# Chapter 1

# Adeno-Associated Virus Biology

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## Abstract

Adeno-associated virus (AAV) was first discovered as a contaminant of adenovirus stocks in the 1960s. The development of recombinant AAV vectors (rAAV) was facilitated by early studies that generated infectious molecular clones, determined the sequence of the genome, and defined the genetic elements of the virus. The refinement of methods and protocols for the production and application of rAAV vectors has come from years of studies that explored the basic biology of this virus and its interaction with host cells. Interest in improving vector performance has in turn driven studies that have provided tremendous insights into the basic biology of the AAV lifecycle. In this chapter, we review the background on AAV biology and its exploitation for vectors and gene delivery.

**Key words:** AAV, rAAV, Adeno-associated virus, Recombinant AAV, Gene delivery, *Dependovirus*, Parvovirus

## 1. Introduction

### *1.1. Life Cycle*

Adeno-associated viruses (AAVs) are helper-dependent members of the *Dependovirus* genus of the parvoviruses that have evolved to replicate under a diverse set of conditions (1). Productive AAV infection requires helper functions that can be supplied either by co-infecting helper viruses or by DNA damaging agents. Helper viruses shown to promote AAV replication include Adenovirus (Ad) and herpes simplex virus (HSV). In each case, the helper induces changes to the cellular environment that can serve to facilitate AAV gene expression and replication. Although ubiquitously prevalent in the human population, AAV has not been associated with any human disease. The success of AAV infection is determined by molecular interactions between the virus and its host cell at every step of the lifecycle. The AAVs are small viruses with

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limited coding capacity, and they are therefore highly reliant on the cellular environment and machinery. In the absence of helper virus, AAV can establish a latent infection in many cell types, from which it can be rescued by subsequent helper virus infection. In the case of AAV2, latency is associated with targeted integration at a specific locus on human chromosome 19 and this requires the viral Rep protein (2–6).

### 1.2. Genome Structure

The most extensively studied serotype of AAV is type 2 (AAV2), which serves as a prototype for the AAV family. Since most experience with vectors has been obtained with AAV2, we will use information about this serotype to describe general features of AAVs. The AAV genome is a molecule of single-stranded DNA of approximately 4.7 kb. The plus and minus strands are packaged with equal efficiency into separate preformed particles. At either end of the genome are inverted terminal repeats (ITRs) that form T-shaped, base-paired hairpin structures, and contain *cis*-elements required for replication and packaging. Two genes (rep and cap) encode for four nonstructural proteins required for replication (Rep78, Rep68, Rep52, and Rep40) and three structural proteins that make up the capsid (VP1, VP2, and VP3). There are three viral promoters that are identified by their relative map position within the viral genome: p5, p19, and p40. Although the transcription profiles vary for different AAV serotypes (7), all transcripts of AAV2 contain a single intron. Unspliced RNAs encode Rep78 and Rep52, while Rep68 and Rep40 are encoded by spliced messages.

The virus can be rescued by transfection of molecular clones into mammalian cells together with Ad helper functions. The basis for the production of rAAV vectors is the fact that the rep and cap genes can be deleted from the viral genome and provided in trans. The viral genes can be replaced by a transgene with transcriptional control elements, resulting in a vector genome of approximately 4.5 kb flanked by the viral ITRs. Various approaches are employed for the production of rAAV vectors and many purification schemes have been developed, some of which are customized to specific serotypes (8). Purification protocols include ultracentrifugation through cesium chloride density gradients, the use of nonionic iodixanol gradients, and various forms of column chromatography.

Some of the inherent limitations of packaging genes into small rAAV genomes have been bypassed by elegant manipulation of the basic vector constructs. These include dual vectors that expand the rAAV packaging capacity (9) and self-complementary (scAAV) vectors that circumvent the requirement for dsDNA conversion (10). Dual vectors consist of two independent viruses that each carry a portion of the transgene cassette which is reconstituted following simultaneous co-infection. Examples of expanded capacity through dual vectors include *cis*-acting vectors in which regulatory elements are separated from the therapeutic gene (11), *trans*-splicing vectors in which exons in separate vectors are reconstituted through

splicing (12–16), and overlapping vectors that exploit homologous recombination (14). These vectors have the potential to double the capacity compared to a conventional single vector approach. The scAAV vectors can be made by reducing the construct size to approximately 2,500 bp, equivalent to half the size of the regular genome. Production of scAAV vectors can be promoted by deleting the terminal resolution site (TRS) sequence from one ITR such that dimeric genomes are generated (17–19). Upon uncoating, the scAAV vector genome can anneal rapidly to form a dsDNA hairpin molecule with a covalently closed ITR at one end, and two open-ended ITRs at the other end, mimicking a conventional rAAV vector genome. These scAAV vectors have a limited capacity for transgene load but in return offer efficient gene expression with a rapid onset of transduction compared to conventional vectors (10, 17–20).

### 1.3. Virion Structure

The AAV virion is an icosahedral nonenveloped particle with an encapsidated single-stranded DNA genome. The AAV2 virion is roughly 20 nm in diameter and is composed of 60 copies of the three capsid proteins VP1, VP2, and VP3 in a 1:1:10 ratio. The VP1 and VP2 proteins share the VP3 sequence and have additional residues at their N-termini. The N-terminus of VP1 has a conserved phospholipase A2 sequence that has been implicated in virus escape from endosomes and is crucial for infectivity (21). The VP2 protein is not essential for assembly or infection (22). The core of the VP3 protein consists of a conserved β-barrel motif composed of antiparallel β-sheets (23). This motif is found in other parvoviruses but the interstrand loops are variable, and it is these that determine receptor usage and serology. Structural information combined with genetic data has been important for understanding the molecular interactions of the virus particles. Structural images of several AAV capsids have been determined by X-ray crystallography and cryo-electron microscopy (23–26). There are extensive interactions between capsid subunits at the threefold axis where proteins come together to form three clusters of peaks on the surface topology of the virion (23).

### 1.4. AAV Serotypes and Variants

AAV serotypes were isolated as contaminants of adenovirus preparations from primates and other species (e.g., avian and bovine). AAV serotypes 2, 3, 5, and 6 were discovered from human cells, while AAV serotypes 1, 4, and 7–11 have nonhuman primate origins (27, 28). As testament to the high prevalence of AAV in humans and other primates, 108 new AAV isolates were identified and classified into various clades based on phylogenetic similarities of capsid sequences (27). The general organization of the parvovirus genome is conserved across the different serotypes, with a similar configuration of replication and structural genes, although there are some differences in transcription profiles (7). Comparing the capsid proteins of the various serotypes revealed 12 hypervariable surface regions, with most of the variability mapping to the threefold proximal peaks (29).

Vectors derived from naturally occurring AAV variants have demonstrated diverse tissue tropisms (30–32). Cross-packaging vectors that use the same vector genome in different capsids have allowed direct comparisons of different serotypes and have shown that the tropism can vary among different tissues in vivo (33–35). Comparisons between the structures of AAV capsids suggests that it is variation in surface topologies that determines the differences in cell surface attachment and receptor usage, intracellular trafficking pathways, and antigenicity between closely related serotypes (23–25). For example, the patches of residues used by AAV2 for heparan sulfate binding are absent from AAV5 and AAV8. Although much of the human population possesses antibodies to AAV capsids, the epitopes may vary between serotypes and not all neutralizing antibodies will cross-react (36).

The structural and functional knowledge obtained from natural AAV variants has enabled rational design of the capsid to improve specific properties (34, 37). The capsid proteins of different serotypes have been mixed to generate mosaic vectors (38), or recombined to generate chimeric virions (39, 40). Combinatorial libraries of capsid proteins have been generated by DNA shuffling and error-prone PCR and these enable directed evolution to isolate vectors with improved functionality (41–43). Targeted transduction and immune evasion have also been achieved through capsid manipulation by site-directed mutagenesis, peptide insertion, and chemical conjugation (44, 45).

### 1.5. Human AAV Infection

Despite the fact that AAVs have been studied for the past 50 years, little is known about the natural infection by this virus. This observation might be further surprising in light of the finding that approximately 80% of the population is seropositive for anti-AAV antibodies. These antibodies have been determined to be against serotypes 1, 2, 3, and 5. It has become apparent that approximately 60% of the population has neutralizing antibodies at age 10, which generally will persist into adulthood. However, together with the absence of any discernable pathology associated with AAV infection, our limited insights into the viral life cycle in vivo possibly underline the close link between the study of viruses cycles with disease. In this context, it has been noted that AAV might have evolved an ideal relationship with its human host. AAV is inherently replication-defective in normal, healthy cells. In the presence of conditions that can be described as adverse to the host (i.e., helper virus co- or super infection) AAV replicates to significant levels (100,000–1,000,000 copies per cell). A consequence is that cells affected by helper viruses (adenoviruses, herpes viruses, papilloma viruses) likely die as a result of AAV replication. Thus, it is reasonable to assume that AAV can indeed have a protective effect on its host.

In search of an in vivo life cycle, AAV particles have only been isolated in the context of acute adenovirus infections (46).

In addition, it has been proposed that AAV can be sexually transmitted (47, 48), possibly together with either herpes or papilloma viruses. It remains unclear, however, if the latent AAV infection that has been well studied in tissue culture, is in fact an essential component of the in vivo life style.

Our current understanding of effects by AAV infection on the host is limited to correlative studies. Consistent with a protective effect hypothesized above, epidemiological studies have suggested that while 85% of healthy women showed to be seropositive for AAV, in cervical cancer patients AAV antibodies could only be detected in 14% of the cases (49). Consistent with this observation is the finding that in healthy women the AAV titers were significantly higher than in women who had cervical cancer (50). More recently, this finding has been extended by the observation that individuals infected with HPV are less likely to develop cervical neoplasia when AAV is present, highlighting a potential protective effect (51). To date, none of these phenomena have been addressed mechanistically and only few hypotheses have been put forward that attempt to explain these findings.

In summary, very little is known about the life style of AAV in human hosts. In addition, an entire arm of the viral life cycle – the latent infection – which has extensively been studied in tissue culture, remains elusive in the context of the host.

### *1.6. A Brief History of Vectors*

Knowledge of the basic biology of AAV and its interactions with the cell has helped to drive the development of rAAV vectors and has guided production and applications (52). The first vectors were generated in the 1980s by replacing the viral genes with a transgene and transfecting Ad-infected cells with this vector plasmid together with a complementing plasmid that provided Rep and Cap functions. rAAV genomes are packaged into virus particles which can be used to deliver the genome for transgene expression in target cells. The most commonly used protocol for the production of rAAV vectors involves transient transfection (53). Other strategies explored for the production of rAAV vectors include the use of producer cell lines, combining viral features into Ad-AAV hybrids (54), the use of herpesvirus systems (55), and production in insect cells using baculoviruses (56).

There are many settings where rAAV vectors have demonstrated efficient gene transduction in preclinical gene therapy studies (52) and have entered clinical trials (57, 58). Transduction efficiency varies depending on the target cell type and serotype capsid being utilized. A number of rate-limiting steps have been identified in the transduction process (59, 60). These hurdles include cytoplasmic trafficking to the nucleus, the rate of uncoating, vector genome instability, and conversion of the single-stranded DNA vector genome into a transcriptionally competent double-stranded DNA molecule.

## 2. Viral Replication

### 2.1. Entry and Trafficking

Much of our current knowledge of the AAV life cycle must be seen in the context of the extraordinary promiscuity of this virus. As learned mainly from recombinant viruses, taken together the serotypes of AAV can infect a wide range of tissue culture and a panoply of tissues. It is thus not surprising that some ambiguity remains with regard to viral entry and trafficking pathways. It is of note that, likely, each of the serotypes isolated to date might share, but more importantly, differ in a number of aspects of virus host interactions, including the use of specific receptors as well as particular trafficking pathways. Ultimately, only the in-depth characterization of each serotype will result in a meaningful understanding of the relevant host factors and pathways involved in AAV infection. An additional point of caution is provided by the limitation that most of the studies to date have been conducted with tissue culture-adapted viruses. It is thus possible, if not likely, that the adaptation process has selected for viral characteristics which cannot be assumed to be found in natural isolates. This point is supported by the finding that while tissue culture-adapted AAV2 readily binds heparin, isolates from human tonsils do not (61).

Arguably the first step in viral tropism is the attachment to the target cell. For the best-studied serotype (AAV2), heparan sulfate was first identified as the cellular docking partner (62). On the viral capsid, the docking site has been mapped to amino acids 585–588 with two relevant arginine residues at positions 585 and 588 (63, 64). Interestingly, these two residues are missing in the isolates from human tissue samples (61). In addition to heparan sulfate proteoglycans, fibroblast growth factor receptor 1 (FGFR1) has been shown to be bound by AAV2 and, in the context of recombinant viruses, its presence has been associated with enhanced transduction (65). An additional molecule identified to enhance AAV2 infection and transduction is the avb5 integrin, supporting a role in AAV2 binding and uptake (66). The interesting aspect of this finding is that the AAV2 capsid does not contain the RGD motif that typically binds to these molecules, suggesting that alternative viral binding sites are used for this interaction. In addition to HSPG attachment by AAV2, sialic acid have been identified as attachment moieties for AAV serotypes 4 and 5 (67, 68). Using an intriguing approach, Di Pasquale and colleagues identified platelet-derived growth factor receptor (PDGF-R) as the uptake receptor for AAV5 (69).

Despite the discovery of a number of attachment partners and protein receptors, to date little is known about the possible downstream effects of receptor binding. The question as to whether particular signaling pathways are involved in enhancing virus uptake and trafficking thus remains unanswered.

Subsequent to receptor binding AAV is thought to enter via clathrin-coated vescicles (70). Following the uptake, AAV2 has been observed in many different cellular compartments and it must be noted that it is still a matter of discussion whether – whatever pathway is followed – the intact virus ends up in the nucleus for uncoating (71, 72). Not unlike the complications experienced in the studies of a number of other viruses, the experimental setup generally used relies on the visualization of virus particles, thus making these approaches vulnerable to detection sensitivities. More importantly, wild-type viruses used in these experiments typically display a particle over infectivity ratio of approximately 10:1; recombinant viruses (required to perform functional studies) are in the range of 40 to 1. A consequence of this notion is the as yet unmet challenge to demonstrate that the visualized particles in trafficking studies indeed reflect the infectious virus rather than the noninfectious majority. Nevertheless, it has been demonstrated that AAV2 can be found in late endosomes as demonstrated by the use of drugs that block endosomal trafficking steps (70, 73, 74). It must be noted that these studies were performed in the absence of helper virus, thus modeling the initiation of the latent life cycle of AAV and the pathway likely to be relevant for gene therapy applications. However, it is reasonable to hypothesize that AAV trafficking follows the route of helper viruses (i.e., early endosome escape in the case of adenovirus) (75).

AAV replication initiates in the nucleus. However, it remains somewhat controversial how, and where, the DNA is released from the capsids. Nevertheless, it has been recognized that one of the rate-limiting steps in viral infection and vector transduction is the uncoating of the extraordinary stable AAV virion (70, 74, 76).

Overall, it has become apparent that many question remain with regard to trafficking of the virus from the cell surface ending up as functional DNA within the nucleus. A challenge will be the identification of relevant cells and tissues as well as the further characterization of the viral reagents used in the dissection of AAV uptake and trafficking pathways (77).

### 2.2. DNA Replication

The current model of the AAV replication can be divided into several steps. In the absence of helper virus factors limited expression of Rep 68/78 occurs, with three main consequences. AAV gene expression is then repressed (78); thus AAV DNA replication is inhibited and AAV DNA can be integrated into the host genome. Co-infection – or super infection of a latently infected cell – with a helper virus leads to rescue of the AAV genome and DNA replication (79).

In a simplifying attempt to summarize the data to date, the requirement for the productive replication of AAV can be divided into three steps: The single-strand genome of AAV must be extended into a double-strand template for transcription (of the *rep* gene)

(17, 80–84). It is not clear yet if Rep plays a significant role in this step. Subsequently *rep* genes are transcribed. This step appears to involve several levels of regulation. In general, co-infection with adenovirus activates the p5 (Rep68/78) promoter as well as the p19 (Rep52/40) promoter (78, 85–88). Analysis of a variety of mutations of the AAV genome has shown that Rep is involved in both, negative (in absence of helper effects) and positive regulation of transcription. Extensive DNA replication then occurs. Rep activities are essential for this step (89). Furthermore, the cell-free assays were able to demonstrate that bacterially expressed Rep68 is capable of mediating AAV DNA replication in an adenovirus-dependent manner in the presence of HeLa cell extracts (90–93).

The model of AAV DNA replication has been developed over the past few decades and has largely remained unchallenged. Key components of this model consist of the notion that the general mode consists of unidirectional strand-displacement replication (92, 94, 95). The AAV genome is flanked on either side by structural elements known as the inverted terminal repeats, or ITRs. The ITRs contain motifs that serve as the viral origin of replication, namely the Rep binding site (RBS) and TRS. The ITR's ability to self-anneal provides the basis for the model of AAV DNA replication. Through its self-complimentary sequence and the resulting secondary structure, the ITR provides a base-paired 3′ hydroxyl group for unidirectional DNA synthesis. This synthesis is believed to be mediated through the host replication machinery including polymerase d (90). However, it is likely that during replication, which is supported by herpes viruses, some components of the cellular replication machinery are replaced by those from the helper virus (96, 97). Once the AAV template has been copied, a remaining step is the terminal resolution, the replication of the ITR, which has self-annealed to form the initial replication primer. This end of the genome is faithfully replicated through the actions of the Rep protein, which specifically binds to the RBS motif (98) within the ITR and regenerates a 3′ hydroxyl end by exacting a site- and strand-specific nick at the TRS. This nick then provides the necessary 3′ hydroxyl group for the replication through the viral ITR. Rep endonuclease activity directed at the double-stranded TRS is indirectly ATP-dependent; Rep helicase activity is required to render the TRS site single-stranded and thus accessible to the nicking active site (99). This replication cycle can result in two possible products, a double-stranded full-length AAV genome and a single-stranded full-length AAV displacement product. It is as yet unknown whether these distinct replication products can serve as templates for distinct downstream activities. It is intriguing to speculate that while the single-stranded AAV genome might serve as a template for further replication, the double-stranded replication product would be directed to serve as a template for genome packaging.

### 2.3. Helper Functions

Productive AAV infection requires co-infection with helper viruses that provide functions that aid in AAV replication, including larger DNA viruses such as Ad and HSV. Genes from Ad that provide helper functions for AAV have been defined as E1a, E1b55K, E2a, and E4orf6, together with the viral associated RNA (VAI RNA). The E1a gene product activates other Ad promoters and also binds to YY1 to relieve repression for the AAV p5 promoter (100, 101). The product of the E2a gene is a single-strand DNA binding protein DBP that is found at AAV replication centers (102) and stimulates processivity of AAV replication in vitro (103). The E1b55K and E4orf6 proteins function together to promote AAV replication and second-strand synthesis (104, 105). The E1b55K/E4orf6 proteins function as an ubiquitin ligase, and degradation targets include the DNA repair proteins that make up the MRN complex, which limits rAAV transduction (106). The VA RNA stimulates expression of AAV proteins, possibly by preventing phosphorylation of the eIF2alpha translation factor (107). In general, the functions of Ad helper proteins are to enhance production of AAV proteins and to alter the cellular environment to promote AAV replication. In the context of this helper virus, it has been shown that cellular DNA polymerases perform AAV replication. High titer rAAV can be generated in the absence of helper virus by transfection of plasmids that provide the Ad helper genes (108).

In contrast to Ad, the helper proteins provided by HSV-1 were initially defined as a subset of HSV-1 replication proteins: the helicase/primase complex (UL5, UL8, and UL52) and the DNA binding protein ICP8 encoded by the UL29 gene (109). Although these minimal proteins are sufficient to replicate AAV (110), other HSV-1 proteins have been shown to enhance helper functions in combination with the replication proteins. These include the ICP0 transactivator that activates rep gene expression (111), and the ICP4 and ICP22 proteins that can augment activity (112). The HSV-1 DNA polymerase complex (the polymerase UL30 and its co-factor UL42) can also contribute to efficient AAV replication (112). In vitro studies have also shown that UL30 can replicate the AAV genome without the requirement for the helicase–primase complex (96).

AAV replication can also be stimulated in the absence of helper viruses by treatments that cause cellular and genotoxic stress (113). These agents include hydroxyurea, topoisomerase inhibitors, and UV irradiation. Exactly how these treatments create a favorable environment for AAV replication remains unclear. AAV replication has also been suggested to occur autonomously in certain cells, such as a skin raft model of keratinocyte differentiation (114).

### 2.4. Cellular Proteins

Cellular proteins that are involved in AAV replication have been identified by biochemical methods, genetic screens, and through the use of cell-based assays. AAV can replicate in cell extracts

(115, 116) and in vitro assays have enabled purification of host cell proteins involved in AAV DNA replication (117). Amplification of AAV DNA can be reconstituted in vitro with purified Rep proteins and the following cellular enzymes: DNA polymerase d, proliferating cell nuclear antigen (PCNA), replication factor C (RFC), and the minichromosome maintenance complex (MCM) (90). In vitro replication can also be enhanced by the addition of single-strand DNA binding proteins from the cell (RPA) and helper viruses (DBP of Ad and ICP8 of HSV) (103, 110).

Cellular proteins that bind to the viral Rep protein or directly to the terminal repeats may either be required for replication or may regulate aspects of the viral lifecycle. A recent proteomic analysis of multiprotein complexes that interact with Rep during productive infection with Ad helper virus identified 188 interacting proteins by tandem affinity purification (118). These factors included those involved in DNA replication, transcription, translation, protein degradation, and RNA splicing. How these host proteins regulate Rep functions or contribute to the AAV lifecycle is still unknown in many cases. Some of the known interactions that affect Rep functions include the importin alpha receptor that mediates nuclear import (119), the Sp1 factor that mediates transcriptional activation (120), the high mobility group protein 1 (HMG1) that stimulates Rep activities (121), and PKA/PrKX that may be involved in inhibition of adenovirus (122).

A number of cellular factors have been shown to regulate AAV through binding to the AAV ITR. The chaperone-associated protein FKBP52 is a single-stranded DNA binding protein that recognizes the D-sequence within the AAV ITR and has been suggested to block second-strand synthesis (83, 123). A one-hybrid screen designed to identify cellular proteins that recognize the Rep binding sequence within the AAV ITR found that the cellular zinc-finger protein ZF5 is a negative regulator of AAV2 replication (124). Other proteins have been shown to inhibit AAV transduction and replication, including human APOBEC3A (125), although the mechanisms of most are not yet known (126). The AAV ITRs are also recognized by cellular DNA repair proteins that process the viral genome to inhibit replication or recombine the termini (106, 127–130).

During infection there is temporal and spatial regulation of AAV DNA replication, capsid assembly, and genome packaging (102, 110, 131, 132). In a similar way to other viruses, AAV replication takes place at discrete sites within the nucleus. It has been suggested that one of the helper functions provided by proteins from HSV is to form a scaffold for the recruitment of cellular proteins (110). Rep proteins colocalize with viral DNA at viral replication centers (102). Cap proteins are seen transiently in nucleoli and they later accumulate with Rep in the nucleoplasm (131, 132).

### 2.5. Gene Expression and Transcriptional Regulation

The AAV genome is highly compact, with complex overlapping coding regions. The transcriptional profiles of a number of AAVs have been examined and allows them to be divided into three main groups that differ based on their use of internal polyadenylation sites (7). The transcription and regulation has been most extensively studied for the prototype AAV2 genome. Since the Rep proteins inhibit cell proliferation (133), AAV2 gene expression is tightly regulated through the repressive effects of cellular factors (100) and the viral Rep protein (134). The Rep proteins can mediate both activation and repression of AAV transcription (78, 85). In the absence of helper virus co-infection, the larger Rep proteins can suppress their own transcription through a binding element in the p5 promoter (78, 134). However, in the presence of Ad, Rep serves to activate all three promoters (78, 135) through direct binding to the viral DNA or through interactions with transcription factors such as Sp1 (120, 136). Inhibition of p5 can be relieved by the E1a transactivator of Ad (101) and the ICP0 protein of HSV (111). There is also evidence to suggest that the ITR can act as both an enhancer and an initiator for transcription (78, 137, 138), implying that it is recognized by cellular proteins that regulate transcription.

The ratios of the various Rep and Cap proteins is determined by the level of splicing, which is stimulated by helper virus co-infection (139). In the presence of helper proteins, the large Rep proteins increased the ratio of spliced to unspliced RNA. Rep enhancement of splicing is dependent on Rep binding to DNA but is independent from effects on transcriptional initiation (140). Further steps in processing of AAV RNAs are unclear. For example, it is not known how the unspliced messages are exported from the nucleus.

### 2.6. The Rep Proteins

A considerable number of studies indicate that the largest of the Rep proteins, Rep78/68, are required for virtually every step of the viral lifecycle. These include DNA replication (89, 141, 142), site-specific integration (4, 143, 144), rescue of integrated genomes (145, 146), and the regulation of both viral and cellular promoters (78, 85, 88, 120, 134, 142, 147–149). The smaller Rep proteins, Rep52 and -40, appear to be required for the accumulation and packaging of single-stranded genomes, thus explaining the prevalence of these transcripts during productive infection (139, 150, 151).

Consistent with their multifunctional role during the viral lifecycle, the products of the *rep* ORF, Rep78, -68, -52, and -40, possess a variety of biochemical activities. The three major functional domains are all present in the largest of the Rep proteins, Rep78. The Rep amino terminus possesses specific DNA binding and endonuclease activity (152, 153), the central domain bears motifs necessary for ATPase and helicase activity as well as the nuclear localization sequence, and the carboxy-terminal Zn-finger domain has been

implicated in interacting with a myriad of cellular factors. The remaining Rep isoforms, Rep68, -52, and -40, are a combination of these functional domains arising from alternative splicing schemes and differential promoter usage within the *rep* ORF. Notably, all four isoforms possess the helicase domain, with Rep40 representing the minimum AAV helicase. Overall, this central domain is the most highly conserved region among parvoviral nonstructural proteins. The AAV Rep helicase falls into the super family 3 (SF3) class, as do several other viral helicases including that of papillomoviruses, poliovirus, and simian virus 40 (SV40). Members of this family contain four highly conserved regions: motifs A, B (also known as Walker motifs A and B), and C essentially make up the core of the helicase active site consisting of an NTP-binding site, divalent metal cation coordination site, and sensor-1 site, respectively. Motif B′ is located between the Walker motifs and the C′ motif. Its role in catalysis was not yet known but the X-ray structures of members of the family solved recently imply a possible role in DNA interactions. A beginning toward understanding the molecular details and mechanism of Rep proteins during the viral life cycle has been the determination of the crystal structure of Rep40 (154, 155). The structure shows that Rep40 is structurally more similar to the $AAA^+$ class of cellular proteins than to DNA helicases from other superfamilies. Rep40 that contains the minimal helicase domain is bimodular with a small helical bundle at the N-terminus and a large $\alpha/\beta$ ATPase domain at the C-terminus. This domain has the classical topology of P loop ATPases, with a central β-sheet flanked by four helices on one side and two on the other. Comparison with the helicase domain of SV40-Tag points out important structural differences that might point to the unique the dynamic oligomeric nature of Rep proteins. Finally, the homology to papilloma virus E1, SV40 Tag and $AAA^+$ proteins in general have led to the suggestion that at least the larger Rep isoforms likely function as hexamers. However, recent cryo-electron microscopy studies revealed that Rep68 represents the only known helicase which forms bi-directional double-octameric rings when bound to a helicase substrate (156). This study further highlighted that the DNA substrate dictates the oligomeric form of Rep68, thus proposing a possible mechanism by which the multiple activities of this protein could be distinguished and/or regulated.

Finally, it must be noted that although a considerable number of biochemical studies have been conducted to characterize Rep, only little is known as yet with respect to the molecular mechanisms that are underlying the various biological functions of these unique proteins.

### 2.7. Genome Integration

To date, wild-type AAV remains the only known eukaryotic virus with the unique ability to integrate site-specifically into the human genome (146, 157). In this context it must be noted that all the

studies addressing this intriguing phenomenon have been conducted with cultured cells and no information is available about the significance of the latent arm of the AAV life cycle in the human host. However, recently the potential significance of the underlying mechanism to the development of targeted gene addition as a novel component of therapeutic gene delivery has been recognized and a number of approaches are under development that aim to apply aspects of wtAAV integration to clinically relevant disease models. Among those aspects are the unique characteristics of the human target site for integration as well as the seemingly sophisticated mechanism, which AAV has evolved in order to insert its genome into the human chromosome.

Site-specific integration was first documented by Kotin and colleagues, who identified viral-cellular junctions from numerous latently infected cell lines, and found that these junctions consistently mapped to one region on human chromosome 19 (3, 158, 159). Using an independent approach, Samulski and colleagues further confirmed this finding (6). The human target locus, termed *AAVS1*, was then isolated and the sequence was determined in order to assess potential characteristics of the cellular sequence that might account for the unusual integration site preference (2). In search for determinants of site-specific integration, the observation by Walsh and colleagues that recombinant viruses devoid of the rep gene did not show evidence of site-specific integration hinted at a role for AAV Rep in site-specific integration (160). More direct evidence was then provided by the observation that when provided *in trans* the large Rep proteins could restore *AAVS1* targeted integration (144, 161). A final and more mechanistic insight was provided when functional RBS and TRS motifs were identified within the human target sequence, suggesting that Rep played a key role in the initiation of site-specific integration (162, 163). Finally, it was demonstrated that in a functional integration assay using episomes carrying *AAVS1* fragments that the RBS-TRS motifs were necessary and sufficient for site-specific integration (143). Based on these findings and together with observations from cell-free integration assays, an initial model for the molecular mechanism of Rep-mediated site-specific integration was proposed (4). The core of this proposal was that AAV site-specific integration parallels AAV DNA replication. In a manner equivalent to the actions of Rep at the viral origin, it was proposed that Rep targets the AAV genome for integration at *AAVS1* by initiating replication at this chromosomal origin, i.e., specific DNA binding at the RBS followed by ATP-dependent endonuclease activity directed at the TRS. Once DNA replication is initiated, strand-switching between the chromosomal and proximal viral DNA template was thought to allow for the eventual incorporation of multiple copies of the AAV genome into the chromosomal locus. This model,

although consistent with the characteristics of the viral-cellular junctions and the organization of the integrated genomes remained hypothetical.

The potential utility of AAV-mediated targeted transgene integration relies on the safety of this viral approach. On the background of the observation that AAV is a nonpathogenic virus, it was somewhat counter-intuitive that the virus appeared to have evolved a mechanism to integrate its genome into a highly gene-rich genomic environment (164, 165), thus making insertional mutagenesis a near certainty. However, the discovery of the mouse ortholog of the human integration target sequence that closely resembled the genetic makeup of *AAVS1* (166) enabled studies to address possible functional consequences of Rep-mediated targeted integration. Henckaerts and colleagues used this system to establish a site-specific transgene integration assay in mouse embryonic stem cells. With this system, it became possible to address several questions. It became clear that targeted integration of a marker gene resulted in strong transgene expression, which persisted throughout in vitro differentiation of these cells into multiple lineages (167). In addition, when injected into blastocysts, the resulting animals demonstrated strong contribution by the site-specifically marked cells to all tissues in the absence of any discernable phenotypic effect throughout the lifetime of the animals. Taken together these experiments demonstrated that *AAVS1*-targeted gene addition could result in the safe genetic modification of relevant cells. These studies were further taken into human embryonic stem cells, where *AAVS1*-targeted maker gene expression aided in the identification of human cardiac progenitor cells that were able to functionally integrate into a mouse heart (168). Molecular characterization of integrants in numerous human and mouse cells then revealed a surprising aspect of the Rep-mediated integration mechanism. It was found that the initial integration mechanism needed to be amended by the finding that in all cases analyzed the mechanism involved a partial duplication of the target sequence, suggesting the intriguing possibility that AAV might have evolved a mechanism for targeted gene addition in the absence of functional perturbation of the disrupted target genes (167).

To date, we begin to understand numerous aspects of this unique viral mechanism. However, it is apparent that many questions remain unanswered. In particular, we do not yet know whether integration is at all a significant component of the viral life style in vivo. In addition, for the development of targeted gene addition in therapeutically relevant approaches (e.g., X-SCID treatment), many remaining questions need to be addressed, including the identification of safe delivery strategies for the Rep protein and – possibly more importantly – the efficiency of successful integration events.

## 3. Summary

### 3.1. Impact of Biology of Vector Development

It is apparent that our, albeit, partial understanding of the biology of AAV has greatly aided the development of AAV vectors. The isolation of a panoply of capsid variants from animals, e.g., has provided a large range of vector types with specific transduction characteristics. In addition, the determination of a number of capsid crystal structures has opened doors that will ultimately allow for the design of vectors with specific and exclusive tropism, to name just a few. On the other hand, it is also clear that many additional discoveries on the biology of AAV are needed in order to improve our current vectors. For example, to date it is unclear why recombinant viruses exhibit a several-fold lower infectivity than wild-type viruses. A further dissection of the requirements for genome packaging as well as the identification of pathways involved might result in vectors with considerably higher infectivity, thus allowing for the administration of much lower vector doses. Finally, a more comprehensive understanding of the integration mechanism could result in vectors for stem cell applications that could overcome the apparent concern for consequential insertional mutagenesis.

### 3.2. Impact of Vectors on AAV Biology

In addition to the basic biology driving the development of rAAV for gene delivery, vectors have also served as tools to study the cell biology of infection and have advanced our knowledge of the basic parvovirus biology. They have been useful in elucidating the early steps in virus infection, including receptor identification (69) and highlighting barriers to intracellular trafficking and uncoating (75, 76, 84, 169). Vectors have also been useful for examining the fate of viral genome during transduction in cell culture and in vivo models (170). These studies revealed that the majority of the vector genomes can persist as circular episomes of monomers or concatemers (5, 170, 171) and identified host DNA repair factors involved in processing AAV genomes (127–129, 172). It is likely that the dynamic relationship between understanding basic biology and developing vectors will continue, and advances in each area will propel the applications that are possible for gene therapy.

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