in order to generate replication-competent wtAAV. An additional strategy for the improvement of rAAV production that allows for reduced levels of contaminating wtAAV involves the addition of intronic sequences to the RepCap helper vectors (18, 77, 159). This method takes advantage of the inability of AAV to efficiently package genomes greater than approximately 6 kilobases (kB). These RepCap helper vectors are sufficient to generate the Rep and capsid proteins required for packaging; in addition, they have the added benefit of being too large to be adequately

packaged into the small AAV capsid should a recombination event between the RepCap vector and the ITR-containing vector occur.

Most recently, a number of studies have involved the use of baculovirus expression systems to generate high titers of rAAV, and in some instances the levels of virus obtained rival those attainable during wtAAV infection (97, 158). However, not all labs (particularly those in the academic setting) are well equipped for using baculovirus as a means to produce rAAV; in addition, it has been suggested that this method is not always efficient and is in further need of study before becoming more mainstream. In the meantime, improved methods to produce rAAV that allow for the continued use of more readily-available reagents, cell lines, and equipment will be of substantial benefit to the rAAV community; in particular, to smaller academic labs that cannot afford to drastically change or update their current methods.

Although there have been a number of useful advancements in the generation of safe, high levels of rAAV for use in human clinical trials, there is still significant room for improvement. Future studies to improve upon these systems, however incremental, will ultimately enable the large-scale quantities of recombinant virus required for widespread use in the treatment of a number of human diseases. In chapter 4, we address a novel strategy to significantly enhance vector production.

Intron definition and the splicing of AAV2 pre-mRNAs.

Alternative splicing is a critical means by which extensive proteomic diversity is established from relatively fewer numbers of genes. Intuitively, the splicing of parvovirus introns is important for the expansion of the coding capacity of these smallest of viruses. All AAVs contain a single intron, consisting of a single 5'-splice site donor and two 3'-splice site acceptors. In AAV2, unspliced pre-mRNAs from the P5 and P19 promoters encode the non-structural proteins Rep78 and Rep52, respectively. The usage of the small internal AAV intron encodes the Rep68 and Rep40 proteins, respectively. The splicing of pre-mRNAs generated by the P40 promoter is required for the production of capsid proteins (7, 22, 153), and the relative usage of the two acceptor sites partially determines the relative ratios of the individual capsid proteins from one another. It is unknown whether unspliced P40 messages serve as bona-fide templates for translation. Interestingly, there is a small open reading frame (ORF) within the AAV intron that is completely removed during splicing. Whether this small ORF actually codes for a functional polypeptide is unknown at this time; however, the presence of several (nine in AAV2) AUGs out-of-frame from the capsid reading frame in unspliced P40 pre-mRNA may be partially responsible for the prevention of VP1 translation from the unspliced message.

The three capsid proteins: VP1, VP2, and VP3, are 87, 73, and 62 kilodaltons (kD) in size, respectively. The capsid protein VP1 utilizes an alternative splice acceptor site at nucleotide (nt) 2201, whereas VP2 and VP3 utilize the second acceptor site at nt 2227. Although they are derived from the same transcription unit, the VP2 protein uses a non-traditional, weaker ACG triplet as its initiation codon; therefore, it is usually read-

through to the downstream AUG triplet to encode VP3. The relative ratio of VP1:VP2:VP3 in the intact capsid is approximately 1:1:10.

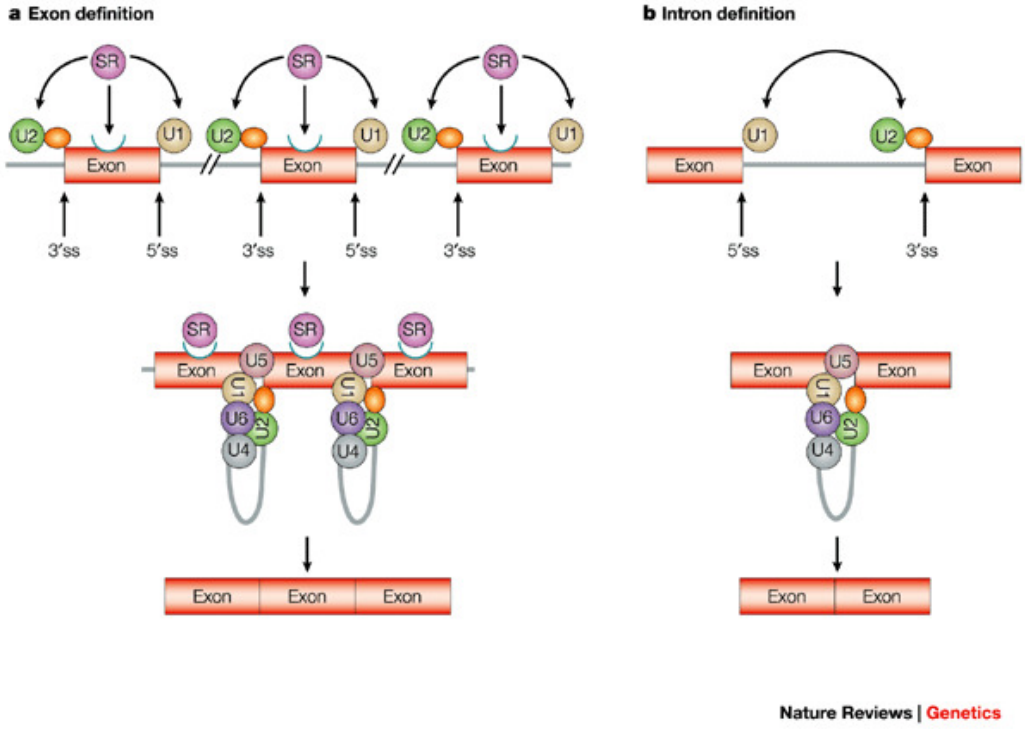
Most vertebrate pre-mRNA splicing involves the excision of large introns, which are flanked by much smaller exons (23, 175) **(Figure 1-12)**. In this model, termed exon definition, the vast majority of the *cis* signals required for pre-mRNA splicing are contained within the exons. In contrast, the excision of small internal introns that are flanked by much larger exons (such as those in lower eukaryotes or in some viruses such as MVM) is governed by a less-understood model known as intron definition (15, 19, 88, 89, 148). For example, efficient splicing of the small internal MVM intron requires the presence of a G-rich intronic enhancer sequence (52). It is unknown whether the small internal AAV intron contains intronic splicing enhancer sequences necessary for proper splicing; indeed, the 5'- and 3'-splice-sites of the AAV intron are quite poor. Therefore, it seems likely that there may be *cis* sequences within the intron that serve to modulate splice-site recognition in AAV. In chapter 4, we demonstrate that there appears to be a *cis*-acting sequence within the AAV2 intron that is required for splicing enhancement in the presence of adenovirus, and that this element is lacking in AAV1 and AAV6. Conversely, it is possible that there exists an intronic splice silencer within AAV1 and AAV6 that does not exist in AAV2.

Figure 1-12: Exon definition versus intron definition.

(A) Basic model of exon definition. In exon definition, the choice whether to include or exclude an exon depends upon the strength of the 3'-splice signal from the upstream intron, the strength of the 5'-splice signal from the downstream intron, as well as the presence and usage of exonic splicing enhancers or inhibitors. In exon definition, most of the *cis* signals required for the mediation of splicing of the upstream and downstream introns are within the exon.

(B) Basic model of intron definition. In intron definition, the choice whether to include or exclude an intron depends upon the strength of both the 5'- and 3'-splice sites of that intron, as well as the presence and usage of intronic splicing enhancers or inhibitors. In intron definition, most of the *cis* signals required for the mediation of splicing of the intron are within the intron itself. Figure from (4).

Figure 1-12: Exon definition versus intron definition.



II. E4ORF6-E1B-55K-DEPENDENT DEGRADATION OF *DE-NOVO*-GENERATED AAV5 REP52 AND CAPSID PROTEINS EMPLOYS A CULLIN 5-CONTAINING E3 UBIQUITIN LIGASE COMPLEX.

ABSTRACT

Degradation of de novo-generated AAV5 Rep52 and capsid proteins is part of the limited target specificity displayed by Ad5 E4orf6/E1b-55k as part of a cullin 5-containing E3 ubiquitin ligase complex. The adenovirus E3 ubiquitin ligase complex mediates the K48-linked poly-ubiquitination of AAV Rep and capsid proteins. In addition, in 293 cells, AAV5 small Rep proteins are modified via K63-linked poly-ubiquitination in a manner that is independent on the presence of adenovirus E4orf6.

INTRODUCTION

The E4orf6 protein is one of five adenovirus gene products (together with E1A, E1B-55k, E2A and VA RNA) required for efficient AAV virus production (14, 164). The E4orf6 protein plays a number of critical roles essential for adenovirus infection, having effects primarily on viral and cellular RNA export and cellular DNA repair pathways (34, 123, 135). At least one aspect of the mechanism of its action is as a component of an E3 ligase complex (122). E4orf6, together with E1b-55k, has been shown to cause the ubiquitination of cellular p53 and Mre11 and their subsequent degradation, which promotes efficient adenovirus replication (122, 144). E4orf6 and E1b-55k form an E3 ligase complex with the cellular adaptor proteins elongin B and elongin C, cullin 5, and the ring finger protein Rbx1(122, 124). Elongins B and C are thought to bring the substrate recognition molecules E4orf6 and E1B-55k into association

with cullin 5 (11, 122, 167). The contact points between E4orf6 and elongins B and C occur through two essential BC box motifs (11). Interestingly, the E4orf6-E1B-55k complex isolated from infected cells is larger than predicted from its known minimal components, and has been shown to contain additional proteins (51).

The E4orf6 protein has been shown to be required for the conversion of AAV2 genomic ssDNA into the double-stranded DNA (dsDNA) replication intermediate (42, 135, 163, 164), and its role in degrading Mre11 has recently been shown to also be important for AAV2 replication in an as yet undetermined manner (135). In addition to these important functions, we have recently shown that E4orf6 together with E1B-55k can promote the degradation of *de novo*-generated AAV5 capsid and small Rep proteins (96). We have also shown that one of the important roles that VA RNA plays in promoting AAV5 infection is to overcome the effect that E4orf6/E1b-55k have in reducing the accumulated levels of AAV5 proteins (96).

Note: Two of the experiments and their figures presented here (Figure 2 and Figures 4A and 4B) were conducted, generated and designed by Ramnath Nayak. However, since these figures are featured as part of the initial manuscript (of which I was 2nd author), and they are critical to the overall scope of the project, they are included in this work. I would like to thank Ram for his assistance in the preliminary stages of this project. As part of this dissertation, I have also included additional data regarding the ubiquitination of AAV5 proteins and their interactions with components of the E4orf6/E1b-55k/Cullin 5 E3 ubiquitin ligase complex.

RESULTS

E4Orf6-E1B55k-dependent degradation shows limited target specificity.

Only three cellular proteins have previously been shown to be targets of the E4orf6/E1b-55k E3 ubiquitin ligase complex: Mre11, p53, and DNA ligase IV (6, 123, 135, 144). While we have previously demonstrated E4orf6/E1b-55k-dependent degradation of AAV proteins (96), our current analysis indicates that the target number of this complex remains small. As shown in Figure 2-1, in experiments in which E4orf6 and E1b-55k directed the degradation of *de novo*-generated AAV5 capsid proteins as detected by immunoblot following transient transfection of E1A- and E1b-55k-expressing 293 cells **(Figure 2-1A, lanes 1-2),** other randomly chosen, transiently-expressed cellular proteins, including APOBEC 3A **(Figure 2-1A, lanes 3-4**), canine parvovirus VP2 **(Figure 2-1A, lanes 5-6)**, the RNA processing factors TIA-1 **(Figure 2-1A, lanes 7-8; 11-12; 17-18)** and SMN (data not shown), and APOBEC 3G (data not shown), as well as endogenous actin **(Figure 2-1A)** and 14-3-3 **(Figure 2-1A and 2-1B)**, were found to be resistant to degradation. Thus, AAV Rep and Cap proteins joined part of a restricted target population of proteins degraded in a manner directed by Ad5 E4orf6 and E1b-55k. Interestingly, the capsid proteins of the highly related goat (Go.1) AAV **(Figure 2-1A, lanes 9-10)**, and the small Rep proteins of AAV2 **(Figure 2-1A, lanes 15-16)** and AAV5 **(Figure 2-1A, lanes 13-14)** were also degraded in 293 cells in an E4orf6-dependent manner. Insertion of a premature termination codon in the N-amino terminal region of E4orf6 abrogated its ability to direct the loss of AAV5 proteins (data not shown).

Degradation of AAV5 Rep52 **(Figure 2-1B),** as well as AAV5 capsid proteins (data not shown), was dependent, in a dose-dependent manner, upon the amount of DNA used in the transient transfection. At high concentrations of Rep52 and E4orf6-expressing plasmid DNA, degradation was not apparent **(Figure 2-1B, compare lanes 7-10 to 1-6)**. These results suggested that degradation depended upon both the relative and absolute levels of participating proteins. It is not yet clear, however, whether this indicates that the cellular degradative machinery can be saturated at the high levels of protein expression for which degradation cannot be demonstrated, or whether over-expression by co-transfection leads to dominant-negative E4orf6-E1b-55k complexes that lack cellular components required for ubiquitination. Supplementation of additional E1b-55k-expressing plasmid to transfections in 293 cells of high amounts of E4orf6 and Rep52 did not result in increased degradation of Rep52 (data not shown).

E4orf6-E1b-55k-directed degradation of *de novo*-generated AAV5 capsid proteins requires both BC Box motifs.

It has been previously shown that E4orf6 function within the E3 ubiquitin ligase complex depends upon its interaction with elongins B and C, *via* motifs of E4orf6 surrounding positions 46 and 122. A number of mutations within these sites that affect degradation have been characterized (11). Single amino-acid mutations of leucine to glycine in BC box 1 (L47G), and leucine to serine in BC box 2 (L122S), have been shown to have little effect on degradation of p53 (3), while double mutations changing leucine and cysteine to glycine and valine in BC box 1 (L47G/C51V), and leucine and

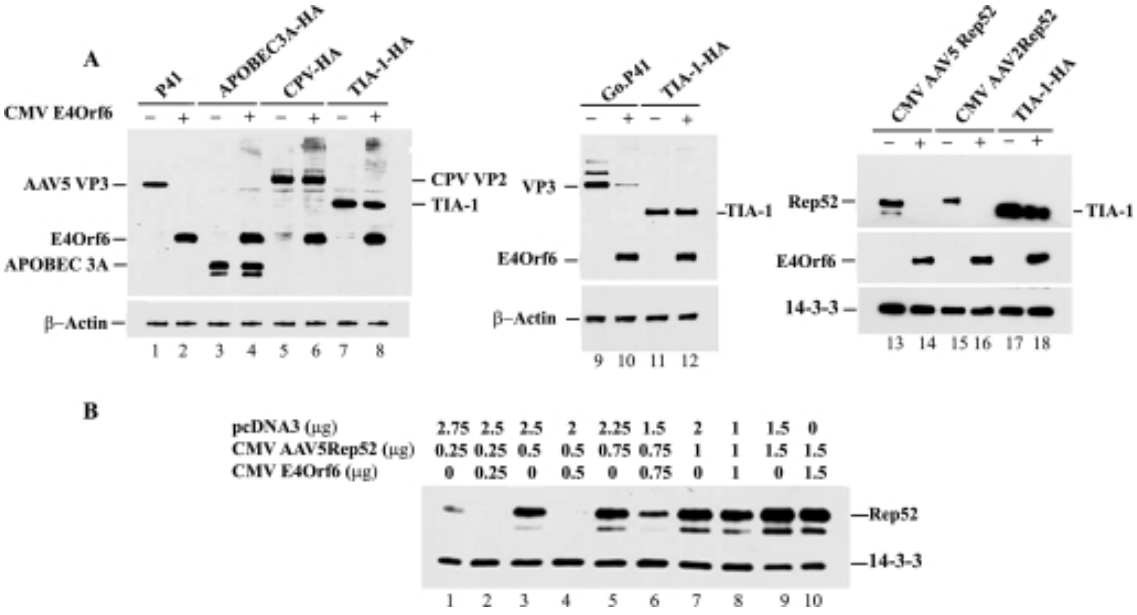
FIGURE 2-1. **The degradative effects of E4orf6 show limited target specificity and act in a dose-dependent fashion.**

(A). An immunoblot following electrophoresis in a denaturing 10% polyacrylamide gel (96), using anti-HA antibody (clone HA-7, Sigma, St. Louis, MO), anti-AAV5 capsid antibody (B1, American Research Products, Inc., Belmont, MA), or anti AAV Rep antibody (259.5, American Research Products, Inc., Belmont, MA), top panel, or anti-actin or anti-14-3-3 antibody (Santa Cruz, Santa Cruz, CA), bottom panel, of protein extracts taken 48 hours post-transfection of 293 cells (96) with a capsid protein-expressing AAV5 minimal capsid gene plasmid [P41, (173)] (lanes 1-2), an HA-tagged APOBEC 3A expression construct (APOBEC 3A-HA, gift of M.D. Weitzman) (lanes 3-4), a construct expressing HA-tagged canine parvovirus VP2 (CPV-HA) (lanes 5-6), a construct expressing HA-tagged TIA-1 (TIA-1-HA, gift of B. Blencowe) (lanes 7-8; 11-12; 17-18), a plasmid expressing the Go.1-AAV capsid proteins [(116), lanes 9-10], the AAV5 Rep52 protein (CMV AAV5Rep52, lanes 13-14), and the AAV2 Rep52 protein (CMV AAV2Rep52, lanes 15-16), together with either a plasmid expressing HA-tagged E4orf6 (E4orf6-HA) in lanes marked (+), or with empty vector pSK (Invitrogen, Carlsbad, CA). The positions of the individual proteins are indicated.

(B). An immunoblot following electrophoresis in a denaturing 10% polyacrylamide gel using anti-Rep antibody and antibody to 14-3-3, of protein extracts taken 48 hours post-transfection of 293 cells with increasing amounts of CMV AAV5Rep52 alone (lanes 1, 3, 5, 7, 9), or increasing amounts of CMV AAV5Rep52 plus increasing amounts of CMV E4orf6 (lanes 2, 4, 6, 8, 10), as shown. The total amount of

DNA in each transfection was brought to 3 μg per 60mm^2 dish with the bacterial plasmid pcDNA3.1. The positions of the individual proteins are indicated.

FIGURE 2-1. The degradative effects of E4orf6 show limited target specificity and act in a dose-dependent fashion.



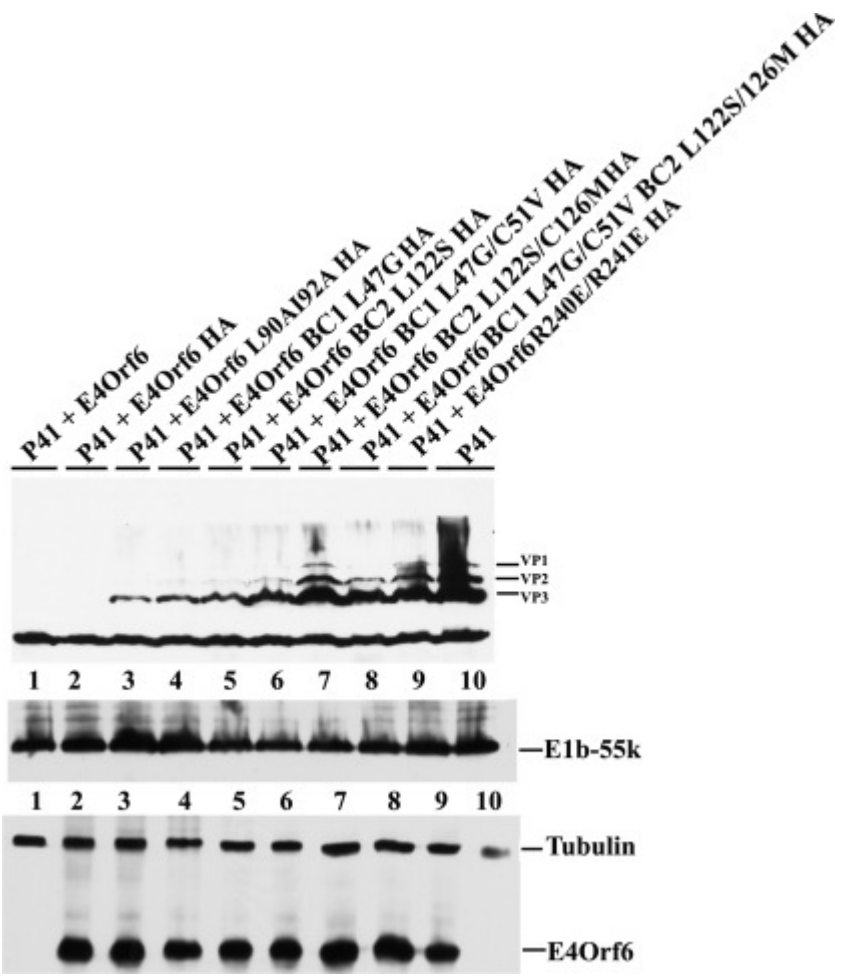
cysteine to serine and methionine in BC box 2 (L122S/C126M) abrogated E4Orf6-E1B-55k-dependent degradation of p53 (11). Consistent with these findings, we found that the same single amino acid mutations in either of the E4orf6 BC box motifs (L47G or L122S) had little effect on the ability of E4orf6 to participate with E1b-55k in directing the degradation of AAV5 capsid proteins transiently co-expressed in 293 cells, as assayed by immunoblot **(Figure 2-2, compare lanes 4 and 5 with lanes 1 and 2**). Mutations that changed two amino acids in BC box 1 (L47G/C51V) had a modest effect on E4orf6-E1b-55k-dependent degradation of AAV5 capsid proteins **(Figure 2-2, compare lane 6 to lanes 1 and 2)**, while the double mutations in BC box 2 (L122S/C126M), or double mutations in both boxes 1 and 2 (L47G/C51V+L122S/C126M) more significantly impaired E4orf6-E1b-55k-dependent degradation **(Figure 2-2, compare lanes 7 and 8, with lanes 1 and 2)**. An E4orf6 mutant previously characterized as lacking a functional nuclear retention signal (NRS) and unable to degrade p53 [R240E/R241E, (124)] was also significantly impaired in its ability to direct the degradation of AAV5 capsid proteins **(Figure 2-2, compare lane 9 with lane 10**), suggesting that a functional nuclear retention signal in E4orf6 was critical for its ability to degrade AAV5 capsid proteins. Consistent with this, an E4orf6 mutant previously suggested to lack a putative nuclear export signal and able to degrade p53 [lL90AI92A, (124)], retained its ability to direct degradation of capsid proteins **(Figure 2-2, compare lane 3 to lanes 1 and 2).**

FIGURE 2-2. **E4orf6/E1b-55k-directed degradation of *de novo*-generated AAV5 capsid proteins requires both BC Box motifs.**

An immunoblot following electrophoresis in a denaturing 10% polyacrylamide gel, using either anti-AAV5 capsid antibody, top panel, antibody to Ad5 E1B-55k (obtained from A.J. Berk, UCLA), middle panel, or anti-tubulin (clone TUB2.1, Sigma Co., St. Louis, MO) and anti-HA antibody, bottom panel, of protein extracts taken 48 hours post-transfection of 293 cells (96) with a minimal AAV5 P41-driven AAV5 capsid protein expressing plasmid (96) by itself (lane 10), or together with a plasmid expressing wild-type E4orf6 (E4orf6, lane 1), wild-type E4orf6 tagged with HA (E4orf6-HA, lane 2), or various HA-tagged E4orf6 mutants as described in the text (lanes 3-9). The positions of the individual proteins are indicated.

An additional thank-you to Ramnath Nayak for conducting the experiment shown here as part of our manuscript.

FIGURE 2-2. E4orf6/E1b-55k-directed degradation of *de novo*-generated AAV5 capsid proteins requires both BC Box motifs.



The degradative effects of E4orf6 in 293 cells on AAV5 small Rep and capsid proteins are similar among the adenovirus serotypes.

For Mre11, it has been shown that there is no significant difference in the degradative capacity of the E4orf6 proteins from various adenovirus serotypes (145). However, given the importance of the BC-box motifs for the degradation of AAV5 proteins (96) and the observation that sequence alignments of the BC-box motifs for the E4orf6 proteins from the adenovirus serotypes Ad4, Ad5, Ad9, and Ad12 differ greatly, we set out to determine whether the E4orf6 proteins from these different serotypes had differential effects on the degradation of AAV5 small Rep proteins in 293 cells.

Previous work in our lab using adenovirus E4orf6 for degradation studies were performed using E4orf6 from adenovirus serotype 5 (Ad5). In 293 cells, AAV5 small Rep and capsid proteins were all sufficiently degraded in the presence of E4orf6 from Ad4, Ad5, and Ad9 **(Figure 2-3A, compare lane 1 to lanes 2-4)**. In addition, AAV2 Rep52 was also degraded in the presence of E4orf6 from Ad4, Ad5, Ad9, and Ad12, although the E4orf6 from Ad12 was less efficient **(Figure 2-3B, compare lane 1 to lanes 2-5)**. Therefore, the E4orf6 gene products from all adenovirus serotypes tested were sufficient to mediate the degradation of AAV small Rep and capsid proteins by the proteasome in 293 cells.

E4orf6 and E1b-55k form a cullin 5-containing E3 ligase complex together with AAV5 small Rep proteins and *de novo*-generated AAV5 capsid proteins.

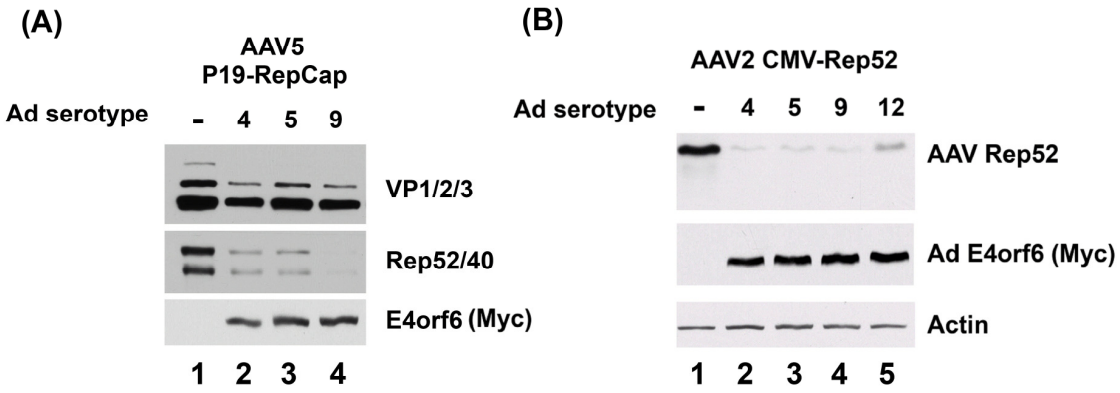
Following transient co-expression of AAV5 capsid proteins and wild-type HA-tagged E4orf6 in 293 cells, both E4orf6 and E1b-55k could be immunoprecipitated under

FIGURE 2-3: The degradative effects of E4orf6 in 293 cells on AAV5 small Rep and capsid proteins are similar among the adenovirus serotypes.

(A) An immunoblot following electrophoresis in a denaturing 10% polyacrylamide gel, using either anti-AAV5 capsid antibody, top panel, antibody to the small Rep proteins, middle panel, or myc-tagged E4orf6 (as assayed by the anti-myc antibody) of protein extracts taken 48 hours post-transfection of 293 cells (96) with a minimal AAV5 P19-driven small Rep/capsid protein expressing plasmid (96) by itself (lane 1), or together with a plasmid expressing wild-type E4orf6 from adenovirus serotypes 4, 5, or 9 (lanes 2-4). The positions of the individual proteins are indicated.

(B) An immunoblot following electrophoresis in a denaturing 10% polyacrylamide gel, using anti-AAV Rep antibody, top panel, antibody to myc-tagged Ad5 E4orf6 (as assayed by the anti-myc antibody), middle panel, or anti-actin, bottom panel, of protein extracts taken 48 hours post-transfection of 293 cells (96) with a minimal AAV2 CMV-driven Rep52 protein expressing plasmid by itself (lane 1), or together with a plasmid expressing wild-type E4orf6 from adenovirus serotypes 4, 5, 9, or 12 (lanes 2-5). The positions of the individual proteins are indicated.

FIGURE 2-3: The degradative effects of E4orf6 in 293 cells on AAV5 small Rep and capsid proteins are similar among the adenovirus serotypes.



non-denaturing extraction conditions (90) using α-capsid antibody, and the sequestered levels of E1b-55k were greater if degradation was inhibited with the proteasome inhibitor MG132 **(Figure 2-4A, lanes 1-2**). These results suggested that the E4orf6 and E1b-55k proteins form a stable complex along with *de novo*-generated capsid proteins prior to proteasomal degradation**.** Complex formation was dependent on the addition of E4orf6 suggesting that E1b-55k and AAV5 capsid proteins may not interact directly **(Figure 2-4A, lanes 9-10)**, however, such E1b-55k-containing complexes were seen to be relatively less abundant in the presence of the HA-tagged E4orf6 mutant previously mentioned **(Figure 2-4A, lanes 5-6)**, in which a single amino acid in BC box 2 was altered, and which moderately affected degradation of capsid proteins **(L122S, Figure 2-2, lane 5)**. These complexes were undetectable in the presence of either the HA-tagged E4orf6 mutant which lacks a functional nuclear relocalization signal (NRS) **(Figure 2-4A, R240E/R241E, lanes 3-4),** or in which two amino acids within BC box 2 were altered **(Figure 2-4A, lanes 7-8)**, and for which degradation of AAV5 capsid proteins was more severely abrogated **(Figure 2-2, L122S/C126M, lanes 9 and 7, respectively)**. Whether interaction between AAV5 capsid proteins and E4orf6 can occur without participation of E1b-55k [in which case the inability of E4orf6 R240E/R241E HA to bind AAV5 capsid protein **(Figure 2-4A, lanes 3-4)** may play a role in its inability to degrade this substrate **(Figure 2-2, lane 9)**], or whether these results reflect the inability to isolate a less stable complex is currently being investigated. Additionally, it remains unclear whether the immunoreactive material at the top of the gel in Figure 2-4A is aggregated E1b-55k. Although impaired in its ability to direct degradation, the E4orf6 BC box 2 mutant L122S/C126M was still able to associate with the capsid proteins both in the presence

and in the absence of MG132 **(Figure 2-4A, lanes 7-8)**, suggesting that interaction with elongins and subsequent inclusion of the E1b-55k protein into the complex was critical for degradation of AAV5 capsid proteins.

The E4orf6/E1b-55k E3 ligase complex that ubiquitinylates p53, DNA ligase IV, and Mre11 utilizes cullin 5, which acts as a scaffold protein bringing E4orf6, which is linked to elongins B and C, to an E2 ubiquitin conjugating enzyme (51, 122, 167). Similarly, in our co-transfections, when cullin 5 was depleted by siRNA treatment, E4orf6 was no longer able to bring about the degradation of AAV5 capsid proteins **(Figure 2-4B, compare lanes 4 with 3)**. These results confirmed that degradation of *de novo*-generated AAV capsid proteins occurs via an E3 ubiquitin ligase complex containing E4orf6, E1b-55k and cullin 5.

AAV5 small Rep proteins could be co-immunoprecipitated with adenovirus E1b-55k in the presence or absence of adenovirus during transient transfection of 293 cells with a Flag-tagged E1b-55k **(Figure 2-4C, lanes 3 and 5)**. However, the small Rep proteins of AAV5 were unable to be co-immunoprecipitated with transfected HA-tagged cullin 5 (Cul5) in the absence of E4orf6 **(Figure 2-4D, compare lanes 3 and 5)**. This data suggests that similarly to p53, E1b-55k interacts with its protein substrate and then acts as a chaperone to bring the substrate to the rest of the cullin 5 E3 ubiquitin ligase complex, where it and the substrate (in this case, Rep52) are anchored to the ubiquitin ligase through E4orf6 and its interactions with the elongins B and C.

FIGURE 2-4. E4orf6 and E1b-55k form a cullin 5-containing E3 ligase complex together with *de novo*-generated AAV5 capsid and small Rep proteins.

(A). An immunoblot following electrophoresis in a denaturing 10% polyacrylamide gel, using either antibody to E1b-55k, top panel, or HA, bottom panel, of immunoprecipitations (90), using anti-AAV5 capsid antibody, of protein extracts taken at 48 hours post transfection of 293 cells with the minimal AAV5 P41-driven AAV5 capsid protein expressing plasmid either by itself (lanes 9-10), or together with a plasmid expressing wild-type E4orf6 tagged with HA (lanes 1 and 2), or various HA-tagged E4Orf6 mutants as described in the text (lanes 3-8), either in the presence of 10 μM MG132 (+), or DMSO vehicle control (-), added 6 hours prior to harvesting the cells. The positions of individual proteins are indicated.

(B). An immunoblot following electrophoresis in a denaturing 10% polyacrylamide gel, using either using either anti-AAV5 capsid antibody and anti-actin antibody, top panel, antibody to cullin 5 (clone H-300, # sc13014, Santa Cruz, Inc., Santa Cruz, CA), middle panel, or antibody to tubulin and HA, bottom panel, of protein extracts taken 48 hours post-transfection of 293 cells. Transfections were performed with either the minimal P41-driven AAV5 capsid protein-expressing plasmid (lanes 1-2), or this plasmid together with a plasmid expressing wild-type E4orf6 tagged with HA (lanes 3-4), either with (lanes 2-4) or without (lanes 1-3) additional transfection of 50 pmol of siRNA to cullin 5a (# 37574, Santa Cruz, Inc., Santa Cruz, CA) using the siRNA transfection reagent (#sc-29528, Santa Cruz, Inc., Santa Cruz, CA), 24 hours before transfection with the above mentioned plasmids. The locations of the individual proteins are shown.

(C) An immunoblot following electrophoresis in a denaturing 10% polyacrylamide gel, using either anti-flag antibody to detect E1b-55k, top panel, or HA to detect AAV5 Rep52, bottom panel, of immunoprecipitations (90), using anti-E1b-55k antibody 2A6, of protein extracts taken at 48 hours post transfection of 293 cells with a flag-tagged E1b-55k construct (a generous gift from Jianming Qiu, lanes 2-5) and/or a minimal HA-tagged CMV-driven AAV5 Rep52 protein expressing plasmid either by itself (lane 2), or together with a plasmid expressing wild-type E4orf6 (lane 5) in the presence of 10 μM MG132. The positions of individual proteins are indicated.

(D) An immunoblot following electrophoresis in a denaturing 10% polyacrylamide gel, using anti-HA antibody to detect HA-tagged cullin 5, top panel, and HA-tagged AAV5 Rep52, bottom panel, of immunoprecipitations (90), using anti-cullin 5 antibody, of protein extracts taken at 48 hours post transfection of 293 cells with an HA-tagged cullin 5 construct (a generous gift from Paola Blanchette, lanes 2-5) and/or a minimal HA-tagged CMV-driven AAV5 Rep52 protein expressing plasmid either by itself (lane 2), or together with a plasmid expressing wild-type E4orf6 (lane 5) in the presence of 10 μM MG132. The positions of individual proteins are indicated.

Additional thank-you to Ramnath Nayak for conducting the experiments shown in Figure 2-4A and 2-4B as part of our initial manuscript.

FIGURE 2-4. E4orf6 and E1b-55k form a cullin 5-containing E3 ligase complex together with *de novo*-generated AAV5 capsid proteins.

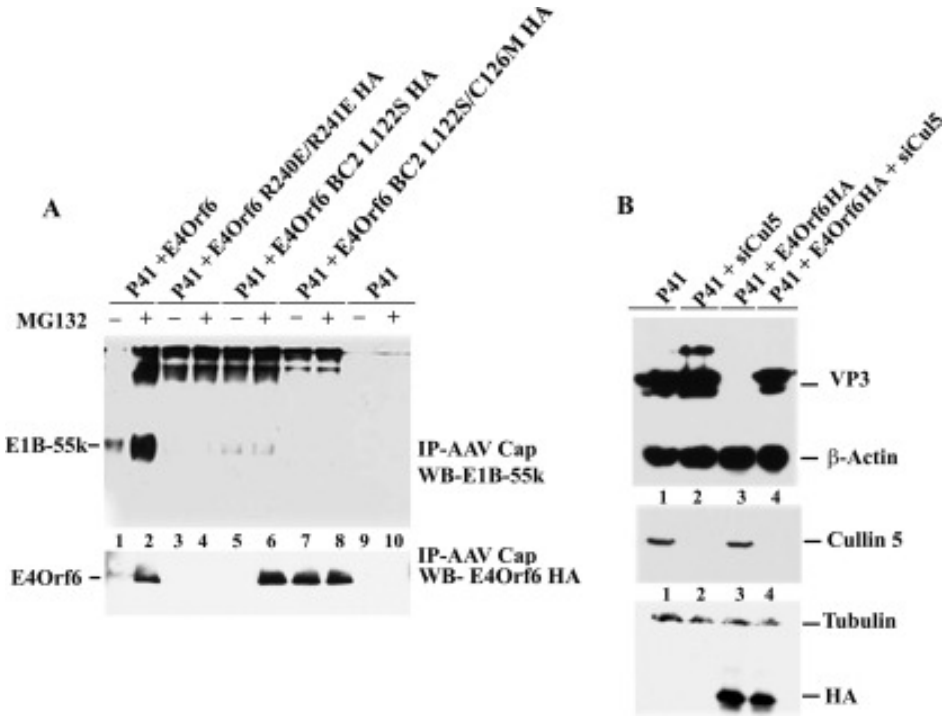
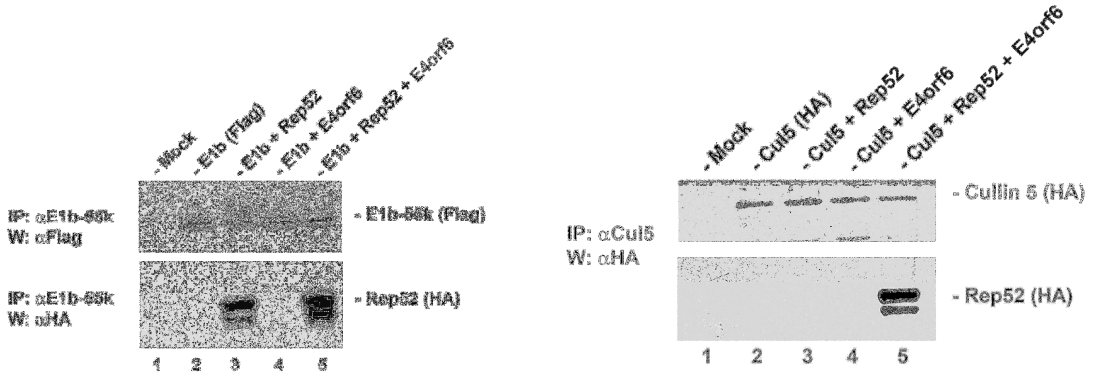


FIGURE 2-4. E4orf6 and E1b-55k form a cullin 5-containing E3 ligase complex together with *de novo*-generated AAV5 small Rep proteins.

(C) **(D)**



E4Orf6-E1B-55k-dependent degradation of AAV5 capsid and Rep52 proteins requires ubiquitin chain elongation.

E4orf6-E1b-55k-dependent degradation of *de novo*-generated AAV5 capsid proteins and Rep52 is inhibited by both MG132 and lactacystin (96), suggesting that it occurs in a proteasomal-dependent manner, which typically is mediated by ubiquitination of target proteins. As shown, E4orf6-E1b-55k-dependent degradation of both AAV5 capsid proteins **(Figure 2-5, compare lane 4 to lane 3)**, and Rep52 **(Figure 2-5, compare lane 8 to 7)**, was inhibited by a dominant negative, lysine-less ubiquitin UBR7 (137), which prevents the ubiquitin chain elongation required for targeting to, and processing by, the proteasome.

In 293 cells, the AAV5 small Rep proteins are ubiquitinated in the presence or absence of E4orf6.

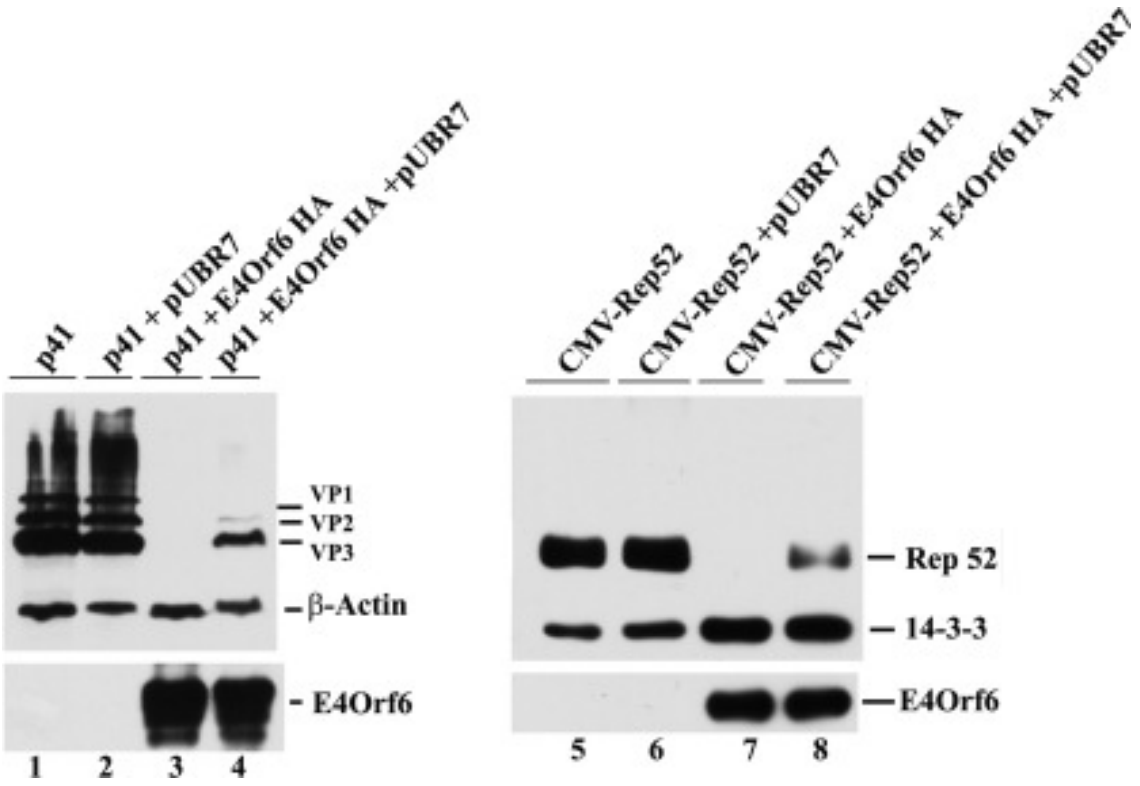
The adenovirus E1b-55k/E4orf6/Cullin5 E3 ubiquitin ligase complex targets substrate proteins p53, Mre11, and DNA Ligase IV for degradation by adding poly-ubiquitin chains to the proteins. We utilized an HA-tagged wild-type ubiquitin to determine whether the small Rep proteins were also ubiquitinated in the presence of E1b-55k and E4orf6. 293 cells were co-transfected with HA-tagged ubiquitin, Myc-tagged AAV5 Rep52, and/or adenovirus E4orf6 in the presence or absence of the proteasome inhibitor MG132.

In vivo ubiquitination assays demonstrated significant levels of high molecular-weight ubiquitinated isoforms of AAV5 Rep proteins in the presence of HA-ubiquitin, Rep, E4orf6, and MG132 **(Figure 2-6A, lane 5)**. As expected, a marked decrease in the

FIGURE 2-5. E4orf6/E1b-55k-directed degradation of *de novo*-generated AAV5 capsid and Rep52 proteins requires ubiquitin chain elongation.

An immunoblot following electrophoresis in a denaturing 10% polyacrylamide gel, using either using either anti-AAV5 capsid antibody and anti-actin antibody, top panel, or antibody to HA, bottom panel, of protein extracts taken 48 hours post-transfection of 293 cells with the minimal P41-driven AAV5 capsid protein expressing plasmid (lanes 1-4), or a plasmid expressing AAV5 Rep52 (lanes 5-8), or either of these plasmids together with a plasmid expressing wild-type E4orf6 tagged with HA (lanes 3-4, and 7-8, respectively), either with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) additional co-transfection of a plasmid expressing the dominant negative, lysine-less ubiquitin UBR7 described in the text. The positions of the individual proteins are indicated.

FIGURE 2-5. E4orf6/E1b-55k-directed degradation of *de novo*-generated AAV5 capsid and Rep52 proteins requires ubiquitin chain elongation.



levels of ubiquitinated substrate was observed in the absence of MG132 **(Figure 2-6A, lane 4)**. However, significant, albeit lower levels, of ubiquitinated Rep were observed even in the absence of E4orf6 **(Figure 2-6A, lane 3)**, suggesting that AAV5 small Rep proteins are ubiquitinated naturally in 293 cells through an E4orf6-independent mechanism. A similar outcome was observed when all ubiquitinated proteins were immunoprecipiated and subsequently western blotted for the presence of Rep **(Figure 2-6B, lanes 3-5)**.

In 293 cells, *de-novo*-synthesized capsid proteins are ubiquitinated only in the presence of adenovirus E4orf6.

Upon entry into an infected cell, fully-assembled AAV capsid particles are ubiquitinated and degraded, a process that is thought to downregulate transduction by preventing efficient trafficking of the assembled viral particle to the nucleus (37). However, it is unknown whether *de-novo*-synthesized capsid proteins are also ubiquitinated. Given that AAV5 capsid proteins are degraded by the proteasome in an E4orf6/E1b-55k-dependent manner (96), we hypothesized that newly-formed capsid proteins could also be ubiquitinated. *In vivo* ubiquitination assays confirmed that in the presence of E4orf6 and MG132 in 293 cells, *de-novo*-synthesized capsid proteins are ubiquitinated **(Figure 2-6C, lane 5)**. As expected, the levels of higher molecular-weight isoforms of the capsid proteins are reduced in the absence of MG132 **(Figure 2-6C, lane 4)**. However, in contrast to the small Rep proteins, we were unable to detect significant amounts of ubiquitinated capsid proteins in the absence of E4orf6, even in the presence of MG132 **(Figure 2-6C, lane 3)**. Therefore, although fully assembled capsid proteins

Figure 2-6: In 293 cells, the AAV5 small Rep proteins are ubiquitinated in the presence or absence of E4orf6; however, AAV5 capsid proteins are only ubiquitinated in 293 cells in the presence of E4orf6.

(A) In-vivo ubiquitination immunoblot following transient co-transfection of 293 cells of a myc-tagged, CMV-driven AAV5 Rep52 construct, a construct featuring an HA-tagged ubiquitin (a generous gift from Mark Hannink), and a wild-type E4orf6 expression vector in the presence of MG132 (lane 5). Transfections were also conducted in the absence of Rep (lane 1), HA-tagged ubiquitin (lane 2), E4orf6 (lane 3), or MG132 (lane 4). After a 48-hour co-transfection, cells were lysed and boiled in a buffer containing 1% SDS before diluting five-fold in an identical buffer lacking SDS. Immunoprecipitations were conducted using the anti-myc antibody. Immunoblots were carried out using either the HA-antibody, top panel, to detect ubiquitin-conjugated Rep substrates, or the myc-antibody, bottom panel, to detect AAV5 Rep52 as a control for the immunoprecipitation.

(B) In-vivo ubiquitination immunoblot following transient co-transfection of 293 cells of a myc-tagged, CMV-driven AAV5 Rep52 construct, a construct featuring HA-tagged ubiquitin, and the wild-type E4orf6 expression vector in the presence of MG132 (lane 5). Transfections were also conducted in the absence of Rep (lane 1), HA-tagged ubiquitin (lane 2), E4orf6 (lane 3), or MG132 (lane 4). After a 48-hour co-transfection, cells were lysed and boiled in a buffer containing 1% SDS before diluting five-fold in an identical buffer lacking SDS. Immunoprecipitations were conducted using the anti-HA antibody. Immunoblots were carried out using either the HA-antibody, top panel, to detect total cellular ubiquitin-conjugated substrates as a control for the immunoprecipitation, or the myc-antibody, bottom panel, to detect AAV5 Rep52.

(C) In-vivo ubiquitination immunoblot following transient co-transfection of 293 cells of the minimal AAV5 P41-Cap construct, a construct featuring an HA-tagged ubiquitin, and the wild-type E4orf6 expression vector in the presence of MG132 (lane 5). Transfections were also conducted in the absence of Cap (lane 1), HA-tagged ubiquitin (lane 2), E4orf6 (lane 3), or MG132 (lane 4). After a 48-hour co-transfection, cells were lysed and boiled in a buffer containing 1% SDS before diluting five-fold in an identical buffer lacking SDS. Immunoprecipitations were conducted using the anti-capsid antibody. Immunoblots were carried out using either the HA-antibody, top panel, to detect ubiquitin-conjugated Rep substrates, or the anti-capsid antibody, bottom panel, to detect AAV5 capsid proteins as a control for the immunoprecipitation.

Figure 2-6: In 293 cells, the AAV5 small Rep proteins are ubiquitinated in the presence or absence of E4orf6.

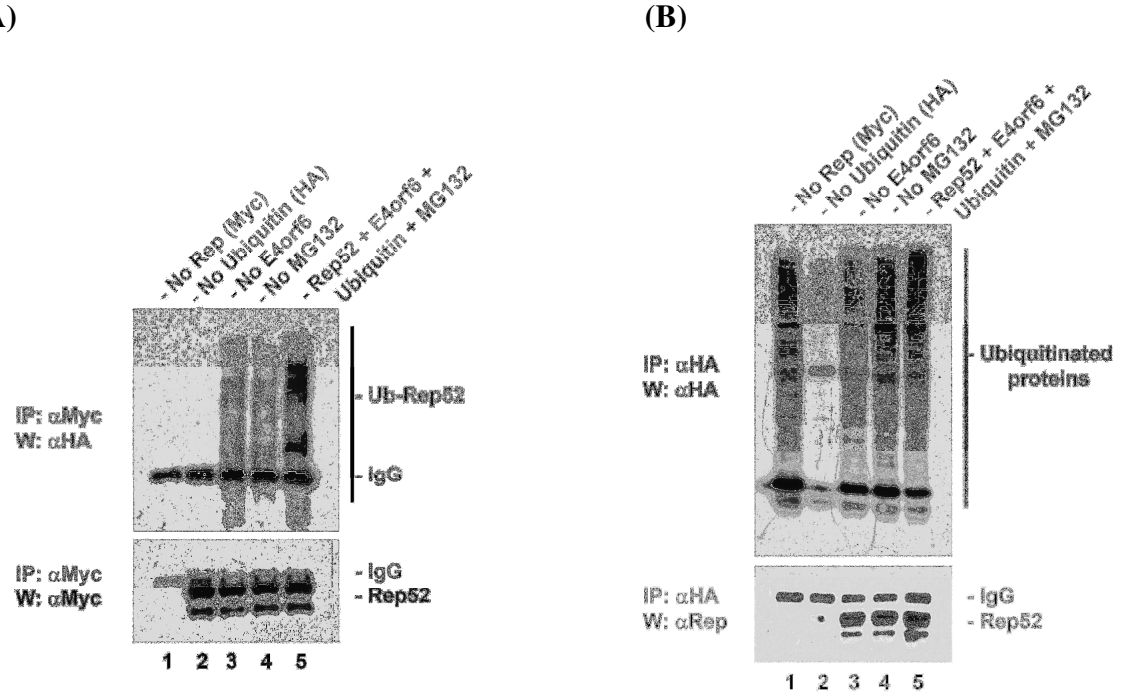
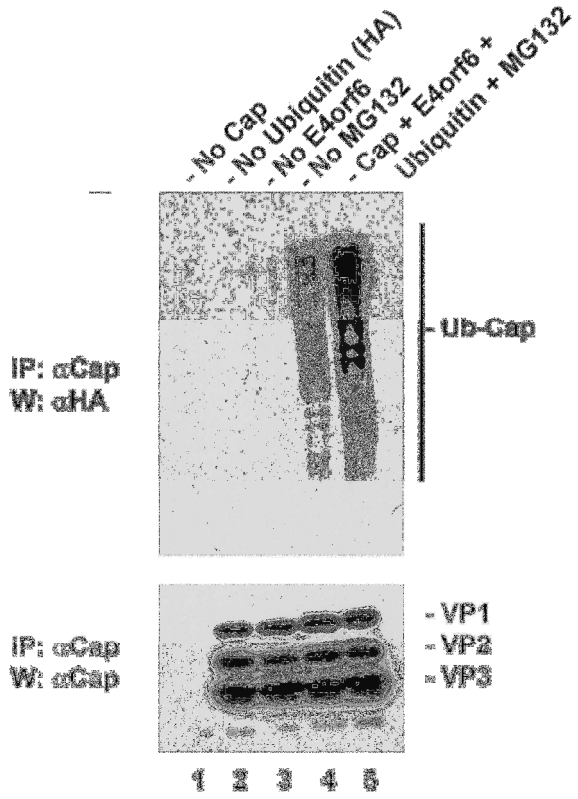


Figure 2-6: In 293 cells, *de-novo*-synthesized capsid proteins are ubiquitinated only in the presence of adenovirus E4orf6.

(C)



are ubiquitinated upon entry to an infected cell, *de-novo*-synthesized capsid proteins are only ubiquitinated in 293 cells in the presence of E4orf6. This suggests that the proteasome-mediated degradation of AAV5 capsid proteins is indeed caused by the poly-ubiquitination of capsids by the E4orf6/E1b-55k/Cullin5 E3 ubiquitin ligase complex.

The AAV5 small Rep proteins display multiple ubiquitination patterns.

There are a number of different types of mono- and poly-ubiquitination, and the type of ubiquitination that occurs determines the fate of the ubiquitinated substrate protein. The two major types of ubiquitination are through lysine 48 (K48) and lysine 63 (K63) poly-ubiquitin ligation. Poly-ubiquitination through K48 is the proteolytic form, and is the form that traditionally targets proteins for degradation by the proteasome. Poly-ubiquitination through K63 is non-proteolytic, and the fates of these proteins vary greatly, having effects on protein characteristics such as trafficking, translation, and kinase activation (16, 55, 57, 108, 146). We wondered whether the type of ubiquitination that occurred on AAV5 small Rep proteins was different in 293 cells depending upon the presence of E4orf6.

We utilized two specific HA-tagged ubiquitin mutants: one mutant containing a series of mutations in which all ubiquitin lysines were mutated to arginine except the lysine at position 48 (K48), and another containing a series of mutations in which all lysines except the one at position 63 (K63) were mutated to arginine. As expected, the use of the K48-specific ubiquitin overexpression construct indicated that significant levels of poly-ubiquitinated AAV5 small Rep proteins were detected in the presence of MG132 and E4orf6; however, no significant levels of ubiquitination were detected in the

absence of E4orf6 **(Figure 2-7B, compare lanes 3-5)**. Interestingly, the use of the K63-specific ubiquitin overexpression construct indicated that there was significant ubiquitination of Rep occurring in the presence or the absence of E4orf6 **(Figure 2-7A, compare lanes 3-5)**.

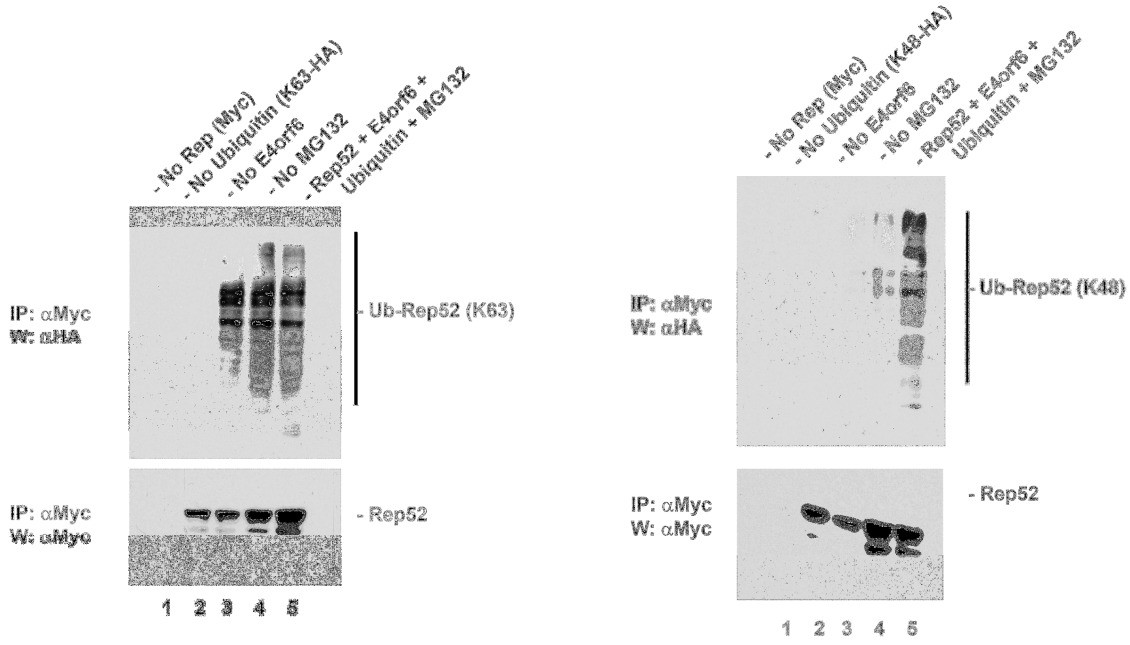
Therefore, as is the case with p53 and the other known substrates of this specific adenovirus E3 ubiquitin ligase complex, the AAV5 small Rep proteins are targeted for degradation by the proteasome via K48-specific poly-ubiquitination by adenovirus E4orf6 and E1b-55k. Additionally, the AAV5 small Rep proteins are also poly-ubiquitinated via K63 in a manner independent of adenovirus E4orf6. This K63-specific poly-ubiquitination likely does not result in targeting of the protein for degradation, but may be required for some as-of-yet unknown function for the small Rep proteins.

Figure 2-7: The AAV5 small Rep proteins display multiple ubiquitination patterns.

(A) In-vivo ubiquitination immunoblot following transient co-transfection of 293 cells of a myc-tagged, CMV-driven AAV5 Rep52 construct, a construct featuring an HA-tagged K63-specific ubiquitin (a generous gift from Mark Hannink), and a wild-type E4orf6 expression vector in the presence of MG132 (lane 5). Transfections were also conducted in the absence of Rep (lane 1), HA-tagged K63-specific ubiquitin (lane 2), E4orf6 (lane 3), or MG132 (lane 4). After a 48-hour co-transfection, cells were lysed and boiled in a buffer containing 1% SDS before diluting five-fold in an identical buffer lacking SDS. Immunoprecipitations were conducted using the anti-myc antibody. Immunoblots were carried out using either the HA-antibody, top panel, to detect ubiquitin-conjugated Rep substrates, or the myc-antibody, bottom panel, to detect AAV5 Rep52 as a control for the immunoprecipitation.

(B) In-vivo ubiquitination immunoblot following transient co-transfection of 293 cells of a myc-tagged, CMV-driven AAV5 Rep52 construct, a construct featuring an HA-tagged K48-specific ubiquitin (a generous gift from Mark Hannink), and a wild-type E4orf6 expression vector in the presence of MG132 (lane 5). Transfections were also conducted in the absence of Rep (lane 1), HA-tagged K48-specific ubiquitin (lane 2), E4orf6 (lane 3), or MG132 (lane 4). After a 48-hour co-transfection, cells were lysed and boiled in a buffer containing 1% SDS before diluting five-fold in an identical buffer lacking SDS. Immunoprecipitations were conducted using the anti-myc antibody. Immunoblots were carried out using either the HA-antibody, top panel, to detect ubiquitin-conjugated Rep substrates, or the myc-antibody, bottom panel, to detect AAV5 Rep52 as a control for the immunoprecipitation.

Figure 2-7: The AAV5 small Rep proteins display multiple ubiquitination patterns.



DISCUSSION

We have demonstrated that E4orf6/E1b-55k-dependent degradation of AAV5 proteins is mediated by a ubiquitinating, cullin 5-containing, E3 ligase complex similar to that previously demonstrated to degrade Mre11, DNA ligase IV, and p53. This effect is specific to AAV5 and AAV2 small Rep and capsid proteins. The BC-box motifs of E4orf6, and hence the interaction between E4Orf6 and cellular elongins B and C, are crucial for E3 activity. Similar BC-box motifs have been found in the E4orf6 proteins of other adenovirus serotypes, and E4orf6 from Ad4, Ad9, and Ad12 can all direct degradation of AAV5 proteins in 293 cells, although the activity of Ad12 is less robust.

The AAV5 capsid proteins are co-immunoprecipitated with adenovirus E1b-55k and E4orf6; however, the details of these interactions are still yet unclear and are being revisited. The AAV5 small Rep proteins, however, are clearly shown here to interact with E1b-55k in the absence or presence of E4orf6. Interactions between AAV5 Rep and cullin 5 (and presumably, the rest of the Cul5 complex) occur only in the presence of E4orf6. This suggests a model in which during transient transfection of 293 cells, AAV5 small Rep proteins are bound by E1b-55k and carried to the rest of the cullin 5 E3 ubiquitin ligase complex, where Rep and E1b interact as part of the complex through interactions requiring E4orf6. In the case of p53, this interaction with E4orf6 is thought to be direct; however, it is unknown at this time whether Rep interacts with E4orf6 directly or if its interaction comes only as part of the complex. We also demonstrate that the E3 ligase complex brings about the degradation of AAV2 and AAV5 proteins via the addition of K48-specific poly-ubiquitin moieties onto the AAV substrate proteins.

Surprisingly, we also found that AAV5 small Rep proteins are ubiquitinated through K63-mediated poly-ubiquitin chain elongation in the presence or absence of adenovirus E4orf6 in 293 cells. Thus, we report here the first example of the ubiquitination of a parvovirus non-structural protein. The implications of this phenomenon will be of great interest in our understanding of the effects of ubiquitination in the life cycle of AAV and of parvoviruses in general. Experiments are currently planned for the expansion of these *in vivo* ubiquitination and co-immunoprecipitation assays on other parvovirus non-structural proteins, such as those from MVM.

E4orf6/E1b-55k dependent degradation of Mre11 and p53 (122, 124, 135, 144), as well as a E4orf6/E1b-55k-dependent degradative function required for RNA export (167), is necessary for efficient replication of Ad5 (144). E4orf6/E1b-55k-dependent degradation of Mre11 has recently also been shown to enhance AAV2 replication (135); however, there are likely to be multiple mechanisms by which E4orf6 supplies help to AAV replication. How can E4orf6/E1b-55k-dependent degradation of AAV proteins be reconciled with their role as a helper functions? We have previously shown that the enhancement of translation that VA RNA provides as part of its helper function is necessary to restore AAV5 protein levels to those necessary for viral replication. It may be that E4orf6/E1b-55k-dependent degradation of AAV5 proteins by E4orf6 may merely be a by-product of its role in targeting the degradation of a cellular protein necessary for viral replication. In this scenario, perhaps only the required levels of AAV5 proteins, and not the cellular target whose degradation is required for viral replication, becomes restored by VA RNA activity. Alternatively, it may be that AAV has evolved to rely on E4orf6 and E1b-55k as regulators of its own gene expression. If unopposed, VA RNA

might enhance excessive amounts of viral Rep and Cap at inappropriate times, which might be detrimental to infection. Another possibility might be that E4orf6/E1b-55k activity in this regard has evolved to aid adenovirus replication in the presence of AAV, targeting the degradation of AAV proteins as a protective measure to temper AAV expression during AAV/Ad co-infection. Whatever its role in promoting AAV infection, E4orf6/E1b-55k-dependent degradation of AAV5 proteins is likely to be an important facet of AAV biology.

III. IN CONTRAST TO AAV2, AAV5 UTILIZES AN ALTERNATE INITIATION EVENT TO GENERATE A SMALL REP40 PROTEIN.

INTRODUCTION

The adeno-associated viruses are small, single-stranded DNA viruses of only approximately 4.7 kilobases (kB) in size. Thus, the genomic coding capacity of AAV is quite limited, and the virus must rely on a number of methods to enhance its protein repertoire. AAV2, the prototype serotype, utilizes three promoters at map units P5, P19, and P40, and the pre-mRNAs generated from these promoters encode all of the AAV proteins.

The alternative splicing of pre-mRNAs generated by the P5 promoter is responsible for the two large Rep proteins, Rep78 and Rep68. A number of functions are associated with the large Rep proteins, including DNA replication, promoter activation, DNA binding and endonuclease activity, integration, and nuclear localization (61, 87, 103, 105, 139, 171). The large Rep proteins also contain domains that have been implicated with helicase and ATPase activity.

Although much is known about the large Rep proteins and their functions, relatively much less is known about the small Rep proteins. In AAV2, Rep52 and Rep40 are encoded by P19 transcripts and differ in the alternative splicing of the intron. The primary role of the small Rep proteins is in the packaging of ssDNA genomes into preformed capsids (71), although some evidence suggests that the expression of these proteins may affect cell proliferation rates (78).

Interestingly, all four Rep proteins share a common domain, a conserved motif common to the superfamly 3 (SF3) helicases (49). The Rep52 and Rep40 helicases proceed in a 3'→5' fashion and are thought to act as motors to "pump" the ssDNA genomes into assembled capsids (71).

There appears to be some functional redundancy between Rep52 and Rep40 in that they both unwind DNA substrates with single-stranded ends and operate with the same polarity. However, Rep40 possesses the additional ability to unwind double-stranded ends of DNA (27). During AAV replication, Rep40 may play a role in the more efficient unwinding of the double-stranded replicative DNA form. Further unwinding may then be assisted by Rep52 and/or Rep40.

Although Rep52 functions as a monomer in solution (138), analyses of the biochemical characteristics and the crystal structure of Rep40 suggest that Rep40 functions as a hexamer when bound to its substrate (27, 62), and that the additional C-terminal portion of Rep52 not shared by Rep40 prevents this oligomerization. Taken together, it is clear that AAV2 requires both Rep52 and Rep40 for efficient packaging.

The transcription profile for AAV5 is quite different from that of AAV2 (118). Rep-encoding transcripts from the P7 and P19 promoters preferentially poly-adenylate in the intron in AAV5, as opposed to transcribing all the way to the distal end of the genome as in AAV2. As a result, these upstream transcripts are predominately poly-adenylated rather than spliced, and would therefore predict that only the unspliced isoforms of the Rep proteins (Rep78 and Rep52) would be made. However, we demonstrate in this work that not only does AAV5 generate a significant Rep40-like gene product, it likely accomplishes this feat through the use of an alternate initiation codon within the Rep52

reading frame. Therefore AAV5, and the other animal AAVs, may have evolved to generate this crucial protein by a method that is functionally distinct from that of AAV2 and the other human and non-human primate AAVs.

MATERIALS AND METHODS

Cell Lines, reagents, and Virus.

293 cells were propagated as previously described (91). AAV2, AAV5, and Goat AAV infections were carried out at an MOI of 10, while Ad5 infections were done at an MOI of 5. A pan-caspase inhibitor (z-VAD-FMK, Biomol) was used at a concentration of 10 μM.

Plasmid Constructs.

The AAV5 RepCap and HIV-Rep5 plasmids were described previously (118). Where utilized, all HA tags were placed in-frame of the AAV5 Rep proteins. AV5MfeHA was generated by placing an HA tag within the MfeI site of AAV5 RepCap. AV5HA380 was generated by using overlapping PCR mutagenesis to place an HA tag, within the intron, after amino acid 380 in the Rep52 protein. CMV-Rep52 was created by one-step PCR amplification of the entire AAV5 Rep52 coding region (AAV5 nts 1019 to 2191), with an HA tag just before the termination codon, into the *Hind*III and *Xba*I sites of pcDNA3.1. CMV-2R52 was generated by one-step PCR amplification of the entire AAV2 Rep52 coding region (AAV2 nts 993 to 2252), amplified from a Rep40-less AAV2 Rep52 silent mutant construct described previously (119), with an HA tag just before the termination codon, into the *Eco*RV and *Xba*I sites of pcDNA3.1. The CMV-Rep52 (HA 10) and CMV-Rep52 (HA 60) expression vectors were generated by overlapping PCR amplification of the Rep52 coding region, with an HA tag after amino acid 10 or amino acid 60, respectively.

The Rep52 deletion mutants (Δ31-60, Δ61-90, Δ91-120, Δ121-150, Δ45-49, Δ50-54, Δ55-59, Δ31-45, Δ31-35, Δ36-40, and Δ41-45) were made by overlapping PCR deletion of the amino acids indicated (with regards to Rep52). CMV-Rep52 mutants M50R, S51R, L52R, T53R, and K54R were created by overlapping PCR mutagenesis of the indicated amino acids in Rep52 to arginine (AGA, Arg). The CMV-Rep52 frameshift mutants (46, 48, 49, 50, and 51) were generated by placing a single base (A) after the indicated amino acids.

CMV-Rep52 (10/45sm, 30/45sm, and 45sm) mutants were made by overlapping PCR mutagenesis to introduce silent mutations in amino acids 10, 30, and/or 45 as to create ATGs out-of-frame with Rep52. CMV-2R52 (30sm, 45sm, and 30/45sm) mutants were made by making silent mutations in the indicated amino acids in the Rep reading frame that eliminated out-of-frame ATGs.

Transfections, RNA isolation, and RNase Protection Assays.

Transfections, using Lipofectamine and the Plus reagent (Invitrogen), were performed as described in (119). Total RNA was isolated 36-42 hours post-transfection or post-infection as previously described (94, 133). RNase Protection Assays were also performed as previously described (94, 133). The AAV2, AAV5, and Goat AAV RP probes were all described previously (91, 116, 118).

Protein isolation and Western Blot Analysis.

Cells were resuspended in SDS lysis buffer 24-36 hours post-transfection or post-infection, and western blots were conducted as described (95). Monocolonal antibody

303.9 (American Research Products, Inc.), which recognizes all AAV Rep proteins, was used at a 1:100 dilution. The mouse monoclonal HA antibody (Sigma-Aldrich) was used at a 1:5000 dilution. Secondary antibody goat-anti-mouse HRP (ARP, inc.) was used at a dilution of 1:5000. A monoclonal antibody to AMDV VP2 amino acids 428 to 446 (hybridoma cell line 282.20.1.4) was used as described (13, 115).

RESULTS

Although the AAV5 and goat AAV pre-mRNAs generated from the P7 and P19 promoters are not efficiently spliced, high levels of a Rep40-like protein product are produced during viral infection.

AAV2 requires both Rep52 and Rep40 for efficient packaging of its ssDNA genome into fully-assembled capsids (27, 62, 71). AAV2 uses alternative splicing of its small internal intron to generate Rep52 (from unspliced pre-mRNA) or Rep40 (from spliced pre-mRNA) from P19 transcripts. We have previously demonstrated that upstream messages from the P7 and P19 promoters in AAV5 and goat AAV are preferentially polyadenylated within the intron, at the detriment of splicing (116, 118, 120). Therefore, inefficient splicing would predict that significant levels of Rep40 would not be generated in AAV5. However, we have shown previously (120), and demonstrate here, that high levels of a Rep40-like protein product are generated during AAV infection despite the relatively low levels of splicing from upstream transcripts **(Figure 3-1).**

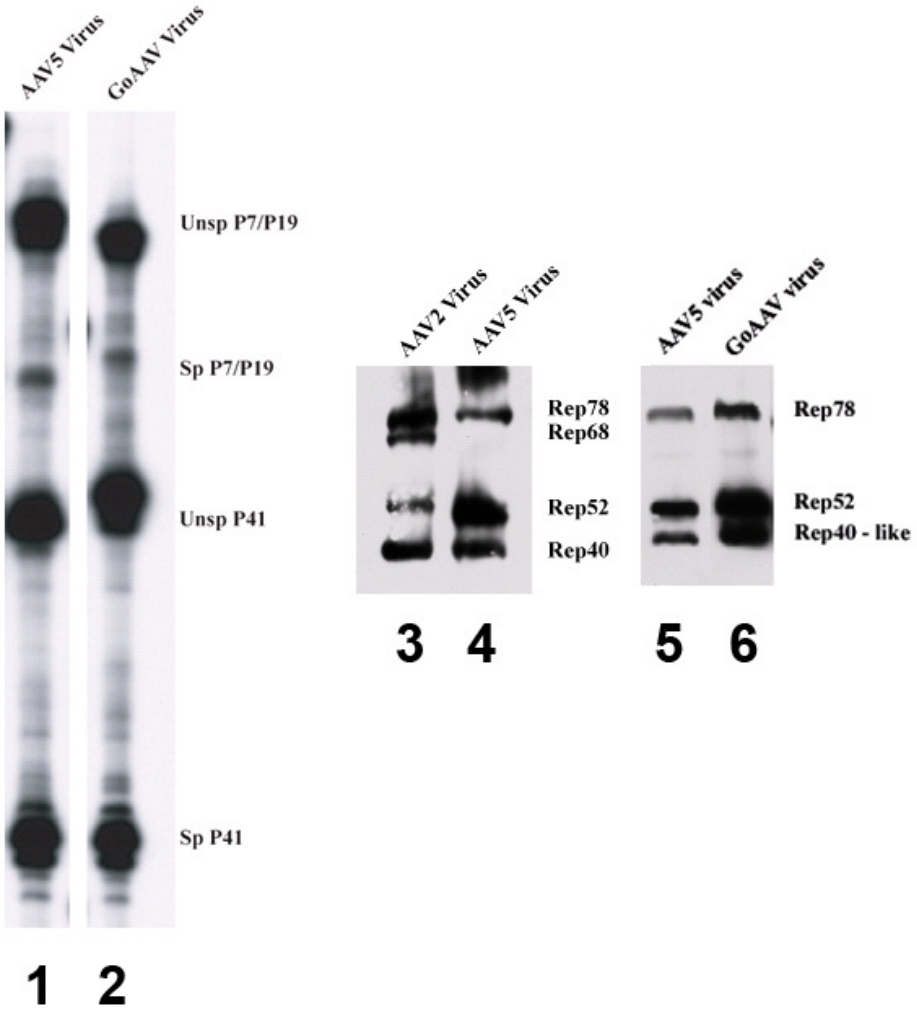
Unlike the parvovirus AMDV, the AAV5 and goat AAV Rep proteins are not cleaved by proteases.

Due to their small sizes, parvoviruses use a myriad of transcriptional, translational, and post-translational modifications to expand their protein repertoires. For example, the aleutian mink disease virus (AMDV) requires caspase cleavage of its capsid proteins to generate a small protein that is required for replication (8, 9). Therefore, we wondered whether AAV5 also utilized caspase cleavage as an alternate method of generating Rep40.

Figure 3-1: Although the AAV5 and goat AAV pre-mRNAs generated from the P7 and P19 promoters are not efficiently spliced, high levels of a Rep40-like protein product are produced during viral infection.

RNase protection assay (lanes 1 and 2) and western blot analysis (lanes 3-6) of AAV5, AAV2, and goat AAV infections in the presence of adenovirus type 5. Infections of AAV and Ad5 were carried out at multiplicities of infection (MOIs) of 5 and 10, respectively. AAV5 (lane 1) and goat AAV (lane 2) protections were conducted using appropriate RP probes, which allow the separation of the capsid-encoding P41-generated pre-mRNAs (Unsp P41 and Sp P41) from the upstream Rep-encoding P7 and P19 pre-mRNAs (Unsp P7/P19 and Sp P7/P19). Western blot analyses were carried out with the anti-Rep antibody 303.9 (American Research Products) that recognizes all four Rep proteins of all AAV serotypes. Locations of pre-mRNA and protein products are indicated. Lanes 1 and 5, and lanes 2 and 6, are from the same experiment.

Figure 3-1: Although the AAV5 and goat AAV pre-mRNAs generated from the P7 and P19 promoters are not efficiently spliced, high levels of a Rep40-like protein product are produced during viral infection.



Addition of a pan-caspase inhibitor during AMDV transfection prevented the protein cleavage necessary for generation of the small protein **(Figure 3-2, lanes 1 and 2)**. However, addition of the inhibitor during AAV5 transfection **(Figure 3-2, compare lanes 3 and 5 to lanes 4 and 6)** or infection **(Figure 3-2, lanes 7 and 8**), failed to prevent the accumulation of Rep40.

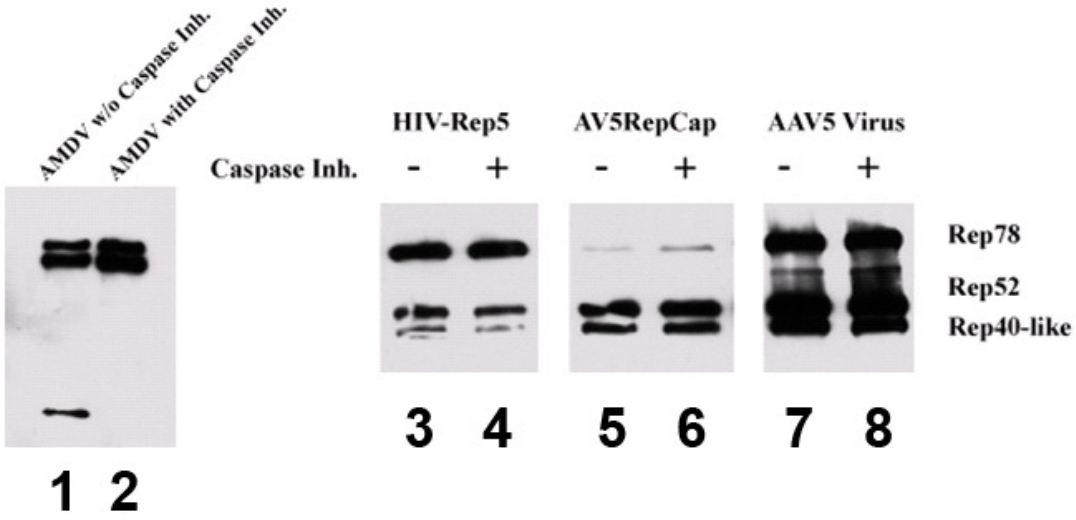
The use of HA tags reveals that AAV5 Rep52 and Rep40 share the same C-terminus.

In AAV2, Rep52 and Rep40 share the same N-terminus and differ in their C-termini by virtue of alternative splicing. As this was likely not to be the case in AAV5 due to inefficient splicing of P19-generated pre-mRNAs, we then set out to define the N-terminal and C-terminal ends of Rep40 to give us a better understanding of its origin. We therefore placed HA tags within the AAV5 RepCap plasmid, one just upstream of the intron **(Figure 3-3, lane 1, termed [AV5MfeHA])** and one within the intron, ten amino acids from the C-terminus of Rep78 and Rep52 **(Figure 3-3, lane 2, termed [AV5HA380**]). If AAV5 utilized alternative splicing to generate its Rep40 protein product in a manner identical to that in AAV2, we hypothesized that the AV5MfeHA tag would allow all three Rep proteins (Rep78, Rep52, and Rep40) to be recognized by the HA antibody, whereas the AV5HA380 tag would only allow Rep78 and Rep52 to be recognized as the tag would be removed during splicing. Surprisingly, the levels of Rep40 protein were unaffected when using the intronic HA tag **(Figure 3-3, compare lanes 1 and 2).**

Figure 3-2: Unlike the parvovirus AMDV, the AAV5 and goat AAV Rep proteins are not cleaved by caspases.

Immunoblots of AMDV capsid and AAV5 Rep protein expression in the presence or absence of the pan-caspase inhibitor z-VAD-FMK. Where indicated, 293 cells were either transfected (lanes 1-6) or infected with AAV5 at an MOI of 5. Ad5 at an MOI of 10 was used to co-infect all AAV5 samples (lanes 3-8). Locations of AAV5 Rep protein products are indicated.

Figure 3-2: Unlike the parvovirus AMDV, the AAV5 and goat AAV Rep proteins are not cleaved by caspases.



We then asked whether AAV5 Rep52 and Rep40 differed in their N-termini. The entire Rep52 coding sequence was placed behind the CMV promoter with an HA tag after amino acid 10 [CMV-Rep52 (HA 10)] or amino acid 60 [CMV-Rep52 (HA 60)]. Interestingly, AAV5 Rep40 was not detected using the HA antibody when the HA tag was placed at position 10 **(Figure 3-3, lane 3**); however, significant levels of Rep40 were detected using the HA antibody when the HA tag was placed at position 60 **(Figure 3-3, lane 4)**.

Taken together, the data indicates that unlike AAV2, AAV5 Rep40 is not generated by alternative splicing, and that Rep52 and Rep40 share the same C-terminus and differ in their N-termini.

Mapping of the N-terminal region of AAV5 Rep52 reveals that Rep40 is likely generated by the usage of an internal methionine ATG initiation codon.

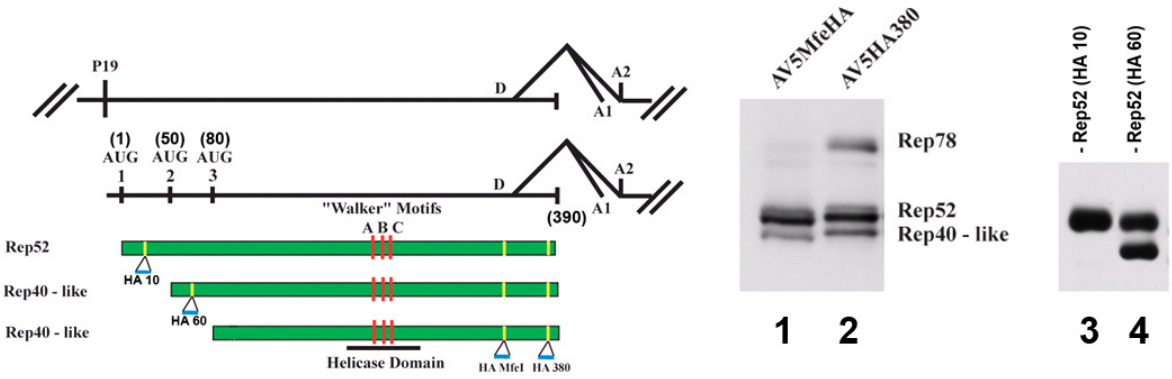
One of the possibilities for the origin of the Rep40 protein in AAV5 is that it is the result of a specific protease cleavage event of Rep52. Our previous data suggested that the N-terminal end of Rep40 resided between residues 10 and 60. Calculations of the size difference between Rep52 and Rep40 further placed the approximate N-terminal end of Rep40 between amino acids 45 and 55. The (HA 10) mutant only yielded a Rep52 product; protease cleavage of the protein may result in a smaller product (5 to 10 kD), but such a fragment was not detected by the HA tag. This did not fully discount the possibility of protease cleavage, however, as this smaller protein fragment may not be stable *in vivo*.

Figure 3-3: The use of HA tags reveals that AAV5 Rep52 and Rep40 share the same C-terminus.

(Left) Diagram of AAV5 Rep52 mutants used in this study. Approximate location of Walker motif residues are indicated. Numbering systems are based on the amino acid sequence of Rep52 (out of 390 amino acids) with the AAV5 Rep52 initiating AUG set at 1.

(Right) Western blot analysis of HA-tagged AAV5 RepCap vectors (lanes 1 and 2) or CMV-driven AAV5 Rep52 vectors (lanes 3 and 4).

Figure 3-3: The use of HA tags reveals that AAV5 Rep52 and Rep40 share the same C-terminus.



The other possibility for the origin of the Rep40 protein is that it is the result of an alternate initiation event. Indeed, an additional methionine start codon resides at position 50; however, this methionine is also within AAV2, which it is not used. Thus, it was imperative that we determine the exact N-terminal amino acid.

First, a series of large, 30-amino-acid deletions in Rep52 were made in order to confirm the approximate location of the N-terminal end of Rep40. As compared to the wild-type CMV-Rep52 construct, the (Δ31-60) mutant resulted in a noticeable shift in the size of the Rep52 protein and an elimination of the Rep40 protein **(Figure 3-4A, compare lanes 1 and 2)**. In addition, the (Δ91-120) and (Δ121-150) mutants both resulted in a noticeable shift in both Rep52 and Rep40, indicating that these regions are shared by both proteins **(Figure 3-4A, compare lane 1 to lanes 4 and 5**). A (Δ61-90) mutant **(Figure 3-4A, lane 3),** as well as the (Δ91-120) mutant, consistently resulted in low levels of Rep expression, indicating that these mutants may render the Rep protein unstable. The lack of Rep40 expression in the (Δ31-60) mutant was consistent with our previous data and again suggested that this region is required for the production of Rep40 in AAV5.

We next attempted to narrow down the Rep40 start region even further by developing a series of smaller, 5-amino-acid deletions as to hopefully generate in a simple deletion mutant that would result in the elimination of Rep40. None of the mutants tested (Δ31-35, Δ36-40, Δ41-45, Δ45-49, Δ50-54, or Δ55-59) resulted in the elimination of Rep40, although they all resulted in a slight shift in the size of Rep52 **(Figure 3-4A, compare lanes 6-8 to lane 1, and Figure 3-4B, compare lanes 4-6 to lane 1)**. Surprisingly, the (Δ50-54) mutant, which eliminated the internal methionine at

position 50, also did not result in the elimination of Rep40 **(Figure 3-4A, lane 7**). However, we hypothesized that it was still possible that this mutant may derive Rep40 from alternate initiation if an additional non-methionine start codon may be utilized in this mutant.

To test the importance of the methionine at position 50 directly, we created a series of point mutations in which the individual amino acids at positions 50-54 were mutated to arginine. The point mutation (M50R) resulted in a clear, reproducible elimination of Rep40 **(Figure 3-4C, compare lanes 1 and 2)** that did not result in the increase of Rep52. Other mutations in this region **(Figure 3-4C, lanes 3-6)** had no effect on the accumulation of Rep40 protein product. The fact that the levels of Rep52 in the M50R mutant were largely unchanged as compared to the levels of Rep52 from the wild-type construct strongly indicated that the loss of Rep40 through this mutation was likely the result of the loss of alternate initiation at this location, and not due to the prevention of a specific protease cleavage event, as this would be expected to increase the levels of Rep52.

The potential use of the internal methionine was tested further by a series of frameshift and ochre mutations around this region. The introduction of premature termination codons to any position prior to the internal methionine resulted in the loss of Rep52 but not Rep40 **(Figure 3-4D, left panel, compare lane 1 to lanes 2 and 3).** Introduction of premature termination codons after M50 resulted in a loss of both proteins **(Figure 3-4D, left panel, lanes 4 and 5**). In addition, the insertion of frameshift mutations upstream of M50 also resulted in the loss of Rep52 but not Rep40 **(Figure 3-4D, right panel, compare lane 1 to lanes 2-4**), and the insertion of frameshift mutations

after M50 resulted in the loss of both proteins (**Figure 3-4D, right panel, lanes 5 and 6**). Taken together, this data indicates that AAV5 likely generates a Rep40 protein product by the additional use of an internal methionine start codon at position 50.

Figure 3-4: Mapping of the N-terminal region of AAV5 Rep52 reveals that Rep40 is likely generated by the usage of an internal methionine AUG initiation codon.

(A) Diagram of locations of the deletion mutants used in this study, top, and immunoblot analysis of deletion mutants (lanes 2-8) in the HA-tagged CMV-driven AAV5 Rep52 vector (lane 1) following transient transfection into 293 cells, bottom. Precise amino acids deleted in each vector are indicated. Note that the Δ50-54 and the Δ31-60 mutants delete an internal methionine from the Rep52 reading frame. In the map, the numbers (1), (50), and (80) correspond to the amino acid position of the first three (1, 2, and 3) in-frame methionines.

(B) Diagram of locations of the deletion and mutants used in this study, top, and immunoblot analysis of deletion (lanes 3-6) or point (lane 2) mutants in the HA-tagged CMV-driven AAV5 Rep52 vector (lane 1) following transient transfection into 293 cells, bottom. Precise amino acids deleted in each vector are indicated.

(C) Diagram of locations of the point mutants used in this study, top, and immunoblot analysis of point (lane 2-6) mutants in the HA-tagged CMV-driven AAV5 Rep52 vector (lane 1) following transient transfection into 293 cells, bottom. Precise amino acids deleted in each vector are indicated.

(D) Diagram of locations of the frameshift and ochre mutations used in this study, top, and immunoblot analysis of ochre **(left, lanes 2-5)** and frameshift **(right, lanes 2-6)** mutants as compared to the HA-tagged wild-type CMV-driven AAV5 Rep52 vector **(lane 1, both left and right)**.

Figure 3-4A: Mapping of the N-terminal region of AAV5 Rep52 reveals that Rep40 is likely generated by the usage of an internal methionine AUG initiation codon.

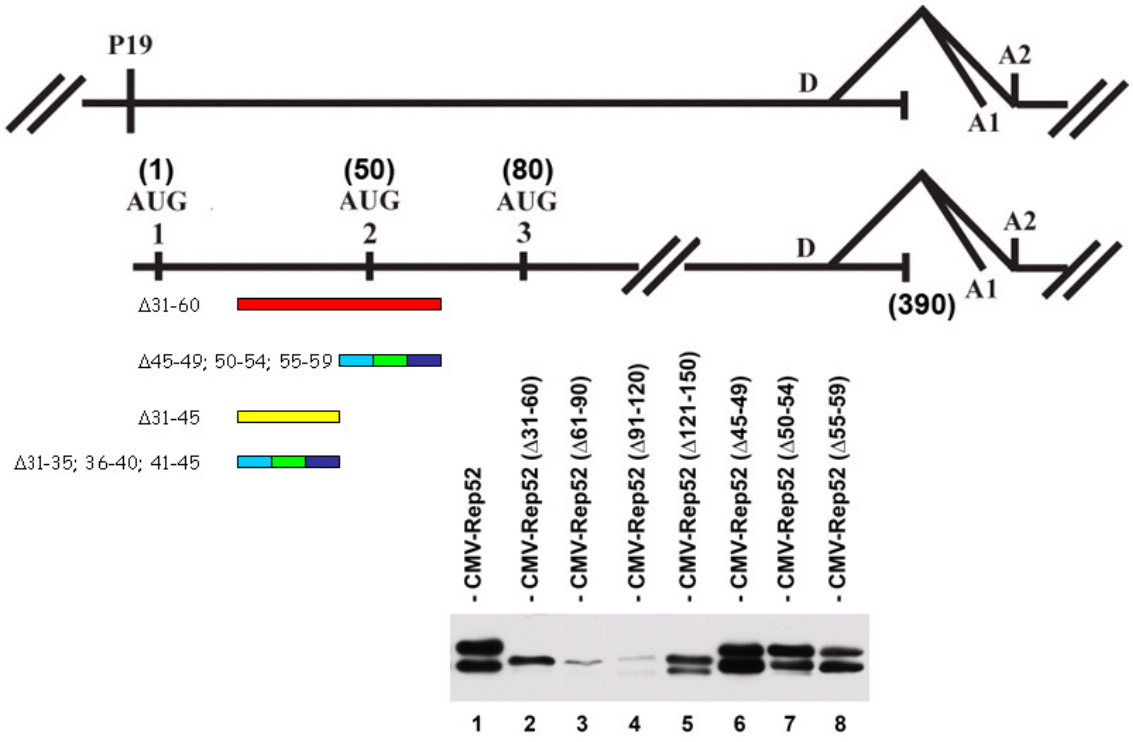


Figure 3-4B: Mapping of the N-terminal region of AAV5 Rep52 reveals that Rep40 is likely generated by the usage of an internal methionine AUG initiation codon.

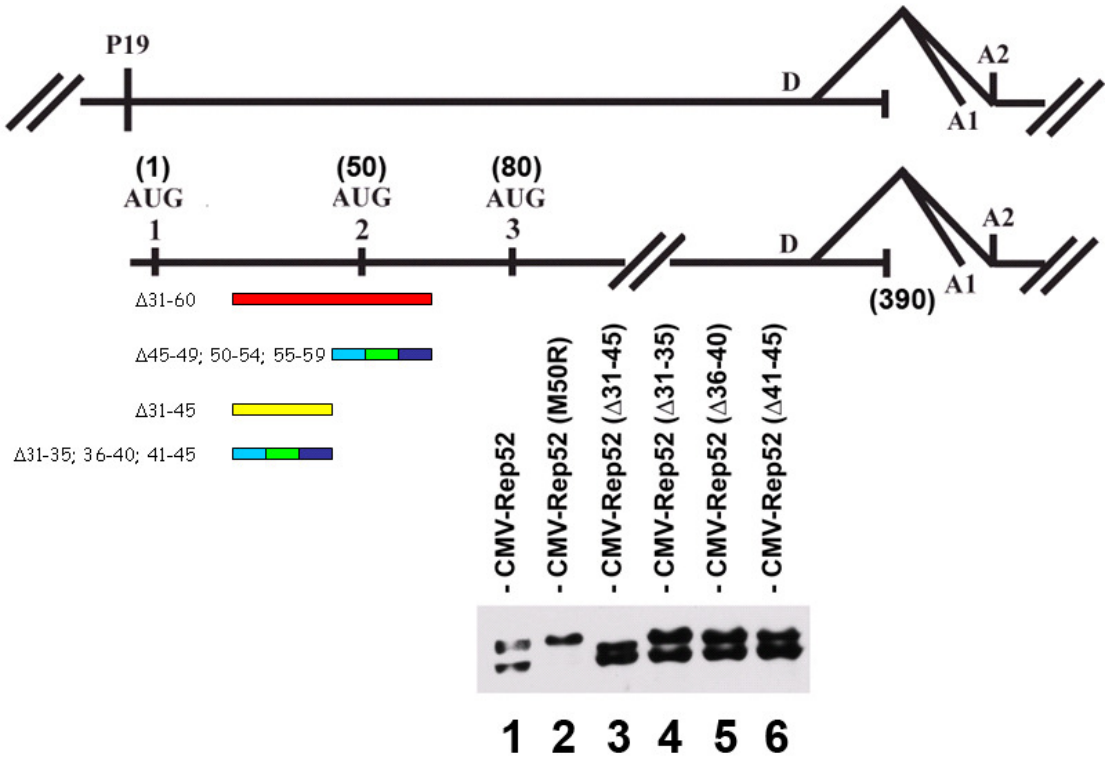


Figure 3-4C: Mapping of the N-terminal region of AAV5 Rep52 reveals that Rep40 is likely generated by the usage of an internal methionine AUG initiation codon.

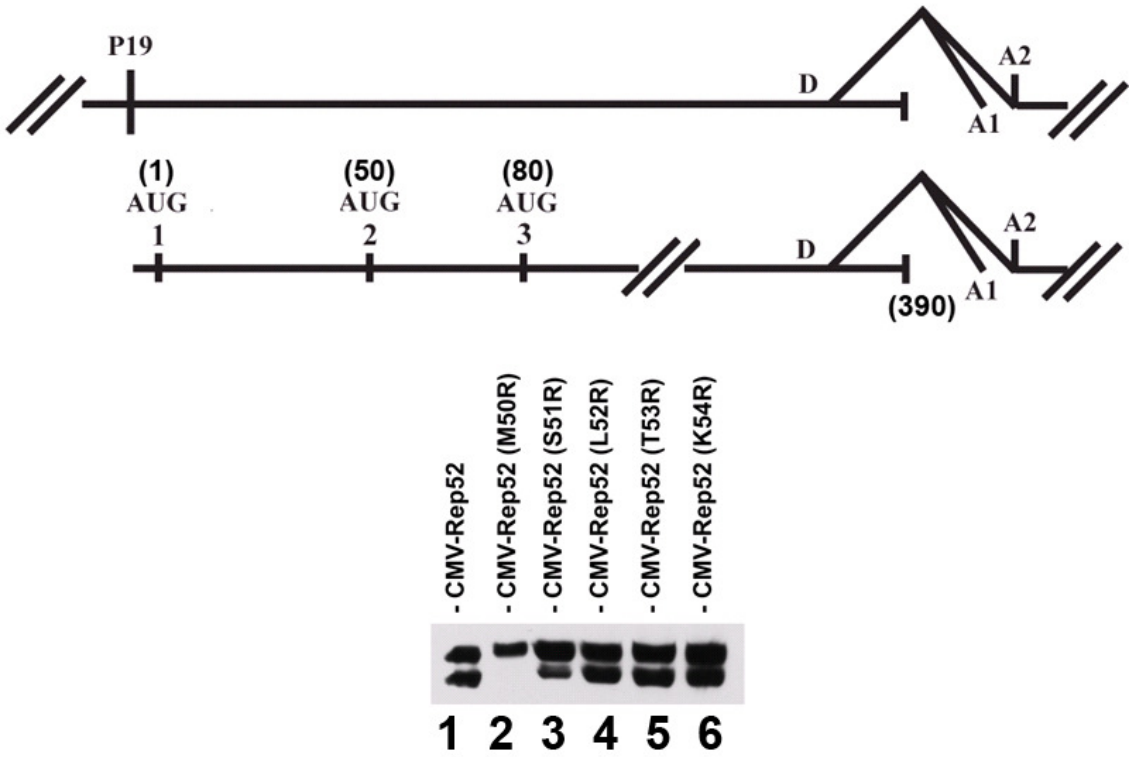
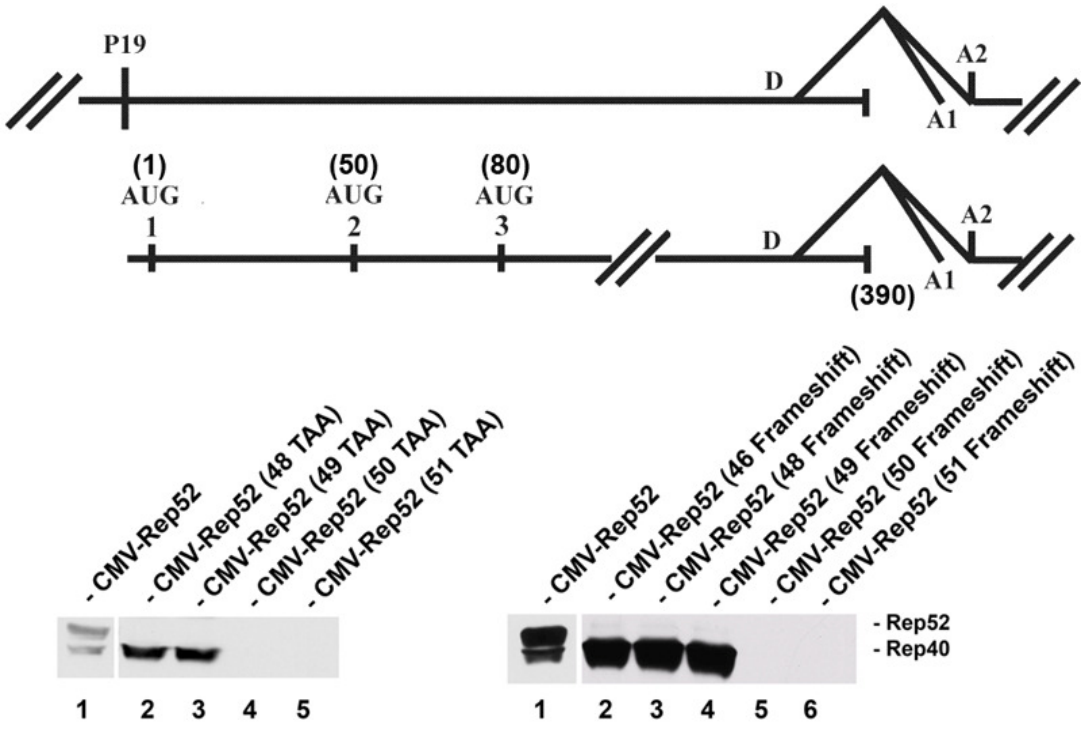


Figure 3-4D: Mapping of the N-terminal region of AAV5 Rep52 reveals that Rep40 is likely generated by the usage of an internal methionine AUG initiation codon.



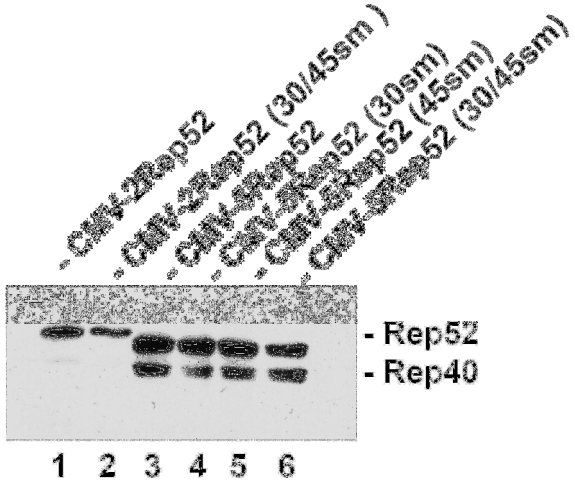
The mechanism by which the M50 start codon may be utilized in AAV5, but not AAV2, is not due to the usage of additional out-of-frame AUG triplets within AAV2 small Rep-encoding pre-mRNAs.

As demonstrated previously, although AAV2 also contains an internal methionine at this position, it is not used (119). The examination of the mechanism by which this occurs in AAV5 will present a fascinating closer look into the biology of AAV. In all AAV serotypes, there are a number of ATGs within the intron out-of-frame of Rep. It is possible that these ATGs prevent the usage of the VP1 ATG codon in unspliced P40 pre-mRNA. In AAV2 P19 mRNAs, there are two ATGs out-of-frame with Rep52, and they lie between the first ATG and Met50. AAV5 does not contain these additional ATGs. Therefore, we wondered whether the presence or absence of these additional ATGs could explain the usage, or prevention, of Met50 as a start codon in AAV5 or AAV2, respectively. Silent mutations were generated in AAV2 such that these internal ATGs were mutated without changing the Rep52 amino acid sequence. Comparable mutations were designed in AAV5 such that additional ATGs were introduced with minimal changes to the Rep52 amino acid sequence. Elimination of the additional ATGs in AAV2 was not sufficient to allow the usage of the internal methionine at position 50 **(Figure 3-5, compare lanes 1 and 2)**. Also, the addition of two ATGs out-of-frame of Rep52 in AAV5 failed to prevent the accumulation of Rep40 **(Figure 3-5, compare lane 3 to lanes 4-6)**. Therefore, the usage of the internal methionine in AAV5 Rep52 at position 50 as the initiation site for Rep40 cannot be explained by the simple absence of upstream ATGs.

Figure 3-5: The mechanism by which the M50 start codon is utilized in AAV5, but not AAV2, is not due to the presence of additional out-of-frame AUG triplets within AAV2 small Rep-encoding pre-mRNAs.

Immunoblot analysis following transient transfection of 293 cells of either a wild-type CMV-driven AAV2 Rep52 (lane 1) construct or a construct featuring silent mutations of amino acids 30 and 45 (lane 2) that eliminate additional AUG codons that exist out-of-frame from Rep52, in addition to a wild-type CMV-driven AAV5 Rep52 (lane 3) construct or constructs featuring silent mutations of amino acid 30 (lane 4), 45 (lane 5), or both (lane 6) that introduce additional AUG codons out-of-frame from Rep52.

Figure 3-5: The mechanism by which the M50 start codon is utilized in AAV5, but not AAV2, is not due to the presence of additional out-of-frame AUG triplets within AAV2 small Rep-encoding pre-mRNAs.



DISCUSSION

Unlike AAV2, the transcription profile of AAV5 predicts that only two Rep proteins, Rep78 and Rep52, can be encoded in significant quantities by P7 and P19 pre-mRNA transcripts. However, we have consistently been able to detect high levels of Rep40 in AAV5 in spite of low levels of splicing. The knowledge that AAV2 Rep40 is required for efficient packaging of replicated ssDNA into preformed capsids led us to examine the mechanism by which AAV5 could make this critical protein. The addition of caspase inhibitors was not sufficient to eliminate Rep40, suggesting that AAV5 Rep52 is not cleaved by caspases to generate Rep40. Mapping of the common regions between AAV5 Rep52 and Rep40 indicated that these proteins share the same C-terminus and differ in their N-termini, unlike the AAV2-like AAVs. Deletion mapping proved to be inconclusive as to the exact location of the N-terminal end of Rep40; however, we demonstrate here by the use of frameshift, ochre, and point mutations that in addition to its typical ATG start site for translation of Rep52, AAV5 also likely utilizes an in-frame, internal ATG (M50) to code for Rep40. Interestingly, this methionine is also in AAV2; however, it is not used. Eliminating additional out-of-frame ATGs from Rep52-encoding pre-mRNA from AAV2 was not sufficient to allow the production of Rep40 through alternate initiation. In addition, the introduction of extra out-of-frame ATGs into the Rep52-encoding pre-mRNA of AAV5 did not eliminate Rep40, indicating that the mechanisms that may control the usage of this internal translational start site are complex and require further study.

It is well known that the structure of the nascent mRNA can play a role in the translation initiation of proteins, including those in parvoviruses (115). Therefore, it is likely that the causes for this event are complex and will demand further study. However, the finding that AAV5 utilizes a method vastly different from AAV2 to generate Rep40 is a fascinating addition to our understanding of AAV biology.

Future studies should be directed toward obtaining a clearer understanding of the parameters surrounding the usage of this internal start site, and whether Rep40 is necessary for efficient packaging in AAV5. Through personal communication with Dr. James Trempe of the University of Toledo, Ohio, we have agreed on a future collaboration to determine the helicase activities of AAV5 Rep40. This may give us insight in its function. Whether AAV5 Rep40 is a monomer in solution or forms a hexamer like the Rep40 from AAV2 is unknown and additional collaborations with Dr. Aneel Aggarwal from the Mount Sinai School of Medicine in New York in determining the crystal structure of this protein may help with our understanding.

Given the small genome size of AAV, and that this pattern of Rep40 expression is a conserved feature of all AAVs, including other animal AAVs such as goat AAV (which displays an identical transcription profile to AAV5), it seems unlikely that AAV5 Rep40 is dispensable for the proper packaging of AAV. Interestingly, AAV5 does not generate significant quantities of Rep68, suggesting that Rep68 is irrelevant to AAV5 replication. However, the conserved nature of Rep40 in all AAVs appears to demand its presence for proper packaging. The M50R mutant, which completely eliminated Rep40, placed into the infectious clone of AAV5, should answer that question. A more immediate answer can be reached by placing the mutant into a RepCap construct for use in generating

recombinant AAV. A drop in the production of rAAV using this construct would suggest that Rep40 is required for efficient packaging of the virus. This can further be supported by re-introducing Rep40 in *trans* from a separate vector to demonstrate that the packaging phenotype can be rescued. It is unknown if the M50R mutation will have detrimental effects on the structure of Rep52 (or Rep78, as any mutant in Rep52 will also be in Rep78). The best way to determine the importance of Rep40 is to create one or more silent mutations that eliminate Rep40 without changing the amino acid sequence of Rep52; however, those mutants will not be possible until the mechanism of the use of the internal methionine in AAV5 is determined.

A thorough understanding of the differences between these viruses will surely enable us to obtain a greater understanding of the complex biology of the parvoviruses. How these genotypic differences translate *in vivo* in the contexts of the host cell and in the contexts of AAV co-habitation with adenovirus is an area of intense study by our lab as well as others in the parvovirus field. Whether these differences between AAV5 and the animal AAVs versus AAV2 and the other human and non-human primate AAVs will allow us to have a greater understanding of the evolution of these viruses remains to be seen. In addition, comparing the mechanisms by which AAV2 and AAV5 generate their Rep40 gene products, and whether these mechanisms can contribute to the field of gene therapy, will certainly be of interest in the future.

IV. IMPROVED SPLICING OF THE AAV CAPSID-PROTEIN-SUPPLYING pre-MRNAs LEADS TO INCREASED rAAV VECTOR PRODUCTION.

ABSTRACT

AAV capsid proteins, thought to be a rate-limiting step in the production of rAAV, are translated from spliced mRNAs. Improvement of the native AAV nonconsensus donor sequence increases splicing yet leaves the relative levels of VP1- and VP2/3-encoding mRNAs unchanged, and thus provides a means to increase delivery of correct ratios of AAV capsid proteins. This effect is independent of the AAV serotype used, and occurs whether the *rep* and *cap* genes supplied in *trans* are on the same or separate expression vectors. In the split-vector system, replacement of the more traditionally-used CMV promoter with that of the AAV5 P41 promoter allowed for even greater levels of splicing, and together with an improved intron donor, led to a 10-15-fold increase in the levels of splicing, rAAV production and transduction compared to levels achieved with current co-transfection methods. Thus, the enhancement of splicing presents a useful method to enhance rAAV production during transient transfection.

INTRODUCTION

Adeno-associated virus (AAV) is a small, non-enveloped, ssDNA virus that was initially discovered as a contaminant of adenovirus stocks (10, 58). AAV is a member of the Dependovirus genera of the Parvovirinae and its efficient replication requires co-infection with a helper virus such as adenovirus or herpesvirus (14). In the presence of the AAV Rep protein, wild type AAV can integrate efficiently into human chromosome 19 (73, 130). In the absence of Rep, recombinant AAV genomes (rAAV) have been shown to persist episomally in the nucleus of many cell types. Because AAV is capable of transducing and persisting in a number of cell types, because it has not been found to be associated with any human disease, and because of its low observed immunogenicity, it has become a useful gene-therapy candidate for a number of diseases (21).

A typical method of generating rAAV involves co-transfection of three plasmids: an ITR-containing plasmid, which has the *rep* and *cap* genes of AAV replaced with that of a promoter and gene of interest to be expressed; an adenovirus (Ad) helper plasmid, which together with host 293 cells provides the minimal gene products required for AAV replication; and finally, the AAV *rep* and *cap* genes, supplied in *trans* from a third vector, for encapsidation. The generation of high levels of rAAV remains a challenge, and production of rAAV to the levels needed for human clinical trials remains costly and labor-intensive. Increased yields of rAAV have been achieved by repressing expression of Rep78 and Rep68 protein levels, either by mutation of their initiating ATG to ACG, or by replacing the endogenous P5 promoter with the weaker mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter; by increasing capsid production through

the use of vectors which express the *rep* and cytomegalovirus immediate early promoter (CMV-IE)-driven *cap* genes individually; and by the use of packaging cell lines or baculovirus expression systems (17, 77, 79, 158-160, 168).

The three capsid proteins of AAV are generated from spliced mRNAs (7, 153), and their relative abundances determine the levels of the individual capsid proteins that are produced. The 5'-splice sites of all human and non-human primate AAVs (with the exception of AAV5) are identical (CAG|GTA*CCA*), and differ from the U1 snRNP consensus binding site CAG|GTA*AGT* (differences between these two sequences are italicized). Therefore, the enhancement of splicing of the capsid gene pre-mRNAs by improving the intron donor presents a potentially useful avenue to significantly increase rAAV titers. Such improvements would not be expected to alter the relative ratios of the mRNAs that individually encode VP1, and VP2 and VP3, which are produced by alternative use of the intron acceptors.

In this report, we describe a simple and novel mechanism to increase the levels of rAAV production during transient transfection. By enhancing the splice donor site of the small internal AAV intron to a sequence closer to the consensus U1 snRNP binding site, we were able to significantly enhance the overall levels of splicing of the capsid-generating P40 pre-mRNA transcripts. Increases in splicing via donor alteration resulted in a parallel increase of both capsid-encoding mRNAs and so did not disrupt the delicate balance between the three capsid proteins. Subsequent increases in rAAV vector production were thus directly proportional to the levels of splicing. We demonstrate that this strategy can be utilized either in a single-vector system, where the *rep* and *cap* genes are on the same vector, or more robustly, in the dual-vector system that uses the two

major AAV open reading frames on separate vectors. In addition, the AAV5 P41 promoter increases both expression and splicing of capsid gene RNAs compared to the cytomegalovirus immediate early promoter, and together with an improved intron donor, results in levels of splicing, rAAV production, and transduction that were further enhanced as much as 10-15-fold compared to levels achieved with current co-transfection methods.

MATERIALS AND METHODS

Cell lines, AAV packaging plasmids, and viruses.

293T cells, which express SV40 Large T-antigen and therefore allow episomal replication of plasmids containing the SV40 origin of replication, were propagated as previously described (91). The AAV2RepCap plasmid, which lacks both ITRs, was described previously (119). The AAV1RepCap plasmid was generated by inserting bases 181 to 4566 from AAV1 into the *Eco* RI and *Xba* I sites of pSK(+) (Stratagene). The AAV6RepCap plasmid was generated by inserting bases 181 to 4560 from AAV6 into the *Eco* RI and *Xba* I sites of pSK(+). The CMV-Cap2 plasmid was previously described (119). AAV1-2-1RepCap and AAV2-1-2RepCap was created by replacing the full intron (bases 1924 to 2254 in AAV1 and 1907 to 2234 in AAV2 with that of the other serotype). "Better" or consensus donor mutations were generated by overlapping PCR mutagenesis of the native AAV donor site (CAG|GTACCA) to that of the "better" donor site (CAG|GTACGT) or consensus donor site (CAG|GTAAGT). The AAV2/8RepCap and CMV-Cap8 plasmids were generous gifts from Dusty Miller. P41-Cap2 and P41-Cap8 constructs were generated by replacing the region of AAV2RepCap or AAV2/8RepCap from the *Sfi* I site at position 544 to the *Hind* III site at position 1883, with a PCR-amplified P41 promoter from AAV5 (position 1681 to 1974) using *Sfi* I for the 5'-end and *Hind* III for the 3'-end.

For generating rAAV, the AAV2 ITR-containing packaging vector pMU2, which contains GFP as a reporter gene described previously (125) was used. For supplying the

four AAV2 Rep proteins in the split-vector method, the HIV-AAV Rep vector used, was also previously described (119).

Transfections, RNA isolation and analysis.

For RNA analysis, transfections were conducted using a modified PEI transfection method as described in (125). Cells were harvested and RNA was isolated 36-42 hours post-transfection (119). RNase protection assays were performed using 10 µg of total RNA as previously described (94, 133).

For rAAV production, cells were plated onto 100-mm dishes the day prior to transfection, and a total of 20 µg of DNA was transfected per plate using the PEI method as described (125). For triple transfections, reactions were carried out at a mass ratio of 1:1:2 (pMU2:RepCap:pHelper). For quadruple transfections, reactions were carried out at a mass ratio of 1:1:1:2 (pMU2:Rep:Cap:pHelper). Recombinant virus was harvested 60 hours post-transfection.

RNA was isolated following guanidinium isothiocyanate lysis and cesium chloride gradient centrifugation as previously described (94, 133). The AAV2 RP probe, which spans nts 1767-1958 and allows identification of the spliced and unspliced P40 transcripts, was described previously (91). AAV1 and AAV6 homologous RP probes were generated by PCR amplification of regions 1781 to 1975 in AAV1 and 1766 to 1960 in AAV6, and subsequent cloning into the *Eco* RI / *Bam* HI sites in pGEM-3Z (Promega). CMV-Cap RP probes were generated by PCR amplification using a forward CMV primer and the appropriate serotype-specific reverse RP primer, as above. P41-Cap

RP probes were generated by PCR amplification using a forward AAV5 P41 primer and the appropriate serotype-specific reverse RP primer, as above.

Recombinant AAV isolation / Quantitative PCR.

Sixty hours post-transfection, cells were collected and processed as described (86). Briefly, cell pellets were resuspended in 50 mM Tris / 1 mM EDTA buffer and subjected to three cycles of freeze/thaw lysis before brief sonication. Lysates were diluted 1:100 and treated with DNase I to remove unpackaged genomic DNA. During DNase I inactivation by heat, samples were diluted further 1:15 prior to quantitative PCR analysis. Quantification of packaged rAAV genomes was calculated using the SybrGreen reagent (BioRad) on the Applied Biosystems 7500 real-time qPCR machine as described previously (86). The forward and reverse GFP primers utilized for the qPCR were also described previously (86).

Transduction assay.

HeLa cells were plated onto 24-well plates 24 hours prior to infection. Following quantification of packaged rAAV genomes by qPCR, aliquots of the crude lysates were standardized, and serial 10-fold dilutions (beginning with 5 x 10^7 packaged genomes per well) were used for infection. GFP-positive cells were counted and approximate transducing units of rAAV were scored as the number of GFP-positive cells relative to the total number of cells. Infectious titers were calculated by dividing the genomic copy

number (as assayed by qPCR) by the amount of transducing units (86).

RESULTS and DISCUSSION

In the presence of co-infecting Ad5, splicing of pre-mRNAs generated by AAV1 and AAV6 constructs was significantly less than splicing of pre-mRNAs generated by AAV2. This difference was governed by cis-acting sequences within the introns of these constructs.

A rate-limiting step in the production of recombinant AAV is the production of capsids (160). We found our yields of rAAV1 and rAAV6 vectors to be consistently less than those of rAAV2 (data not shown) and since AAV capsid proteins are generated from spliced capsid gene mRNA, we wondered whether reduced levels of splicing could contribute to the difference in rAAV production for these serotypes.

We found that similar to AAV2, the basal level of expression and splicing of AAV1 and AAV6 pre-mRNAs generated from RepCap constructs in 293 cells is low **(Figure 4-1, compare lanes 1, 3, and 5)**; however, in contrast to AAV2, enhancement of expression and splicing of pre-mRNAs generated from AAV1 and AAV6 RepCap constructs in the presence of pHelper (supplying adenovirus 5 serotype E2a, E4orf6 and VA RNA), or adenovirus co-infection (data not shown), was quite modest **(Figure 4-1, compare lanes 2 and 6 to lane 4)**.

The splicing of small internal introns is often governed by intron definition, in which all necessary *cis*-acting signals are contained within the intron itself, and this is the case for the internal intron of the parvovirus minute virus of mice (MVM) (52). Therefore, we sought to determine whether the splicing differences seen between AAV1 and AAV2 were mediated by their introns. As suspected, the levels of AAV1 splicing in

the presence of pHelper could be enhanced to approximately AAV2-like levels when its intron was substituted with that from AAV2 **(AAV1-2-1, Figure 4-1, compare lanes 11 to 8)**. Additionally, AAV2 pre-mRNA splicing was not significantly enhanced in the presence of pHelper when its intron was replaced by that from AAV1 **(AAV2-1-2, Figure 4-1, compare lanes 14 to 4)**. Co-transfection of additional wild-type AAV2 Rep78 protein in *trans* failed to rescue the splicing phenotypes of AAV1 or AAV2-1-2 **(Figure 4-1, lanes 9 and 15)**, indicating that there is either a *cis*-acting sequence within the AAV2 intron that governs splicing enhancement in the presence of Ad5 gene products, or a sequence within the AAV1 intron that prevents splicing enhancement. The intron donors and acceptors for all these serotypes are identical, suggesting that such sequences must be within and internal intronic region.

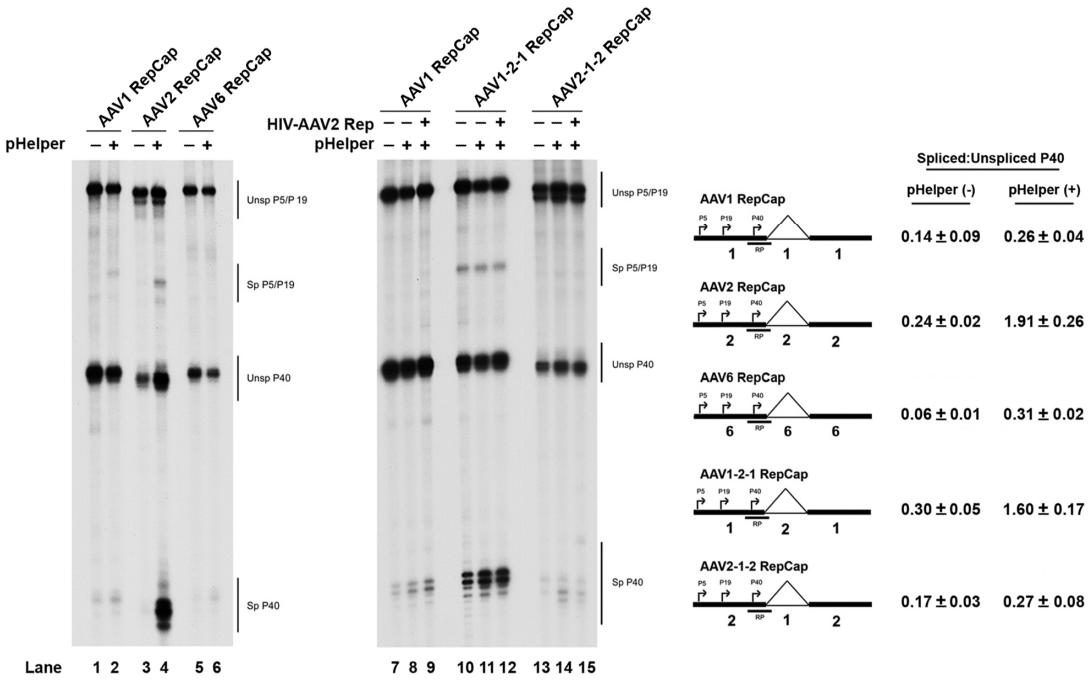
Improvement of the nonconsensus AAV donor significantly enhanced overall pre-mRNA splicing, capsid production, and rAAV production.

To test the hypothesis that splicing levels of the capsid protein-supplying construct contributes to the levels of production of rAAV, we attempted to increase the levels of spliced mRNAs by generating a series of AAV1 and AAV2 RepCap vectors in which the native, nonconsensus donor sites were improved. We made constructs in which the donors were made fully complementary to the endogenous U1snRNP interaction site, which also adds a termination codon in the Rep78 and Rep52 ORFs [CAG|GTACCA to CAG|G**TAA**GT, termination signal in bold, termed consensus donor (cD)], as well as constructs in which the U1snRNP-binding donor sequence was

Figure 4-1: In the presence of co-infecting Ad5, splicing of pre-mRNAs generated by AAV1 and AAV6 constructs was significantly less than splicing of pre-mRNAs generated by AAV2. This difference was governed by cis-acting sequences within the introns of these constructs.

(Left) Representative RNase protection assay of AAV1, AAV2, and AAV6 RepCap plasmids in the presence (+) or absence (-) of pHelper plasmid. **(Center)** Representative RPA of AAV1, AAV1-2-1, and AAV2-1-2 RepCap plasmids in the presence or absence of pHelper plasmid, and the presence or absence of HIV-driven AAV2 Rep78 in *trans*. **(Right)** Quantification of the relative spliced to unspliced ratios of capsid-encoding pre-mRNAs. Data taken from at least three experiments and show standard deviations. The position of the "RP" RNase protection probe is indicated. Unsp, Unspliced; Sp, Spliced.

Figure 4-1: In the presence of co-infecting Ad5, splicing of pre-mRNAs generated by AAV1 and AAV6 constructs was significantly less than splicing of pre-mRNAs generated by AAV2. This difference was governed by cis-acting sequences within the introns of these constructs.



improved but did not generate a termination signal [CAG|GTACCA to CAG|GTACGT introducing a glutamine (CAA) to valine (GTA) substitution, termed "better" donor (bD)]. Wild-type and mutant AAV1 and AAV2 RepCap constructs were then assayed for splicing by RNAse protection assays using homologous probes. As expected, in 293 cells in the presence of pHelper, the overall levels of pre-mRNA splicing of both AAV1 and AAV2 capsid encoding RNAs were increased significantly when the donors were made more consensus. For AAV1, the "better" donor and consensus donor improvements led to approximately 6-fold and 7-fold increases, respectively **(Figure 4-2A, AV1RepCap bD and cD, compare lane 1 to lanes 2 and 3)**, while for AAV2, the "better" donor and consensus donor improvements led to approximately 2-fold increases **(Figure 4-2A, AV2RepCap bD and cD, compare lane 4 to lanes 4 and 5)**. The differences in improvement between AAV1 and AAV2 may reflect a greater dependence for AAV2 of the modified Rep protein. In addition to an increase in P40 pre-mRNA splicing, we also saw a marked increase in the overall levels of pre-mRNA splicing from the upstream P5 and P19 promoters in both AAV1 and AAV2.

The "better" and consensus donor mutants were then used as helper plasmids to supply the Rep and capsid proteins needed, together with pHelper, to generate rAAV in 293 cells. The same "better" and consensus donor mutants were also generated in a hybrid AAV2/AAV8 RepCap vector to examine the effects of increased splicing on the production of rAAV8-encapsidated virions. As shown in the graph in **Figure 4-2**, the use of the "better" donor mutants resulted in a significant increase (approximately 3- to 4-fold) in the numbers of packaged rAAV genomes compared to those generated by the wild-type constructs for all three serotypes. As expected, the consensus donor constructs,

Figure 4-2: Improvement of the nonconsensus AAV donor significantly enhanced overall pre-mRNA splicing, capsid production, and rAAV production.

(Left) Representative RNase protection assay of AAV1 and AAV2 RepCap plasmids (AV1RC and AV2RC, respectively) with wild-type, "better" (bD), or consensus donor (cD) mutations in the presence of pHelper. **(Right, top)** Quantification of the relative spliced to unspliced ratios of capsid-encoding pre-mRNA. Data taken from at least three experiments and show standard deviations. The position of the "RP" RNase protection probe is indicated. Unsp, Unspliced; Sp, Spliced. **(Right, bottom)** Relative levels of rAAV production observed for AAV1, AAV2, and AAV8 with the "better" and consensus donor mutants in relation to levels obtained using the wild-type donors (set to a value of 1.00). Titers of rAAVs generated using the wild-type donor RepCap constructs typically ranged from approximately 4.5×10^9 to 2.0×10^{11} packaged genomes per 100-mm dish.

(B, C, and D) Physical titers of rAAVs produced using serotypes 1, 2, and 8, respectively. Values represent total calculated packaged genomes per 100-mm dish, with standard error.

Figure 4-2A: Improvement of the nonconsensus AAV donor significantly enhanced overall pre-mRNA splicing, capsid production, and rAAV production.

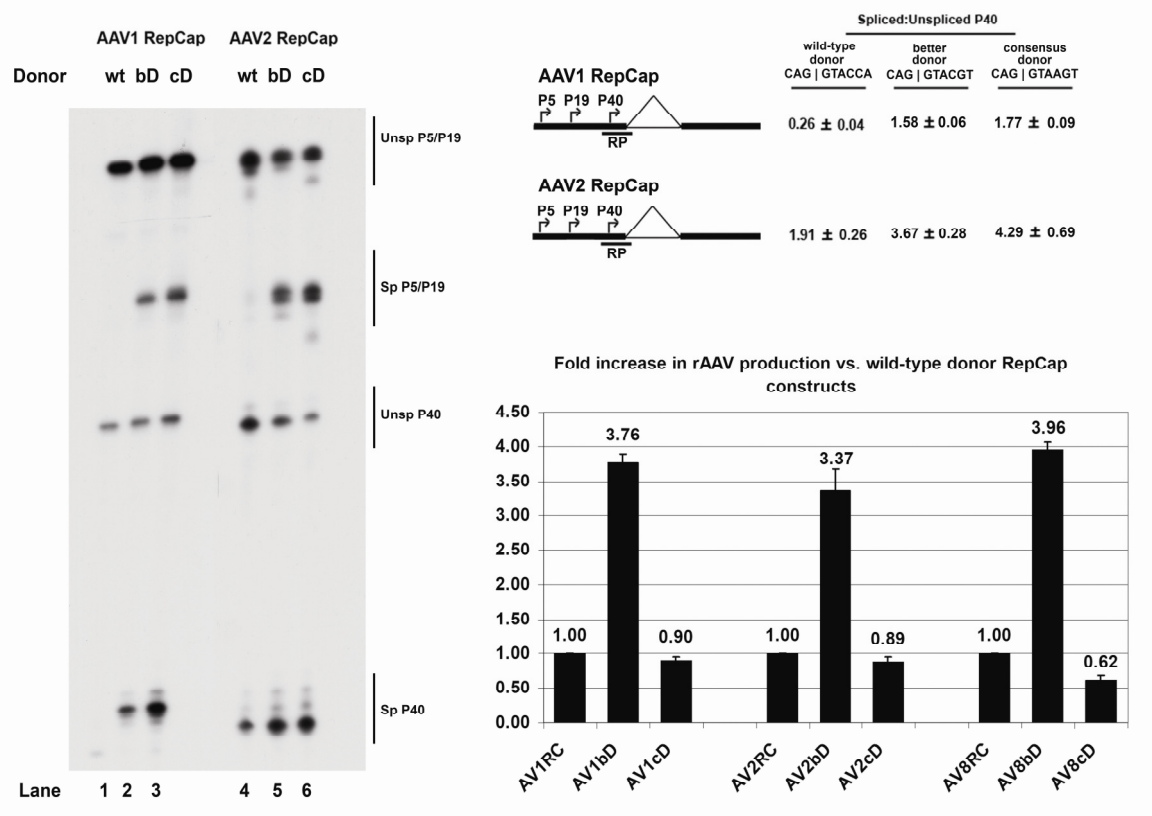


Figure 4-2B: Improvement of the nonconsensus AAV donor significantly enhanced overall pre-mRNA splicing, capsid production, and rAAV production.

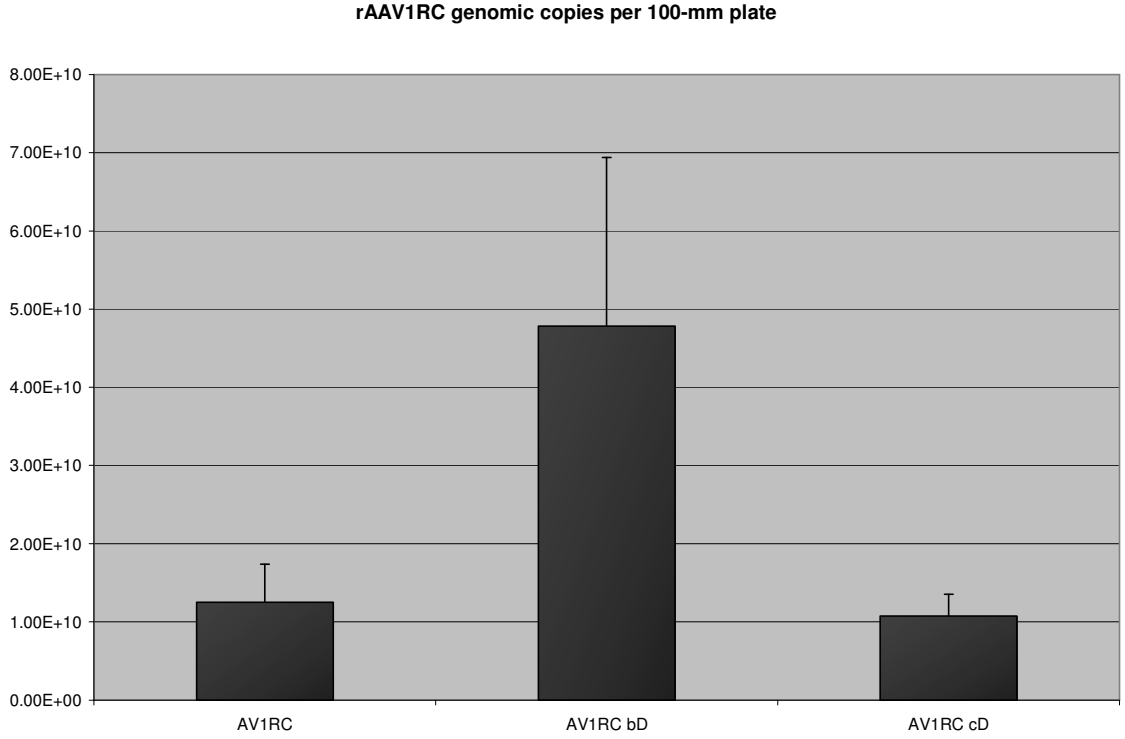


Figure 4-2C: Improvement of the nonconsensus AAV donor significantly enhanced overall pre-mRNA splicing, capsid production, and rAAV production.

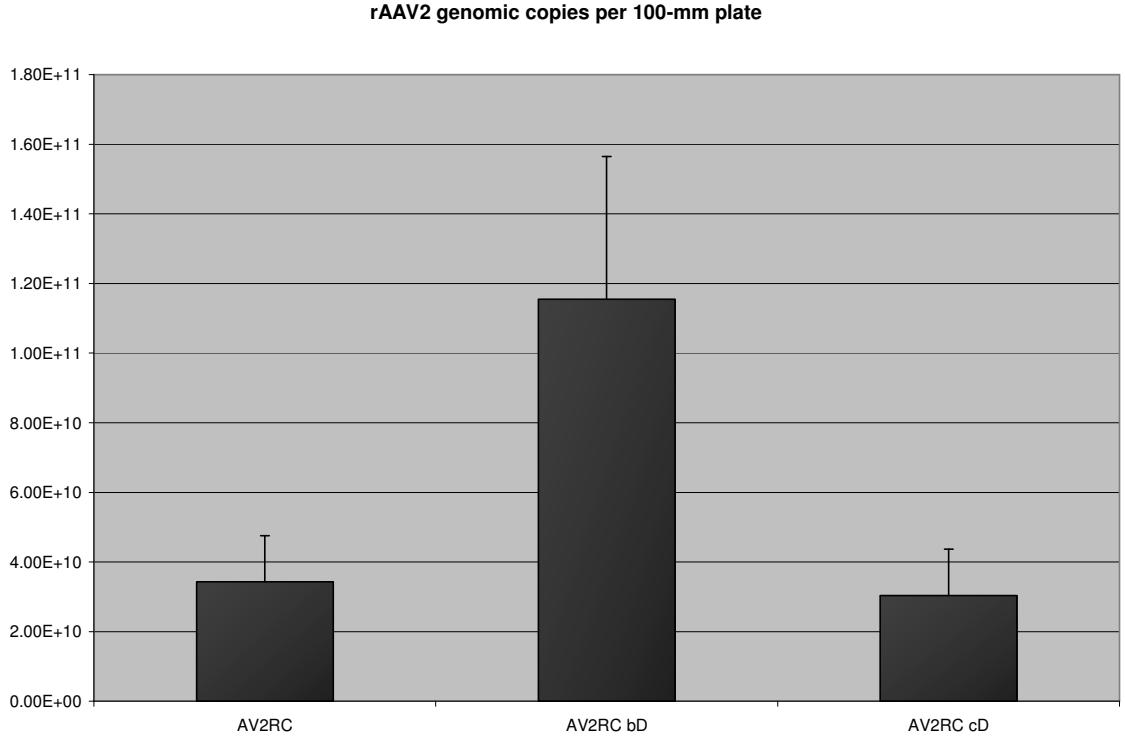
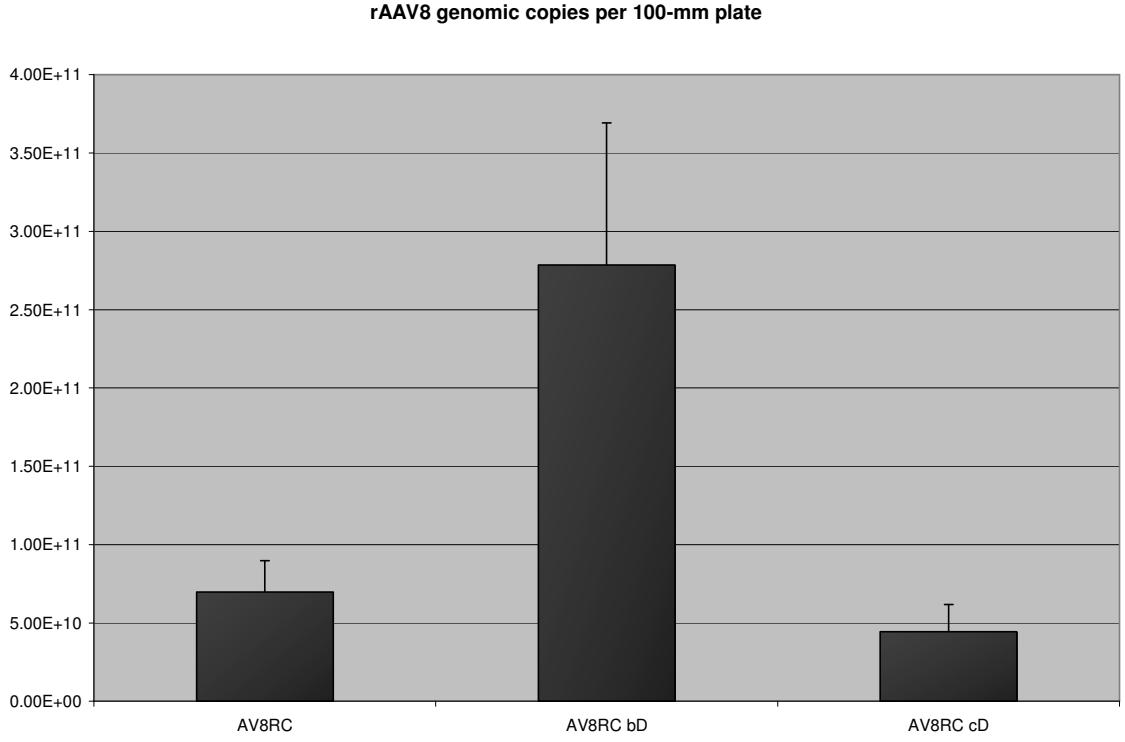


Figure 4-2D: Improvement of the nonconsensus AAV donor significantly enhanced overall pre-mRNA splicing, capsid production, and rAAV production.



which terminate the Rep open reading frame, supported even less packaged genomes than the wild-type RepCap constructs, consistent with previous findings suggesting that full-length Rep78 and Rep52 proteins are required for efficient viral production (25, 32, 63, 93, 152). Titers of rAAVs generated using the wild-type donor RepCap constructs typically ranged from approximately 4.5 x 10^9 to 2.0 x 10^{11} packaged genomes per 100-mm dish **(Figure 4-2B, 2C, and 2D)**.

The use of a split Rep/Cap AAV helper system overcame the negative effects of the consensus donor allowing for even greater levels of rAAV production.

AAV helper vectors in which the Rep and capsid proteins are on the same vector risk wild-type AAV contamination originating from low levels of recombination between the RepCap and ITR-containing plasmids. Several strategies have been used to overcome this problem, including the addition of intronic sequences to the RepCap vectors (1, 18, 77, 162), and the use of a split-vector system in which the *rep* and *cap* genes are on different plasmids (166). We hypothesized that the consensus donor mutants, which introduce a termination codon in the Rep gene of RepCap plasmids, may have a greater enhancing effect on vector production in systems in which Rep is supplied from a separate plasmid.

Currently, a common capsid vector of choice in the split-vector system utilizes the cytomegalovirus immediate early promoter (CMV-IE) to drive expression of the capsid-encoding pre-mRNA (166). Interestingly, we have previously shown (119), and show again in this study **(Figure 4-3A, lane 2)**, that splicing of AAV2 pre-mRNAs expressed from the CMV-IE promoter is relatively inefficient, even in the presence of pHelper. The

addition of Rep78 in *trans* did not further enhance splicing (data not shown). However, substitution of the native nonconsensus AAV2 donor sequence in the CMV-driven cap construct with that of the ”better” donor led to an approximate 3-fold and 4.5-fold increase in the level of splicing in either the absence or presence of pHelper, respectively (Figure 4-3A, lanes 3 and 5); while introduction of the consensus donor led to approximate 10-fold and 9-fold increases in the levels of splicing in the absence and presence of pHelper, respectively **(Figure 4-3A, lanes 4 and 6)**. RNase protection assays using a probe across the intron acceptors that allowed for the quantification of the relative usage of the two AAV acceptor sites revealed no significant alteration of the relative levels of A1/A2 splicing (data not shown).

The “better” and consensus donor mutant AAV2 CMV-Cap constructs were then tested for their abilities to aid in producing rAAV using this split-vector system. In addition, similar donor mutations were made in AAV6 and AAV8 CMV-driven capsid-encoding constructs. Similarly to the RepCap constructs in Figure 2, the “better” donor mutant constructs generated 3- to 4-fold increased levels of rAAV production for all three serotypes **(Figure 4-3A)**. As predicted, the consensus donor mutants increased vector production to even higher levels than the “better” donor mutants (approximately 5- to 6-fold), suggesting that the limitations of using the consensus donor mutants in the RepCap single-vector system were due to truncations in Rep and could be overcome by supplying *rep* and *cap* on separate vectors. Titers of rAAVs generated using the CMV-driven wild-type donor construct typically ranged from approximately 9.5×10^9 to 2.0×10^{11} packaged genomes per 100-mm dish **(Figures 4-3B, 3C, and 3D)**.

Figure 4-3: The use of a split Rep/Cap AAV helper system overcame the negative effects of the consensus donor allowing for even greater levels of rAAV production.

(A) (Left) Representative RNase Protection Assay of AAV2 CMV-driven capsid-encoding pre-mRNAs in the absence or presence of pHelper. **(Center)** Quantification of the relative spliced to unspliced ratios of capsid-encoding pre-mRNA. Data taken from at least three experiments and show standard deviations. The position of the "RP" RNase protection probe is indicated. **(Right)** Relative levels of rAAV production observed in AAV2, AAV6, and AAV8 with the "better" and consensus donor mutants in relation to levels obtained using the wild-type donors (set to a value of 1.00). Titers of rAAVs generated using the CMV-driven wild-type donor construct typically ranged from approximately 9.5×10^9 to 2.0×10^{11} packaged genomes per 100-mm dish.

(B, C, and D) Physical titers of rAAVs produced using serotypes 2, 6, and 8, respectively. Values represent total calculated packaged genomes per 100-mm dish, with standard error.

Figure 4-3A: The use of a split Rep/Cap AAV helper system overcame the negative effects of the consensus donor allowing for even greater levels of rAAV production.

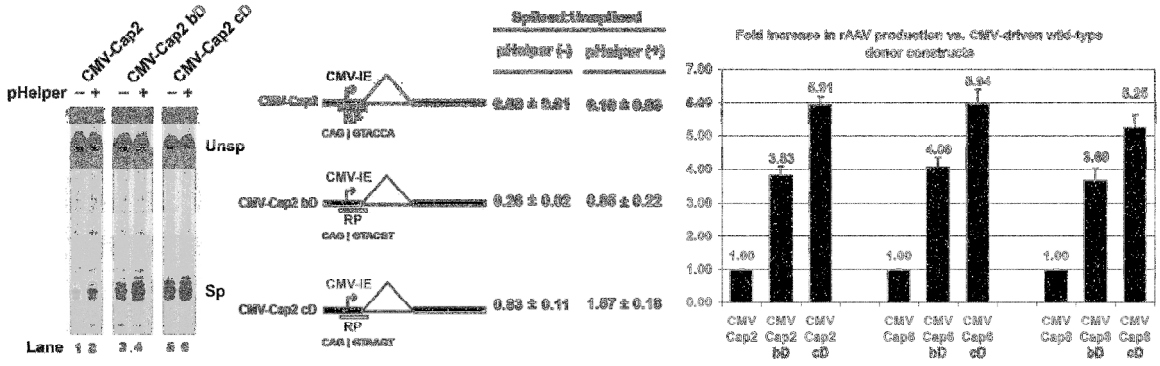


Figure 4-3B: The use of a split Rep/Cap AAV helper system overcame the negative effects of the consensus donor allowing for even greater levels of rAAV production.

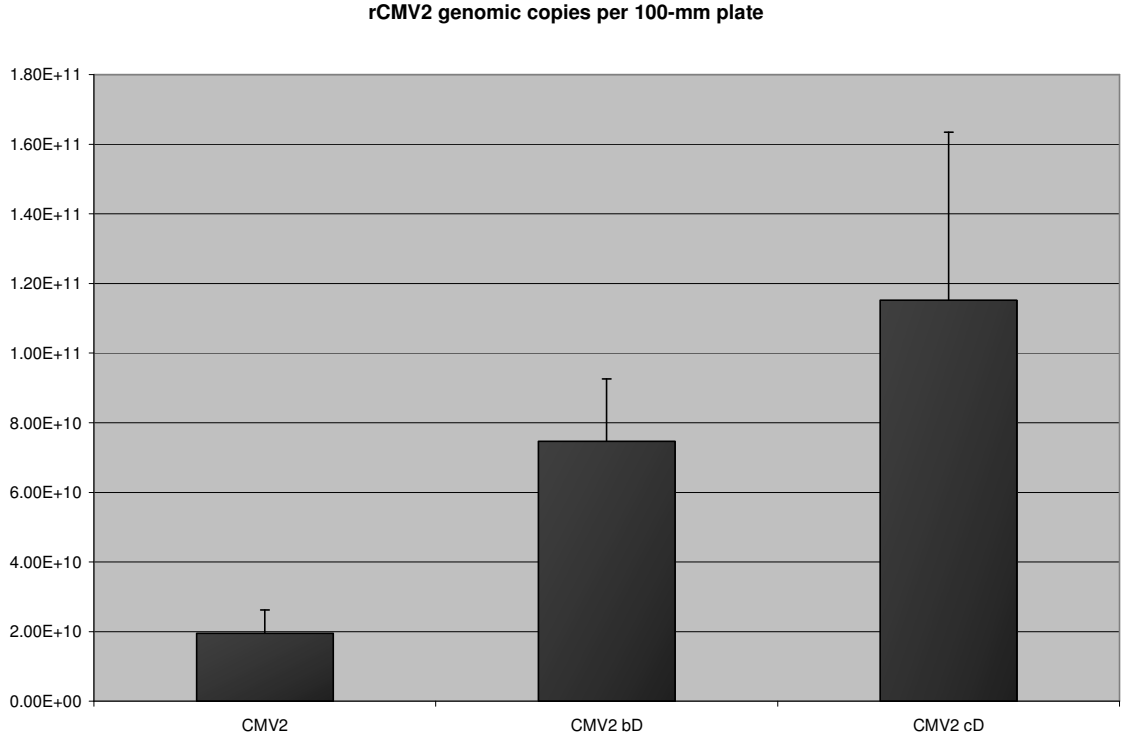


Figure 4-3C: The use of a split Rep/Cap AAV helper system overcame the negative effects of the consensus donor allowing for even greater levels of rAAV production.

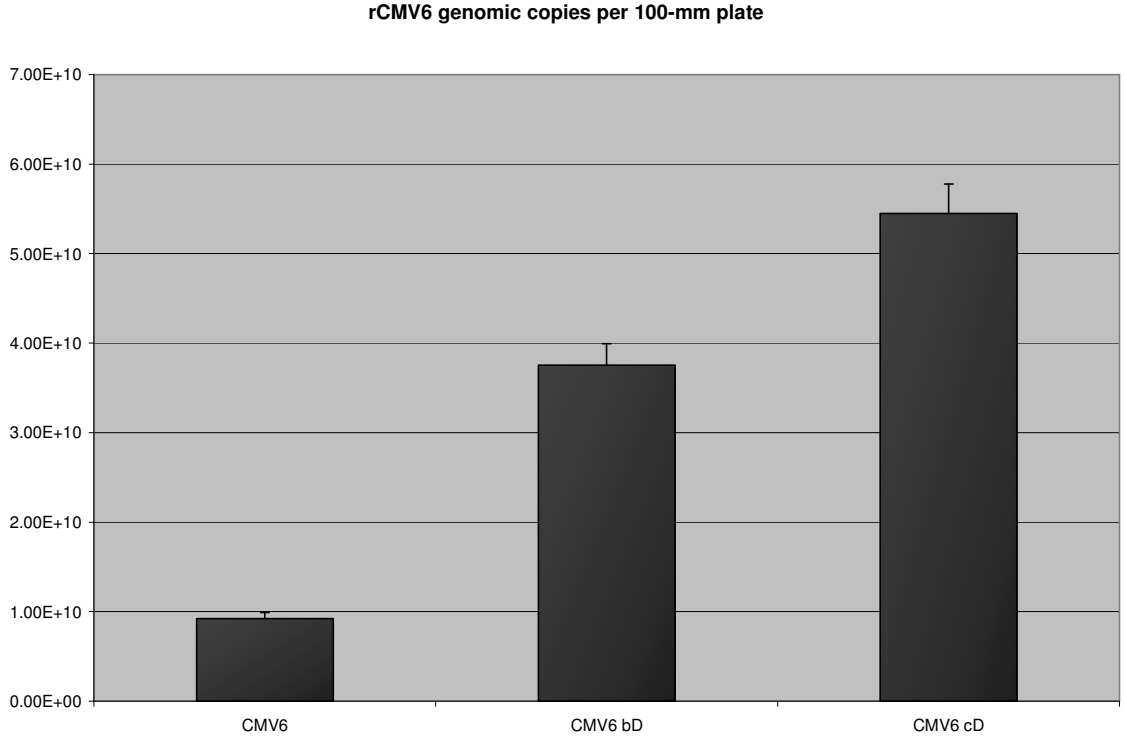
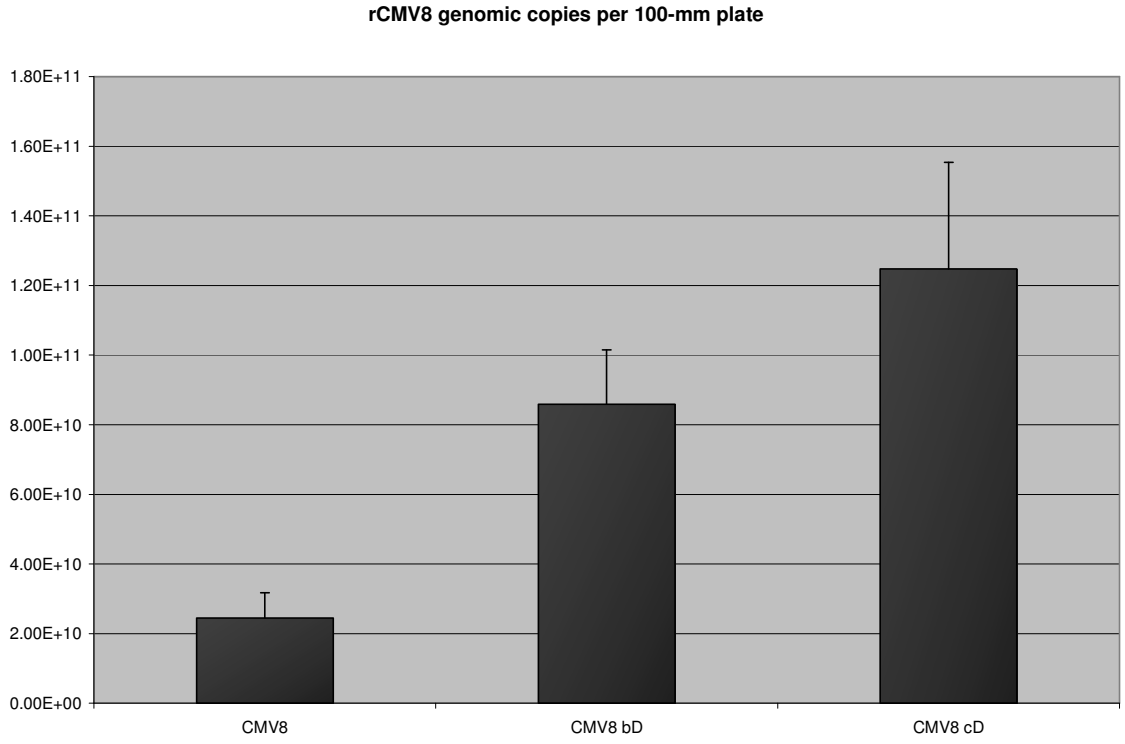


Figure 4-3D: The use of a split Rep/Cap AAV helper system overcame the negative effects of the consensus donor allowing for even greater levels of rAAV production.



The AAV5 P41 promoter allows for more efficient splicing of capsid-encoding AAV pre-mRNA than the CMV promoter, and together with an improved donor, led to the generation of high levels of rAAV.

Although the strong CMV promoter is useful for the production of rAAV from a split-vector system, the majority of the pre-mRNAs generated from these vectors remain unspliced and therefore are poor sources for the capsid proteins. This remains the case even when the donors are improved. We have recently found that in contrast to the AAV2 P40 promoter, the AAV5 P41 capsid-gene promoter generates a high level of spliced mRNAs in 293 cells due to E1A in the absence of added Rep or additional Ad5 gene products (118, 173). Therefore, we hypothesized that although the AAV5 P41 promoter does not contain a Rep-binding element (173), it could be a useful substitute for the CMV promoter in driving the expression of the AAV capsid proteins for rAAV production in 293 cells. This was found to be the case, and more importantly, the levels of spliced pre-mRNA were found to be the predominant species of capsid gene RNA when the nonconsensus AAV2 donor site in these constructs was made consensus **(Figure 4-4, compare lanes 1-3, to lanes 4 and 5)**.

These vectors were then tested in the split-vector system to generate rAAV. Consistent with their relatively high basal expression of spliced capsid gene mRNAs, the P41-driven capsid vectors containing the native donor site generated 5- to 6-fold higher levels of rAAV than the CMV-driven capsid constructs containing the wild-type donor; intermediate between CMV-driven constructs bearing the "better" and the consensus donors. The P41-driven Cap constructs containing the consensus donor produced approximately 10- to 15-fold more rAAV than the CMV-driven capsid-gene constructs

Figure 4-4: The AAV5 P41 promoter allows for more efficient splicing of capsid-encoding AAV pre-mRNA than the CMV promoter, and together with an improved donor, led to the generation of high levels of rAAV.

(A) (Left) RNase Protection Assay of CMV- and P41-driven AAV2 capsid-encoding pre-mRNAs in the presence of pHelper. **(Center)** Quantification of the relative spliced to unspliced ratios of capsid-encoding pre-mRNA. Data taken from at least three experiments and show standard deviations. The position of the "RP" RNase protection probe is indicated. **(Right)** Relative levels of rAAV production observed in AAV2 and AAV8 with the "better" and consensus donor mutants, in addition to the P41 wild-type and consensus donor mutants, in relation to levels obtained using the CMV-driven wild-type donor capsid-encoding construct (set to a value of 1.00). Titers of rAAVs generated using the CMV-driven wild-type donor construct typically ranged from approximately 9.5×10^9 to 2.0×10^{11} packaged genomes per 100-mm dish.

(B and C) Physical titers of rAAVs produced using serotypes 2 and 8, respectively. Values represent total calculated packaged genomes per 100-mm dish, with standard error.

Figure 4-4A: The AAV5 P41 promoter allows for more efficient splicing of capsid-encoding AAV pre-mRNA than the CMV promoter, and together with an improved donor, led to the generation of high levels of rAAV.

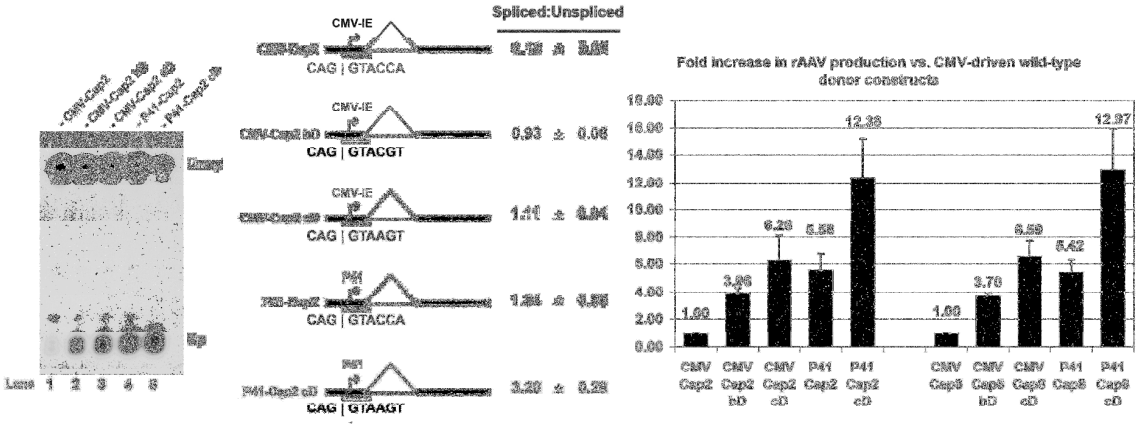


Figure 4-4B: The AAV5 P41 promoter allows for more efficient splicing of capsid-encoding AAV pre-mRNA than the CMV promoter, and together with an improved donor, led to the generation of high levels of rAAV.

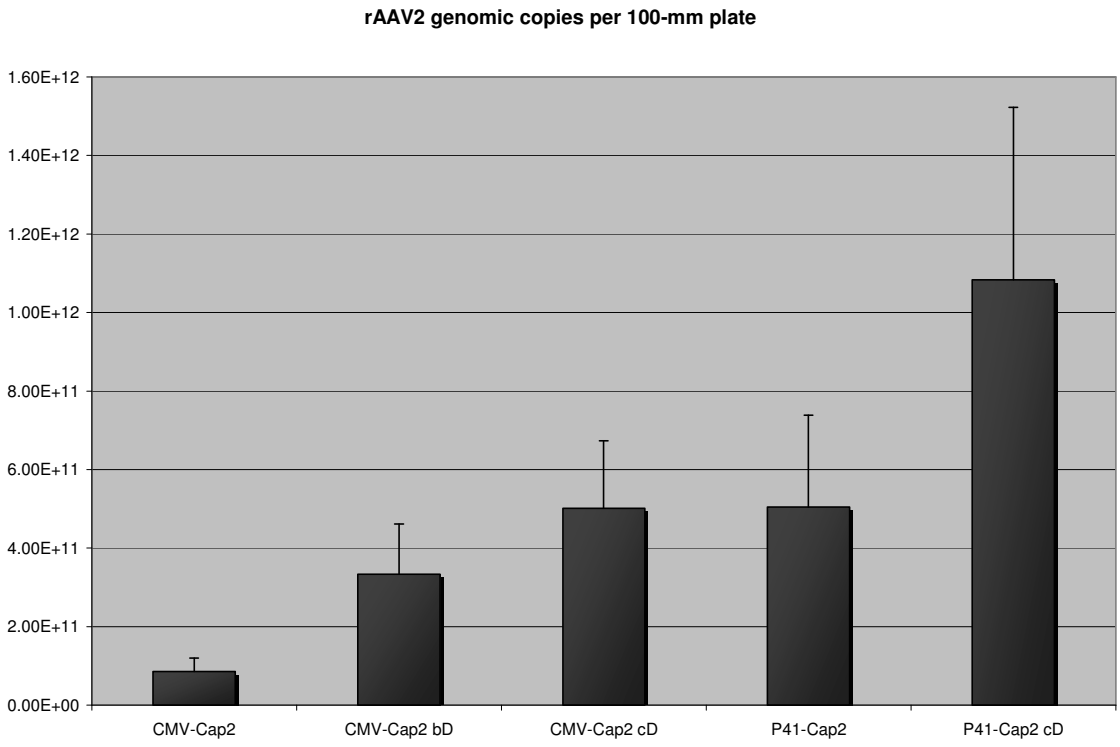
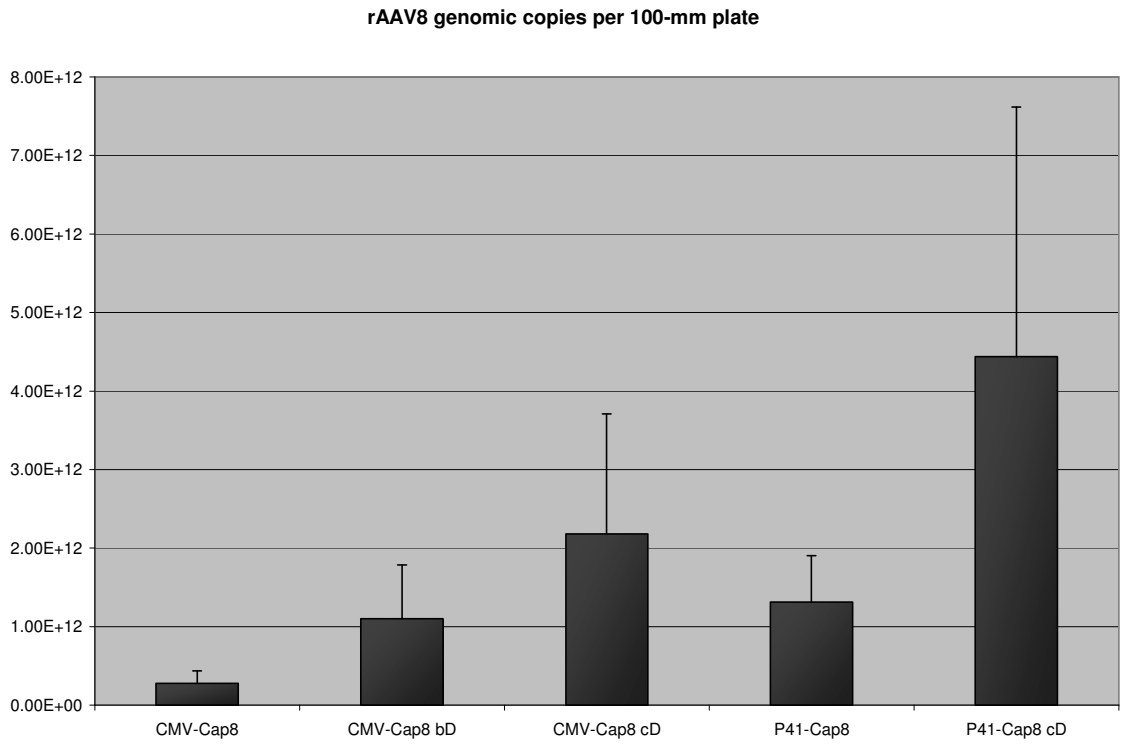


Figure 4-4C: The AAV5 P41 promoter allows for more efficient splicing of capsid-encoding AAV pre-mRNA than the CMV promoter, and together with an improved donor, led to the generation of high levels of rAAV.



bearing the wild-type donor, and twice as high yields as the CMV-driven capsid constructs containing a consensus donor. Similar results were also observed when the P41 promoter was used to drive expression of the AAV8 capsids, again suggesting that this pattern is not serotype specific. Titers of rAAVs generated using the CMV-driven wild-type donor construct typically ranged from approximately 9.5 x 10^9 to 2.0 x 10^{11} packaged genomes per 100-mm dish **(Figures 4-4B and 4C)**.

Levels of transduction-capable rAAV correlate well with the increased levels of packaged genomes.

Levels of transduction-capable rAAV can be determined by calculating the ratio of packaged ssDNA genomes to transducing units (ssDNA/TU). For rAAV, this number is usually between 500 and 1500 ssDNA/TU (86). To determine whether the increase in packaged genomes obtained with the splicing mutants led to a concomitant increase in the numbers of transducing virions, 10-fold serial dilutions of crude cell packaging lysates were used to infect HeLa cells and the number of GFP-positive cells were determined 48-hours post-infection. Recombinant AAV8 generated using the "better" or consensus donor mutants, or generated using the AAV5 P41 promoter were as efficient transducers, on a vector genome basis, as was rAAV8 generated using the CMV-Cap expression vector. The ssDNA/TU for all rAAVs tested ranged from approximately 1400 to 2000 **(Figure 4-5)**.

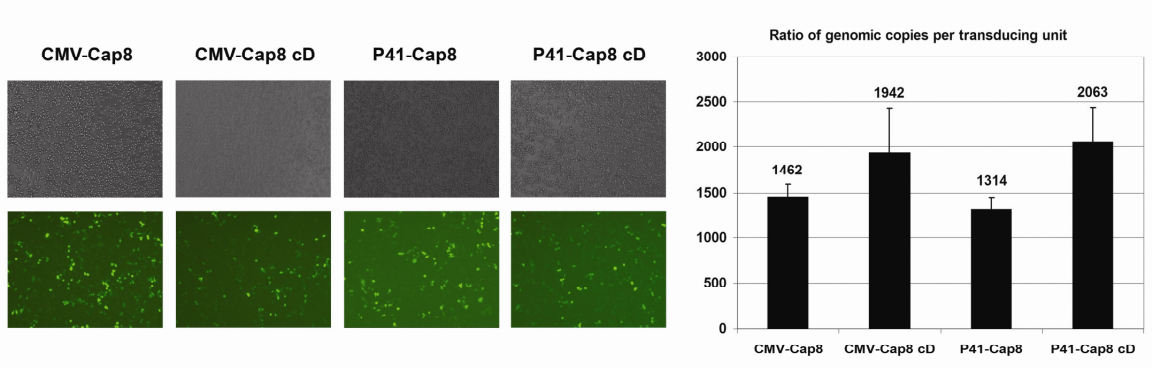
Thus, improving the levels of spliced capsid gene mRNAs is a useful strategy to improve rAAV production. Why the AAV1 and AAV6 introns remain poorly spliced in

the presence of Rep and Ad, even though their donors and acceptors are identical to those of AAV2, is currently under investigation.

Figure 4-5: Levels of transduction-capable rAAV correlate well with the increased levels of packaged genomes.

Transduction assay on HeLa cells, plated on 24-well dishes 24 hours prior to infection with 10-fold serial dilutions of rAAV8 (shown: 5 x 10^7 packaged genomes per well). GFP-positive cells were counted 48 hours post-infection. **(Left, top)**, Phase-contrast microscopy; **(Left, bottom)**, GFP-postitive cells. **(Right)** The numbers of packaged genomes per well were divided by the approximate number of GFP-positive transduced cells to obtain the efficiency of infection. Values indicated are expressed as a ratio of packaged genomes per transducing unit. Data taken from three experiments and show standard deviations.

Figure 4-5: Levels of transduction-capable rAAV correlate well with the increased levels of packaged genomes.



V. SUMMARY AND DISCUSSION

The study of the basic biology of the adeno-associated viruses has given us a much greater understanding of the complex members of the subfamily *Parvoviridae*, their interactions with their hosts, their interactions with other viruses, and has even proven useful in the improved designs of recombinant AAV vectors for gene-delivery and the treatment of serious human diseases.

The adenovirus E4orf6 and E1b-55k gene products are essential for not only adenovirus growth during infection, but also the replication of AAV. Adenovirus E4orf6 and E1b-55k form an E3 ubiquitin ligase complex with the cellular proteins elongin B, elongin C, Rbx1, and cullin 5, to mediate the addition of poly-ubiquitin chains to target substrates such as p53, Mre11, and DNA ligase IV. The subsequent degradation of these proteins is required for full adenovirus growth. In addition, E4orf6 and E1b-55k are required for the preferential export of viral mRNAs at the detriment to cellular mRNAs; however, the exact mechanism by which this occurs is not well understood.

Previous experiments that examined the requirements of the individual adenovirus gene products of AAV5 replication demonstrated that E4orf6 and E1b-55k were capable of mediating the degradation of AAV5 capsid proteins in a proteasome-dependent manner. This result was puzzling, given the well-established role of E4orf6 and E1b-55k in the extention of AAV2 ssDNA to the double stranded replication intermediate. We report here that adenovirus E4orf6 and E1b-55k assemble with AAV structural and non-structural proteins into an E3 ubiquitin ligase complex with the cellular protein cullin 5 and mediate the K48-linked poly-ubiquitination and subsequent degradation of AAV5

small Rep and capsid proteins, as well as AAV2 small Rep proteins, by the proteasome. How can E4orf6/E1b-55k-dependent degradation of AAV proteins be reconciled with their role as a helper functions? We have previously shown that the enhancement of translation provided by adenovirus VA RNA as part of its helper function is necessary to restore AAV5 protein levels to those necessary for viral replication. It may be that E4orf6/E1b-55k-dependent degradation of AAV5 proteins by E4orf6 may merely be a by-product of its role in targeting the degradation of a cellular protein necessary for viral replication. In this scenario, perhaps only the required levels of AAV5 proteins, and not the cellular target whose degradation is required for viral replication, becomes restored by VA RNA activity. Alternatively, it may be that AAV has evolved to rely on E4orf6 and E1b-55k as regulators of its own gene expression. If unopposed, VA RNA might enhance excessive amounts of viral Rep and Cap at inappropriate times, which might be detrimental to infection. Another possibility might be that E4orf6/E1b-55k activity in this regard has evolved to aid adenovirus replication in the presence of AAV, targeting the degradation of AAV proteins as a protective measure to temper AAV expression during AAV/Ad co-infection. It is known that AAV2 large Rep proteins can affect the transcription of adenovirus early genes. Therefore, adenovirus E4orf6 and E1b-55k may be required for the maintenance of Ad replicative fitness in the presence of AAV co-infection. Whatever its role in promoting AAV infection, E4orf6/E1b-55k-dependent degradation of AAV5 proteins is likely to be an important facet of AAV biology.

We also report the initial observation of the poly-ubiquitination of a parvovirus non-structural protein via K63-linked poly-ubiquitination in 293 cells in the absence of E4orf6. This type of ubiquitination does not appear to occur to *de novo*-synthesized

capsids, however, and the specific E3 ligase that mediates this poly-ubiquitination is unknown. It is unlikely that it is mediated by a cullin ligase, as they are traditionally responsible for K48-mediated poly-ubiquitination. Given the hundreds of E3 ligases within mammalian cells, it is likely that its identification will be difficult. However, co-immunoprecipitation of the AAV5 small Rep proteins in the absence of adenovirus, followed by silver-stain and mass spectrometry, may assist in the identification of this factor and other cellular factors that interact with Rep52 *in vivo*.

The K63-linked poly-ubiquitination of many cellular proteins is required for their function. It is likely that the poly-ubiquitination of AAV small Rep proteins in this regard is required for its function as well. Ubiquitination of the small Rep proteins may be required for efficient trafficking of AAV proteins to replication centers during infection, or may be required for interactions with other cellular proteins. The small Rep proteins could necessitate K63-linked poly-ubiquitination for their helicase activities. Future experiments could focus on the specific lysines that are ubiquitinated in Rep52. Are the lysines ubiquitinated in the presence of the adenovirus E3 ubiquitin ligase the same as those that are ubiquitinated naturally by the host cell? This question will likely be difficult to answer. Whether the specific lysines that are ubiquitinated can be determined by deletion or mutation analysis is a question that can only be addressed by trial and error. It is possible that the specific residues ubiquitinated in these contexts can be determined by mass spectrometry; however, mutating these residues to produce a protein that is not ubiquitinated may not be successful, as abberent ubiquitination of cryptic lysines can occur when the preferred lysines are not available. In addition, a lysine-less Rep protein may not be functional. Does the adenovirus E4orf6/E1b-55k E3

ubiquitin ligase complex also possess deubiquitinating activity, similar to that demonstrated with A20 and parkin? It will be interesting to expand the findings reported here to include other parvovirus non-structural proteins. Given that the AAV2 small Rep proteins are degraded in 293 cells in the presence of E4orf6, it is likely that they are ubiquitinated as well. Indeed, ongoing experiments by others in our lab have determined that the AAV2 small Rep proteins are also ubiquitinated in 293 cells in the presence of E4orf6; however, it is not yet known if they are ubiquitinated in the absence of E4orf6.

We have also demonstrated here that *de novo*-synthesized AAV5 capsid proteins are also poly-ubiquitinated in the presence of adenovirus E1b-55k and E4orf6; however, they do not appear to be poly-ubiquitinated in the absence of E4orf6. This finding is interesting considering that AAV capsid proteins are known to be poly-ubiquitinated upon entry to the cell. The capsid transient transfection performed in this report was conducted in less than 24 hours as to minimize the possibility that assembled capsid proteins could exit and re-enter the cell to be poly-ubiquitinated. Although this preliminary result is interesting, a better method to study this phenomenon will be to repeat the experiment in the presence of the cell-surface sialic acid-inhibitor, neuraminidase. As AAV5 primarily utilizes sialic acid as its cell-surface receptor, the addition of this inhibitor will prevent subsequent re-infection of AAV5 capsid proteins following transient transfection. Regardless, the results presented here potentially open the door to a fascinating new chapter in our understanding of parvovirus biology.

Previous reports from our lab have also determined that the transcription profiles of AAV2 and AAV5 differ dramatically. Pre-mRNA transcripts from the upstream P5 and P19 promoters in AAV2 are poly-adenylated at the 3'-end of the genome; however,

transcripts from the comparable P7 and P19 promoters in AAV5 are preferentially poly-adenylated in the intron and are therefore not efficiently spliced. Therefore, we would predict that AAV5 would only generate Rep78 and Rep52 and not the spliced forms Rep68 and Rep40. However, AAV5 is capable of generating significant levels of a Rep40-like protein. The Rep40 protein in AAV2 is required for efficient packaging of replicated AAV genomes into preformed capsids. Its unique ability to form a hexameric structure separates it from the other small Rep protein Rep52. The presence of high levels of Rep40 in AAV5, despite the low levels of splicing, suggests that this protein may also be required for AAV5. We demonstrate in this report that unlike AAV2, the AAV5 Rep52 and Rep40 proteins share the same C-terminus and differ in their N-termini, likely due to the utilization of an internal methionine start codon within the AAV5 Rep52 reading frame. Although this methionine is also present in AAV2, it is not used. The exact mechanism of this phenomenon is currently being investigated and may involve complex mRNA secondary structures in and around the internal methionine. The complex nature by which the parvoviruses utilize multiple strategies to expand the coding capacities of their small genomes deserves closer inspection. The effects of AAV5 Rep40 in its life cycle (presumably, in the packaging of ssDNA into preformed capsids) can be examined by introducing a mutant into the infectious clone such that Rep40 cannot be made. Mutation of the internal methionine to arginine completely eliminates Rep40 and will be a decent start; however, it is unknown if this mutation will have detrimental effects on the function of Rep78 and Rep52 independent on the presence of Rep40. Therefore, silent mutations that do not change the coding sequence yet still eliminate Rep40 will be the most reliable method to determine the importance of Rep40

in AAV5. However, these mutants will not be possible until the precise mechanism of the usage of this internal methionine in AAV5, but not AAV2, is understood. The understanding of this phenomenon will be a fascinating addition to our understanding of the evolution of these most unique of parvoviruses, and may even lead to additional strategies for improving the design of rAAV vectors.

The use of rAAV vectors for gene-therapy applications has become more widespread over the last few years, and it continues to be a safe alternative to other viral-delivery methods. However, one of the larger challenges that remain is the difficulty in generating significant titers of rAAV for use in humans via transient transfection. A number of improvements have been made over the last few years; however, additional improvements to current methods are still necessary. We report a simple method by which to significantly enhance rAAV production during transient transfection. The accumulation of AAV capsids is thought to be a rate-limiting step in the production of rAAV. In contrast to AAV2, we found that the overall levels of pre-mRNA splicing in AAV1 and AAV6 were quite low in the presence of adenovirus. Improvement of overall pre-mRNA splicing by mutating the non-consensus splice donor to that closer or identical to the consensus U1 snRNP recognition site significantly enhanced overall pre-mRNA splicing, capsid production, and rAAV production in all serotypes tested. However, the mutation of the donor sequence of the RepCap single-vector AAV helper plasmids to that of the consensus donor site had detrimental effects on the production of rAAV, presumably because of the introduction of a premature stop codon that truncates Rep78 and Rep52. However, the use of the consensus donor in Rep/Cap split-vector systems overcame this negative effect and allowed for even greater levels (four- to six-fold) of

rAAV production as compared to wild-type vectors. In addition, the replacement of the cytomegalovirus immediate early promoter with that of the AAV5 P41 promoter to drive capsid expression resulted in even greater titers (10- to 15-fold) of rAAV production when the consensus donor was used, as compared to the traditional CMV-driven wild-type donor expression vector. These virions were fully transducible, as the ratios of packaged genomes to transducing units remained in normally acceptable levels. Therefore, the enhancement of capsid production via improved pre-mRNA splicing presents a useful mechanism by which to improve the production of rAAV.

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