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Recombinant Adeno-associated Virus Transduction and Integration

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Abstract

Recombinant adeno-associated virus (rAAV) holds promise as a gene therapy vector for a multitude of genetic disorders such as hemophilia, cystic fibrosis, and the muscular dystrophies. Given the variety of applications and tissue types toward which these vectors may be targeted, an understanding of rAAV transduction is crucial for the effective application of therapy. rAAV transduction mechanisms have been the subject of much study, resulting in a body of knowledge relating to events from virus-cell attachment through to vector genome conformation in the target cell nucleus. Instead of utilizing one mechanism in each phase of vector transduction, rAAV appears to employ multiple possible pathways toward transgene expression, in part dependent on rAAV serotype, dose, and target cell type. Once inside the nucleus, the rAAV genome exists in a predominantly episomal form; therefore, nondividing cells tend to be most stably transduced. However, rAAV has a low frequency of integration into the host cell genome, often in or near genes, and can be associated with host genome mutations. This review describes the current understanding of the mechanisms and rate-limiting steps involved in rAAV transduction.

INTRODUCTION

Adeno-associated virus (AAV) is a single-stranded DNA parvovirus with promising potential as a gene therapy tool. It is a member of the genus *Dependovirus*, and requires genes of a helper virus, such as adenovirus or herpes virus, for successful replication and assembly.¹ The AAV genome spans ~4.7 kilobases (kb) and contains two open reading frames: the *rep* region and the *cap* region.¹ The *rep* region codes for replication-related proteins and the *cap* region codes for the three proteins (VP1, VP2, and VP3) that together form the viral capsid. Inverted terminal repeats (ITRs) flank the viral genome and are the sole requirement for packaging DNA into capsids.² Therefore recombinant AAV (rAAV) vectors may be produced by replacing the wild-type coding regions with any gene or DNA sequence of interest, up to ~5 kb.¹ In addition, a vector genome containing the ITRs from one serotype can be packaged into the capsid of another serotype, producing a recombinant pseudotyped viral vector.³ Although many capsid serotypes, exhibiting a range of tissue tropisms, have been identified, the vast majority of experiments in AAV virology have been performed with vectors of AAV serotype 2.^{1,4–6} In order to transduce cells an AAV vector must enter the cell and deliver its single-stranded DNA

genome to the nucleus, where the genome becomes double-stranded before transcription. This review discusses the routes and mechanisms thus far elucidated in rAAV transduction and genome integration.

CAPSID DIVERSITY AND TISSUE TROPISM

Serotypes

To date, more than 110 distinct primate AAV capsid sequences have been isolated.⁶ Each of those that have unique serological profiles has been named as a particular AAV serotype. To date, 12 primate serotypes (AAV1–12) have been described.^{4,5,7–14} The AAV1 and AAV6 capsids differ by only six amino acids and, subsequent to their naming, they have been found to be serologically indistinguishable.^{4,12} The capsids for which the crystal structures have been determined so far are AAV2, AAV4, and AAV8.^{15–17}

Numerous studies have evaluated and compared serotypes as regards their transduction efficiency in tissues *in vivo*. A consensus of opinion has not been reached regarding the best serotype for each tissue, especially as additional serotypes continue to be isolated. Also, some tissues tend to be more susceptible overall to rAAV transduction, whereas the best transduction of other tissues remains relatively low. Against this background, a brief summary of compiled *in vivo* data is presented here. In striated muscle, early studies achieved high transduction efficiency with AAV1, AAV6, and AAV7.^{11,18–20} More recently, AAV8 and AAV9 have been found to transduce striated muscle with efficiencies at least as high.^{21–26} rAAV8 and rAAV9 are considered to have the highest level of hepatocyte transduction.^{11,22,23} In the pulmonary system, rAAV6 and rAAV9 transduce much of the entire airway epithelium, while rAAV5 transduction is limited to lung alveolar cells.^{27–30} With respect to transduction of the central nervous system, rAAV serotypes 1, 4, 5, 7, and 8 have been found to be efficient transducers of neurons in various regions of the brain.^{31–35} rAAV1 and rAAV5 have also been reported to transduce ependymal and glial cells.^{31,33} In the eye, rAAV serotypes 1, 4, 5, 7, 8, and 9 efficiently transduce retinal pigmented epithelium, while rAAV5, rAAV7, and rAAV8 transduce photoreceptors as well.^{36–38} rAAV1, rAAV8, and rAAV9 have shown the highest reported transduction in pancreas tissue, primarily in acinar cells.^{22,23,39,40} The kidney appears to be a relatively difficult organ to transduce, although proximal tubule cells have been transduced by rAAV2 at low levels,⁴¹ as have glomeruli by rAAV9.³⁰ Finally, rAAV1 has been shown to transduce adipose tissue, albeit with the aid of a nonionic surfactant.⁴²

Capsid engineering

In addition to investigations of the naturally occurring serotypes, efforts are underway to modify AAV capsids for designer tissue tropism and/or immune system evasion.⁴³ One method of achieving this is to produce vector in the presence of *cap* genes for multiple serotypes. Depending on the ratio of capsid proteins from each serotype, the resulting “mosaic” virions can exhibit a combined tropism for cell type or, in some cases, can acquire tropism not exhibited by either serotype individually.^{44,45} Some studies have involved attaching exogenous molecules to the capsid. One experiment utilized a bi-specific antibody obtained by fusing Fc regions of two different antibodies: an anti-capsid antibody and an anti-cell marker antibody, thereby conferring rAAV2 tropism to transduction-resistant megakaryocyte cell lines.⁴⁶ Another study adopted the approach of biotinylating the capsid and subsequently binding it to a streptavidin conjugate carrying epidermal growth factor or fibroblast growth factor. This approach was shown to produce at least a tenfold increase in the transduction of cells that highly express the epidermal growth factor or fibroblast growth factor receptor, respectively.⁴⁷

As an alternative to attaching molecules to the capsid surface, many reports have described engineering the modification directly into the *cap* gene. As a test of capsid flexibility, green fluorescent protein (GFP) (238 amino acids) was inserted into AAV2 VP1 and VP2.⁴⁸ Although the transduction efficiencies of the VP1-GFP and VP2-GFP vectors were 3 and 5 orders of magnitude lower, respectively, than the efficiency of wild-type capsid, the transduction in HeLa cells did occur, suggesting a tolerance for inserted sequences in capsid proteins. For modifying *cap* genes for tissue targeting, a number of researchers have inserted peptide sequences on the basis of known ligand–receptor interactions,^{49–51} or have selected for peptides in phage-display libraries.^{52–54} Another strategy has been to insert random sequences of amino acids, followed by *in vitro* selection of the best performing capsids.^{55, 56} Instead of introducing target-specific peptides, some experiments modified the capsids generically, pending subsequent modification toward targets of choice. For example, a binding site for the Fc portion of antibodies was inserted into the capsid, followed by binding of different antibodies specific for receptors of various cell lines.^{57,58} Another such modification is to insert a biotin-binding site into the capsid, thereby facilitating metabolic biotinylation and allowing flexible targeting with any avidin-conjugated ligands.⁵⁹ Some experiments have taken advantage of peptide insertion as well as mosaic capsids with a virion containing both wild-type capsid proteins and engineered capsid proteins, or a virion containing a combination of multiple different modified capsid proteins.^{58,60} Other techniques are under investigation with a view to evading the immune system, and these include coating capsids with polymer.^{43,61} Depending upon the exact modifications of the capsid in any of these methods, the results can vary from no transduction to high transduction of target cells, provided successful capsid assembly occurs at all. Many iterations of design and/or selection may therefore be necessary in order to develop highly-functional designer capsids. It has proved to be a challenging task to engineer altered capsids that can be produced efficiently and that acquire appropriate transduction profiles, but efforts toward enhancing transduction selectivity and efficiency appear to be promising.⁴³

CELLULAR ENTRY AND TRAFFICKING

Attachment

In order for an AAV vector to enter a cell, it must first attach to the cell and subsequently be internalized. By measuring the interactions between individual fluorescently-labeled virions and the surfaces of HeLa cells, it is estimated that an AAV touches the cell surface an average of 4.4 times before entering or diffusing away.⁶² If the virus does enter the cell, uptake will have occurred within ~100 ms of docking with the cell membrane; however, the penetration efficiency is 13%, as calculated in terms of the ratio of virus-cell contacts to internalization events.⁶²

The primary attachment receptor for AAV2 is heparan sulfate proteoglycan, as determined by competition assays and by heparan sulfate proteoglycan–mutant cell transduction assays.^{63, 64} However, studies of AAV2 capsid binding and cellular internalization suggest that, while heparan sulfate proteoglycan may be a primary cellular receptor for virus binding, interaction with a coreceptor may also be necessary for internalization of the virus. For AAV2, fibroblast growth factor receptor-1, hepatocyte growth factor receptor, and $\alpha_v\beta_5$ integrin can act as coreceptors.^{64–66} Initially, conflicting arguments emerged regarding the role of $\alpha_v\beta_5$ integrin, using different experimental designs.^{65,67} However, subsequent analysis has revealed that anti- $\alpha_v\beta_5$ integrin antibodies dramatically block AAV2 internalization into HeLa cells, thereby supporting its role as a coreceptor.⁶⁴ The blocking of this integrin does not, however, reduce viral binding to the cell, consistent with the paradigm of attachment and internalization receptors.⁶⁴ Further studies have designated the 37/67 kd laminin receptor and the $\alpha_5\beta_1$ integrin as additional coreceptors for AAV2.^{68,69}

Receptors have been identified for other serotypes as well. O-linked 2,3-sialic acid serves as a binding receptor for AAV4, while N-linked sialic acids serve as binding receptors for AAV5, as determined by sialidase treatment of cells and hemagglutination assays.^{70,71} On the basis of gene expression profiles of AAV5-permissive cell lines, followed by inhibition and competition experiments, platelet-derived growth factor receptor has also been identified as a coreceptor for AAV5.⁷² AAV1 and AAV6, whose capsid proteins differ in only six amino acids, have both been determined to utilize N-linked sialic acid for binding and transduction, although this may be partly dependent on cell type.^{28,73,74} Interestingly, AAV6 binds to heparin but, in contrast to AAV2, its transduction is not inhibited by soluble heparin sulfate.²⁷ The 37/67 kd laminin receptor mentioned earlier in relation to AAV2 can also act as a receptor for AAV serotypes 3, 8, and 9, as determined by overexpression and inhibition experiments.⁶⁸ Interestingly, AAV12 appears not to require heparan sulfate proteoglycan or sialic acid for transduction. This distinctive characteristic sets it apart from all other described AAVs.¹⁴

Endocytosis

AAV internalization into cells occurs by endocytosis, primarily in a receptor-mediated manner, through clathrin-coated pits (Figure 1).^{62,75–77} Studies of viral entry into cells have measured a threefold reduction in transduction due to inhibition of dynamin, a GTPase that regulates the pinching-off of clathrin-coated vesicles from the cell membrane.^{75,77,78} However, dynamin knockdown in these studies did not completely abolish transduction. This could be explained either by the presence of alternate entry mechanisms, or by an incomplete inhibition of dynamin. In either case, electron micrographs of HeLa cells infected with AAV5 particles have revealed that AAV occasionally enters cells in noncoated pits.⁷⁶ Therefore, while endocytosis by clathrin-coated vesicles may be the most common entry mechanism for AAV, other minor mechanisms exist as well.

Signaling pathways of endocytosis

Endocytosis of AAV has been associated with a number of signaling pathway molecules. Because integrins can act as AAV coreceptors, integrin-related pathways have been examined in particular. Inhibition of the GTP-binding protein Rac1 in HeLa cells by adenovirus delivery of a dominant-negative N17Rac1 allele decreases cellular uptake of AAV2 by up to 99%, as measured by Cy3-labeled AAV2 confocal microscopy and Southern blot analysis of trypsin-treated cells.⁶⁴ Moreover, Rac1 expression increases in HeLa cells within 5 minutes of infection by AAV2. Similarly, inhibition of PI3K by adding wortmannin decreases nuclear-associated virions 16-fold, and PI3K activity increases during AAV2 infection. N17Rac1 expression inhibits PI3K activation during rAAV2 infection, suggesting that Rac1 precedes PI3K in the signaling pathway.⁶⁴ Notch1 is a transmembrane receptor known to be involved in dynamin interaction and in activation of Rac and PI3K. Knockdown of Notch1 by siRNA is reported to decrease transduction by 97% in 293 cells, while its overexpression increases rAAV2 nuclear trafficking and transduction.⁷⁸ AAV2 has also been found to bind with nucleolin, a nuclear shuttle protein.⁷⁹

Endosomal processing

Endosomal processing of the virions is critical for transduction. Injecting wild-type AAV2 directly into the cytoplasm of HeLa cells results in an infectivity rate 100 times lower than does simple exposure of the cells to the virus.⁸⁰ Similarly, injecting AAV2 directly into the nucleus results in tenfold lower transduction.⁸⁰ In a study of gene targeting by rAAV2 (discussed later in this review), direct injection of vector into the cytoplasm or nucleus failed to result in gene-targeting events.⁸¹ It follows, therefore, that events during endosomal trafficking must play an important role in viral transduction capability.

The N-terminus of capsid protein VP1 contains a phospholipase A₂ (PLA) domain and three clusters of basic residues (BCs) with characteristics of nuclear-localization signals, the latter two (BC2, BC3) being shared with the capsid protein VP2.^{80,82} Mutations to one or more BCs or to the PLA domain decrease or eliminate AAV2 transduction of HeLa cells (depending on the substitutions) but do not affect cellular attachment or internalization.^{80,83} Using a BC3 mutation that eliminates transduction in HeLa cells, fluorescent *in situ* hybridization detects vector genomes in the perinuclear region 24 hours after infection, as compared to wild-type AAV2 capsids that successfully deliver vector genomes into the nucleus.⁸³ Transduction capability in this BC3 mutant is rescued when replaced with a canine parvovirus nuclear-localization sequence, thereby suggesting that BC3 is particularly important for delivering vector genome to the nucleus.⁸³ Interestingly, the simultaneous presence of BC3 on both VP1 and VP2 appears to be redundant, because a capsid assembled with a mutant VP1-BC3 and a wild-type VP2-BC3 is able to transduce HeLa cells.⁸³

In an assembled capsid, the N-termini of VP1 and VP2 are internalized; however they can become externalized by heat treatment.^{80,83} Moreover, immunofluorescent detection of intracellular VP1 and VP2 N-termini is possible within 1 hour of viral exposure to cells, thereby signifying that these motifs have been externalized on the capsid.⁸⁰ Immuno-dot blot analyses of cell lysates after AAV2 infection indicate that, during AAV infection and before uncoating, VP1 and VP2 N-termini become exposed while the number of intact capsids remains stable, thereby suggesting that externalization of the N-termini motifs does not cause capsid disassembly.⁸⁰ Inserting an extra domain into the N-terminus of VP1 can create a fusion protein that, because of size restrictions, cannot be internalized during capsid assembly, thereby achieving externalization of the N-terminus without heat treatment. While capsids composed only of VP2/VP3 are noninfectious, the addition of such a VP1 fusion protein exposing the PLA domain modestly rescues infectivity.⁸⁴ The inclusion of BC3 on the externalized portion of the VP1 fusion protein further increases transduction, reinforcing the suggestion that externalization of both the PLA domain and the BC3 region are required for optimal transduction.⁸⁴ Treatment of AAV2-infected cells with chloroquine or bafilomycin A1 (which increase endosomal pH) decreases the immunodetection of intracellular VP1 and VP2 N-termini and decreases viral gene expression, thereby signifying that endosomal acidification of the capsid is in part responsible for the proper processing of AAV.^{80,85} In addition, the processing by two endosomal cysteine proteases, cathepsins B and L, have also been identified as players in endosomal capsid processing. These cathepsins cleave AAV capsid peptides in a serotype-specific manner; cathepsins B and L cleave the VP3 peptide of AAV2 and AAV8, but not that of AAV5.⁸⁶ Specific inhibition of the cathepsins decreases AAV2 and AAV8 transduction in a dose-dependent manner, while overexpression of the cathepsins increases transduction. Interestingly, AAV particles remain intact after cathepsin cleavage, and are able to transduce cells as well, if not better, than uncleaved capsids.⁸⁶ Taken together, these studies strongly suggest that endosomal processing of the intact capsid is important for downstream events such as endosomal escape, nuclear entry, and viral uncoating.

Route toward nucleus

On the basis of studies performed in AAV2, AAV5, and canine parvovirus, at least five potential trafficking pathways have been postulated, involving multiple types of endosomes, lysosomes, and the Golgi apparatus (Figure 1).⁸⁷ In an experiment in which radiolabeled rAAV2 was added to either the apical or basolateral surface of bronchial epithelial cells, a binding assay measured a four- to sevenfold difference in virions binding to the cell (basolateral > apical).⁸⁸ However, transduction through the basolateral surface was 200-fold greater than that through the apical surface, a difference that cannot be ascribed solely to the differences in binding rate. At 50 days after infection in these experiments, the majority of basolateral-applied viral DNAs had become double-stranded, and only single-stranded DNA viral genomes were

detected in the apical-applied cells.⁸⁸ Further, because polarized cells have different early endosomal compartments for each side of the cell,^{89,90} these findings may suggest that early endosomal trafficking and processing is rate limiting in a cell type-specific manner. It may therefore be prudent to attempt to understand the trafficking patterns of AAV in each type of target cell, in an environment and morphology as similar to *in vivo* conditions as possible.

Additional studies have shed light on the intracellular routing of AAV. Preventing early-to-late endosomal transition with the drug Brefeldin A causes a decrease in rAAV2 transduction by two to three orders of magnitude, suggesting that a high proportion of virions must pass through the late endosomal compartment.⁸⁵ Subcellular fractionation experiments based on endosomal molecular markers have associated AAV2 with both the late endosomal (Rab7+) and the recycling endosomal (Rab11+) compartments, depending in part on the dose of the virus.⁹¹ At low doses (100 multiplicity of infection), AAV traffics primarily through the late endosomal compartment, whereas at high doses (10,000 multiplicity of infection) the recycling endosomal compartment is also utilized, and to a greater degree. At this higher dose, the level of transduction increases at a faster rate, thereby suggesting that transduction may be more efficient through the recycling endosomal compartment.⁹¹ AAV5 particles have been found to accumulate in a perinuclear cap-shaped distribution, further determined to consist of clathrin-coated vesicles, membrane tubules near the Golgi, and cisternae of the trans-Golgi network.⁷⁶ With an increase in multiplicity of infection, AAV5 virions also accumulate in lysosomes.⁷⁶

There is some evidence to suggest that AAV travels along microtubules and microfilaments. Fluorescently labeled AAV2 particles have been observed to travel in a manner consistent with microtubule-dependent directed motion.⁶² The treatment of cells with nocodazole, which depolymerizes microtubules, removes this directed motion.⁶² However, nocodazole has been reported, in two studies, to have little or no effect on transduction rates.^{92,93} In contrast, another experiment suggests that nocodazole reduces nuclear accumulation of AAV2 by 95%,⁶⁴ but the drug doses utilized have been suggested to be toxic to the cells.⁹³ Vinblastine, another drug that disrupts microtubules, does not alter transduction, although treatment with the microtubule stabilizer Taxol has been observed to partially decrease transduction.⁹³ Additional studies of microtubule-facilitated motion modulate the minus-end-directed microtubule motor protein dynein, which can be inhibited in a dominant-negative fashion by overexpression of dynamitin. HeLa cells transfected with a dynamitin-expressing plasmid exhibited no difference in rAAV2 gene expression when compared with untreated HeLa cells, thereby suggesting that dynein-mediated transport is not required for transduction.⁹³ In addition, the treatment of cells with Cytochalasin B, which disrupts microfilaments, has been reported to reduce nuclear accumulation of AAV2 by 91%;⁶⁴ however, drug toxicity has been suggested to confound these results.⁹³ Although there may be some inconsistency in the results and conclusions from these experiments, it appears that AAV probably utilizes a number of routing mechanisms during transduction, including, but not exclusively, microtubule- and microfilament-related movement.

It is currently thought that AAV endosomal escape occurs in the cytoplasm, before entry into the nucleus (Figure 1). As mentioned earlier, the lack of a functional exposed PLA domain of capsid protein VP1 decreases transduction levels, suggesting that its phospholipase activity may be important for efficient endosomal release of virions.^{80,84} In addition, cytoplasmic injection of anti-capsid antibodies against the N-termini of VP1 or VP2, or against intact capsids, almost entirely inhibits viral gene expression.⁸⁰ These antibodies should have little effect on transduction if capsids were not free in the cytoplasm.

Viral uncoating

Viral uncoating is believed to occur inside the nucleus (Figure 1). Many investigators have reported detecting fluorescently labeled capsids inside cell nuclei.^{62,64,75} Moreover, nuclear injection of antibody against intact capsids dramatically reduces transduction.⁸⁰ Viral uncoating may be a rate-limiting step of transduction, dependent in part on AAV serotype. An analysis of subcellular fractions 48 hours after infection with rAAV2 or rAAV6 in cultured cells revealed that most of the viral genomes were present in nuclei in the case of both serotypes.⁹⁴ However, in primary neonatal rat cardiomyocytes, DNase treatment of the nuclear fractions digested the rAAV6 genomes to a much greater extent than the rAAV2 genomes, coincident with higher rAAV6 expression levels.⁹⁴ Experiments in other cell types revealed faster uncoating of rAAV2 than of rAAV6, suggesting that the rate of viral uncoating is serotype- and cell-specific.⁹⁴

Proteasome inhibitors

The administration of different classes of proteasome inhibitors, including tripeptidyl aldehydes and anthracyclines, have been proven to increase transduction *in vitro* and *in vivo*, in a cell-type- and serotype-specific manner.^{85,88,95–98} However, the mechanisms by which these drugs increase transduction have not been defined and are, as yet, a bit contradictory. Both the tripeptidyl aldehyde LLnL and the anthracycline doxorubicin have been found to increase the rate of viral translocation to the nucleus.^{88,96,97} This finding may be related to ubiquitination, given that inhibition of ubiquitin ligase E3 also increases rAAV2 transduction levels, and LLnL results in an increased degree of rAAV2 capsid ubiquitination.⁸⁸ However, LLnL does not appear to prevent the degradation of intracellular viral DNA.^{88,95} Another tripeptidyl aldehyde, MG-132, also increases transduction of multiple rAAV serotypes.^{85, 88,95–97} In contrast to the reports relating to LLnL, however, total cellular rAAV DNA is increased in comparison to untreated cells, suggesting that proteasome inhibition in this case may protect the viral particle from being degraded.⁸⁵ Because doxorubicin is also a DNA topoisomerase inhibitor, its effects may be in part caused by mechanisms similar to those of another topoisomerase inhibitor, etoposide, which has also been found to increase transduction⁹⁹ (discussed below).

THE FATE OF VECTOR GENOMES

AAV genomes, once free in the nucleus, must become double-stranded in order to be transcribed. Wild-type AAV in the lytic phase undergoes second-strand synthesis, utilizing the free end of the ITR hairpin as a primer (see Figure 2a for ITR structure).¹ The resulting replicative form (Rf) of the vector genome exists as a double-stranded monomer (Rfm) or as a dimer (Rfd) in head-to-head or tail-to-tail organization. The manner in which rAAV vector genomes exist as transduction units has been the subject of much study. A number of investigators have reported that the amount of single-stranded vector genome decreases over time, presumably becoming double-stranded, or being degraded.^{100–103}

Concatamers

Some initial reports attempted to describe the molecular characterization of rAAV genomes *in vivo*. Total cellular DNA was collected from muscles injected with rAAV2-lacZ, and probed for the transgene sequence on a Southern blot.^{104,105} After digesting with a restriction enzyme that cut once inside the vector genome, a band equivalent to one vector-length fragment was apparent.^{104,105} This would be consistent with head-to-tail concatamerization of the vector in linear or circular form, or with circularization of a monomer. Studies in liver transduction with a single-vector cutter also detected major bands consistent with head-to-tail concatamerization, as well as minor bands indicative of head-to-head and tail-to-tail

concatamers.^{101,106–108} These studies strongly suggested that concatamers were produced in rAAV transduction events, resembling what had been observed in wtAAV infection.

However, the integration status of these concatamers remained uncertain. In order to clarify this point, a study was performed with an rAAV2 shuttle vector carrying a bacterial origin of replication and an *Amp^r* gene.¹⁰⁹ At timepoints between 0 and 80 days after intramuscular injection of rAAV2, undigested Hirt DNA was extracted and used for transforming *Escherichia coli*. Bacterial colonies would survive antibiotic selection if transformed with circularized DNA containing the shuttle vector sequence. Southern blotting determined that rescued circular DNA was in head-to-tail orientation, with either one or two ITRs. As time increased, larger circular genomes were detected, and the arrays of ITRs in the circles consisted of an increasingly varied number of ITRs. This showed that rAAV genome concatamers did exist as episomes, increasing in size for at least 80 days after injection. The effect of episomes on transduction will be discussed later in this review.

Although both head-to-head and tail-to-tail concatamers had been detected, the majority of concatamers were in a head-to-tail conformation, resembling those reported in wtAAV latent phase infection. In order to determine whether there was a correlation, an examination was performed by infecting HeLa cells with an rAAV2 shuttle vector in the presence of adenovirus deleted for the E4 coding region.¹¹⁰ When compared with superinfection caused by nondeleted adenovirus, the E4-deleted version was shown to result in an increase in abundance of head-to-tail circular vector genomes. Whereas the lytic replicative genome forms Rfm and Rfd are usually easily detectable with adenovirus-caused superinfection, Rfm and Rfd were undetectable in E4-deleted superinfection. In addition, Rfd takes a head-to-head or tail-to-tail conformation. It was therefore concluded that, in the absence of adenovirus, double-stranding of rAAV genomes most likely takes place through a mechanism similar to the wtAAV latent pathway, distinct from lytic pathway Rfs.¹¹⁰

Double-stranded vector genomes

The mechanism by which the single-stranded rAAV genome becomes double-stranded is not fully understood. It was generally assumed that host-cell polymerases would be involved in second-strand synthesis and replication of preintegration intermediates, especially because *in vitro* cells in S-phase had been shown to be transduced at a rate 200 times that of nondividing cells.¹¹¹ However, agents that damage DNA (such as ultraviolet or γ -radiation) or agents that inhibit DNA synthesis (such as hydroxyurea or topoisomerase inhibitors) had also been reported to increase rAAV2 transduction *in vitro*, thereby suggesting that DNA repair mechanisms may be involved in transduction.^{92,99}

Early studies determined second-strand synthesis as a rate-limiting step for rAAV2 transduction.^{112,113} These *in vitro* experiments utilized adenovirus mutants similar to those mentioned above to correlate the rate of transgene expression with the formation of replicative vector genomes (Rfm and Rfd). Both reports identified ORF6 of the Ad E4 locus as the crucial gene for enhancing rAAV2 transgene expression and replicative genome formation, thereby suggesting that rAAV transduction in the absence of a helper virus would be limited by the lack of Rf double-stranded DNA. These arguments were based on the belief that Rf vector genomes would be crucial to transduction; however, the organization of concatamers, discussed earlier in this review, challenges this belief.

Some subsequent studies suggested that double-stranded vector genomes may be created by the annealing of complementary single-stranded vector genomes. An *in vivo* experiment using a methylated vector sequence was performed, and harvested tissue was analyzed with a set of enzymes that cut sequences of varying degrees of methylation (*e.g.*, hemi-methylated, nonmethylated, etc.).¹¹⁴ Whereas double-stranded rAAV genomes created by a host-cell

polymerase would be hemi-methylated, those created by annealing plus-strand and minus-strand single-stranded DNA vector genomes would remain fully methylated. Restriction enzyme analysis revealed that the majority of double-stranded DNA remained fully methylated, thereby suggesting that recombinant double-stranded AAV genomes were predominantly created not by cellular replication mechanisms but by annealing of complementary AAV strands.¹¹⁴ A later report produced rAAV2 in the presence of BrdU, allowing plus- and minus-strands to be separated on a CsCl density gradient.¹⁰² The infection of HeLa cells with either plus-strand or minus-strand rAAV2 did result in transgene expression; however, infection with a mixture of plus- and minus-strand rAAV2 results in greater transduction than with either alone.¹⁰² More recently, single-polarity vectors have been produced by modifying the D element of the vector genome ITR.¹¹⁵ (See Figure 2a for ITR structure.) Experiments performed *in vitro* and *in vivo* with either a single-polarity rAAV2 vector or a conventionally produced rAAV2 vector resulted in similar transduction levels.¹¹⁶ Because transduction occurs with single-polarity vectors alone, host cell-mediated second-strand synthesis must occur. However, it appears that annealing of complementary strands also contributes to the creation of double-stranded AAV genomes. Differences in experimental design, including vector preparation technique and vector dose, may contribute to the continued debate.

Whatever the mechanism of double stranding, a recent report suggests that the stabilization of double-stranded vectors may be more of a rate-limiting step than the actual creation of double-stranded rAAV genomes.¹⁰³ This experiment took advantage of a mouse line in which a *lacZ* gene would be activated by the removal of loxP sites around a premature polyA site.¹¹⁷ Eight weeks after simultaneous systemic injection of rAAV2- or rAAV8-alkaline phosphatase (AP) and rAAV2- or rAAV8-Cre, AP expression remained low and scarce in liver sections, while the expression of β -gal was confluent.¹⁰³ These results suggest that the rAAV genomes were present and expressed in most hepatocytes, but that the double-stranded vector genome is transiently available for transcription.

Mechanism of concatamerization

In conjunction with the mechanism of second stranding, it has been proposed that concatamerization occurs by DNA replication rather than by intermolecular recombination.² However, more recent investigations involving coadministration of different vectors suggest that the latter is more prevalent (Figure 2b). In one study mouse muscle was injected with (i) an rAAV2-GFP shuttle vector for rescue in antibiotic-selected bacterial cells and (ii) an rAAV2-AP vector with no shuttle vector capabilities.¹¹⁸ At various time points after the injection, tissue was harvested and *E. coli* was transformed with undigested low-molecular-weight Hirt DNA. In probing for vector DNA in surviving colonies of the 14-day tissue harvest, only GFP-positive colonies were detected. However, at the time of tissue harvest at 35 days, a higher percentage of colonies were GFP and AP positive, signifying that GFP and AP vectors had ligated *in vivo*. Consistent with previous analyses of rAAV concatamers, structural studies of these hybrid concatamers concluded that head-to-tail, head-to-head, and tail-to-tail junctions were present, and deletions occurred near ITRs.^{100,118}

Another study analyzed head-to-tail junctions in cellular DNA isolated from mouse liver 5 weeks after injection of two different vectors.¹¹⁹ PCR followed by Southern blot probing revealed that, in addition to concatamers of each vector individually, concatamers of the two vectors together were also present.¹¹⁹ Another experiment utilized two rAAV2 vectors with different restriction fingerprints.¹¹⁴ Both were injected into mice, the livers were harvested 6 weeks later, and DNA was obtained. Restriction digestion analysis suggested the presence of large hybrid concatamers of randomly ligated vector genomes containing varying numbers and orders of multiple vectors.¹¹⁴ Further diminishing the case for rolling circle replication, *in*

vivo injection of naked double-stranded circular rAAV genomes does not result in concatamerization; however, injection of naked double-stranded linear rAAV genomes does produce large concatamers.¹²⁰ Interestingly, the presence or absence of ITRs in the linear ds rAAV genomes do not appear to influence the rate of concatamerization.¹²⁰

Episomes versus integration

Thus far in this report, circular episomal vectors have been described without detailing their fate. Given that wild-type AAV integrates into the host cell genome as concatamers, episomes could be preintegration intermediates. Early *in vivo* studies in mouse muscle and liver concluded that the majority of the rAAV2 responsible for transduction was integrated into the host genome.^{100,101,104–106} However, none of these *in vivo* studies provided evidence of integration; the conclusions were based on the inability to detect double-stranded vector genomes in low-molecular-weight DNA fractions extracted from transduced tissue.^{101,104,105} These studies did report concatamers, but did not strongly consider the possibility that they might be episomal.^{100,104–106} Even with the subsequent finding of long-lasting episomes, it was unclear whether the circular structures were preintegration intermediates, or whether they would contribute to transduction as episomes.¹⁰⁹

Some studies investigating related aspects of rAAV transduction hinted at episomal fates of rAAV vector genomes. Performing a partial hepatectomy on mice between 3 and 24 days after injection of rAAV2 caused a drop in vector genome number per diploid genome in comparison with nonhepatectomized mice.¹¹⁹ Another study, which involved partial hepatectomies on mice 6 weeks after injection of rAAV2, found that after liver regeneration the amount of rAAV genome copies per diploid genome dropped up to tenfold.¹¹⁴ If the rAAV genomes had been integrated, they would have been replicated by cellular machinery along with the chromosomes instead of being diluted out by cellular division. It was not known whether these timepoints were too early to allow sufficient time for episomal vectors to integrate.

Longer-term studies further confirmed the long-term role of episomes. Analysis of undigested cellular DNA taken from liver between 10 and 19 months after injection revealed supercoiled double-stranded circular monomers of vector genomes, in addition to vector genomes associated with high-molecular-weight DNA.^{108,121} Another study was also carried out, in which mice underwent a partial hepatectomy 12 months after injection of rAAV2–human clotting Factor IX.¹²¹ By 6 weeks after surgery, serum levels of human clotting Factor IX had dropped 84% as compared to nonhepatectomized mice, and the count of vector genomes per cell had dropped 92%. Similar results have been reported in subsequent partial hepatectomy studies as well.^{103,122}

Another experiment analyzed total cellular DNA from muscle 15 weeks after injection of rAAV2.¹²³ After digestion of DNA with a restriction enzyme that does not cut the vector genome, a Southern blot detected vector genomes at high-molecular-weight bands and at a band indicative of a double-stranded vector monomer. Digestion of cellular DNA with an enzyme that cuts once inside the vector produced a strong band indicative of a double-stranded monomer or of head-to-tail concatamers. After treatment by PS-DNase (which hydrolyzes any linear or single-stranded DNA) these bands were still present, thereby suggesting that the vector genomes detected were circularized episomes.

Although rAAV episomes do contribute significantly to long-term transduction, viral integration occurs as well, albeit at a low level (Figure 1 and Figure 2b). *In vitro* studies utilizing an rAAV2-*neo* vector carried out screening for surviving colonies in a G418 antibiotic selection, and calculated the minimal frequency of occurrence of integration as being in the range of one integration event per 10^3 – 10^4 vector particles.^{111,124} In numerous *in vivo* shuttle vector experiments with rAAV2^{107,125–128} and rAAV8,¹²⁸ sequence analysis of vector-

flanking regions in the rescued proviruses confirmed that the vector had integrated into the mouse genome. In a partial hepatectomy study, the rate of integration was determined as being between 0.06 and 0.2 vector genomes/cell.¹²¹ A subsequent study involved isolating muscle tissue from mice between 6 and 57 weeks after intramuscular injection of rAAV2.¹²³ Taking advantage of the 135-base pairs (bp) Alu-like B1 repeat that occupies 2.66% of the mouse genome,¹²⁹ quantitative PCR was performed to detect and quantify rAAV2 genomes that had integrated between any two B1 repeats. In that experiment no integrated rAAV2 vector sequences were detected.¹²³ Given that rAAV2 integration occurs more frequently in dividing cells,¹¹¹ postmitotic tissues such as muscle may be expected to undergo a minimal number of integration events. Recently, however, rescued shuttle vectors from cardiac and skeletal muscles have also been observed.¹²⁸

Integration sites

There have been a number of studies using shuttle vectors to dissect the anatomy of vector integration sites by sequencing outward from the vector sequence in order to analyze the flanking chromosomal sequence.^{107,124–128,130,131} These experiments have revealed that the locations of vector integration share no significant homology with one another or with the vector, and that integration occurs throughout the genome in a somewhat random distribution rather than at a specific locus, as can occur with wtAAV. However, microhomologies (1–5 bp) between the vector and chromosomal sequences usually occur at the junction site.^{125,131,132} Moreover, chromosomal deletions, most of which are <1 kb, occur in up to 70% of integrations.^{125–127,131,132} Some of the deletions were measured as being in the megabase range.^{126,127} Insertions or duplications also occur at about 35% of the integration sites, usually <100 bp long.^{125–127,131,132} Further, chromosomal rearrangements have been reported in 1–2% of integration events.^{107,126,131} Deletions within the ITRs are ubiquitously observed at integration sites, demonstrating that the sites of integration within rAAV vectors lie most often within or near the ITRs.^{107,124,125,127,130,132} Although integration junctions have been observed scattered throughout the ITRs, the hairpin structure of the ITR appears to contain hotspots of integration, especially at the turns and corners (Figure 2a).^{107,124,126–128,132}

With the human and mouse genome sequences becoming available,^{129,133,134} large overall analyses of integration sites became possible. Earlier, rAAV2 had been found to integrate preferentially within genes.^{125,131} A study of rescued shuttle vectors from mouse liver found that 53% of integrations took place within genes, 27% within 1 kb of a transcription start site, and 25% within a CpG island.¹²⁶ A survey of integration patterns in primary human fibroblasts transduced with rAAV2 found that 38% of the integrations were in the genes, and 4% within a CpG island.¹²⁷ The discrepancies between these two reports may be attributable to a number of factors, including the species or type of tissue studied, and the state of the respective genome builds that were analyzed. In any case, there does appear to be some level of preference for integration within genes—especially within 1 kb of the transcription start site—and within CpG islands. Studies using rAAV2 and rAAV8 report that, in addition to these regions, the vectors display a preference for integration into ribosomal DNA repeats,^{126–128} and near palindromes of at least 20-bp arm length.¹²⁸

In studies of vector–chromosomal junctions, up to 10% of vector-flanking regions have been confirmed as having the sequence of: vector, vector plasmid, and helper plasmid, or production cell genome.^{107,126–128,131} Much of the vector–vector junctions may be the result of transduction-related concatamerization, but unintended integration does occur at a low level during vector production.

Mechanism of integration

Given the lack of homology between vector sequences and chromosomal junction points, nonhomologous recombination is considered the most likely mechanism of integration.¹²⁴ In a study involving fibroblasts, when the DNA synthesis inhibitor hydroxyurea or the topoisomerase inhibitor etoposide was added immediately before rAAV2 infection there was an increase in the number of surviving fibroblast clones on an antibiotic background.⁹⁹ Assuming that the surviving clones were the result of stable vector integration, and given that DNA synthesis was inhibited during transduction, it was hypothesized that integration events occur through a mechanism other than replicative DNA synthesis, such as DNA repair.⁹⁹ This does not conflict with an earlier study which found that cells undergoing S-phase are transduced to a 200-fold greater extent than stationary cells are,¹¹¹ because DNA repair mechanisms are thought to be more active during DNA synthesis. In order to further explore this possibility, double-strand breaks were induced in HT-1080 cells and in primary human fibroblasts using either the I-SceI endonuclease or the DNA-damaging agents, etoposide or γ -irradiation.¹³² Cells transgenic for an I-SceI recognition site were treated with I-SceI, followed by rAAV2 infection. It was found that 7.4% of the integrations in HT-1080 cells and 7.7% of the integrations in primary human fibroblasts were located at the I-SceI site. These are much higher rates of such integration than would be expected in random integration events. As in the earlier study, etoposide had the effect of increasing integration 3-fold, and irradiation increased integration 14-fold. Cells induced to have double-strand breaks exhibited vector-chromosomal junctions similar to those in un-induced cells, suggesting that the primary mechanism of rAAV integration may be through double-strand breaks (Figure 2b).¹³² The integration junctions consisted of the characteristics described earlier, including vector-chromosome microhomologies, chromosomal insertions, and chromosomal and/or vector ITR deletions. Further, it is likely that rAAV may depend on preexisting double-strand breaks, because it is observed to integrate into locations where these breaks occur more often, such as transcribed regions.

Consequences of random vector integration

Despite the infrequency of vector integration, the consequences of random integration into a host cell genome must be considered, especially because chromosomal insertions, deletions, and translocations are characteristic of viral integration. Mice treated at the neonatal stage with rAAV2 have been reported to develop hepatocellular carcinoma at rates between four- and sevenfold higher than untreated mice.¹³⁵ An analysis of integration sites in the tumors found four unique sites into which the vector had integrated, all within a 6-kb region of chromosome 12, while adjacent normal tissue revealed no integration into these sites.

Gene targeting

Some genetic diseases are the result of a relatively small number of nucleotide changes, insertions, or deletions. In these cases, an approach involving the modification of a few nucleotides might be preferred to replacing or adding an entire gene. A number of *in vitro* experiments have achieved site-specific rAAV2 recombination in up to 1% of cultured cells, as compared to a total integration frequency of 10%.^{81,136–139} Initial experiments targeted selectable reporter genes that had been inserted throughout the genome in different clones, each with a mutation of a different type. In these studies, an rAAV vector genome was produced that was homologous to a portion of the gene of interest, differing only in the nucleotides to be modified, inserted, or deleted. Using this method, mutational insertions of up to 14 bp, deletions of up to 4 bp, and substitutions up to 2 bp were all amenable to successful correction.^{81,136,137,139} Deletions appear to be more efficiently corrected than insertion mutations.¹³⁸ In almost all instances of successful gene targeting, the sequence flanking the corrected mutations matched the expected functional sequences. Deletions and insertions common to

rAAV integration sites are not usually observed in these gene-targeting events. In addition to the corrections of ~15 bp, sequences of up to 1.6 kb have also been site-specifically inserted with success.^{139–141} The rate of targeting increases with longer homology to the targeted sequence, with increased vector doses, and at later timepoints after infection.^{81,138,142} In addition, the targeting frequency is higher when the corrective nucleotides of the vector are located nearer to the center of the rAAV sequence than when the corrective nucleotides are closer to the ends of the vector genome.¹³⁸

However, given that the overall frequency of random integration is ~10% of the total cell population, *ex vivo* gene targeting may be more appropriate than *in vivo* in many cases. The insertion of a disruptive sequence into a dominant-negative allele has been shown to be effective in cultured human mesenchymal stem cells taken from patients with osteogenesis imperfecta.^{140,141} The resulting corrected clones are effectively hemizygous because the vector targets each allele with equal frequency. Therefore *ex vivo* selection is important for choosing correctly targeted clones and for avoiding random integrations.

In addition to increasing the rate of random integration, the introduction of double-strand breaks increases the rate of site-specific integration events.^{143,144} As opposed to random integration, however, gene targeting is not enhanced by the addition of etoposide or hydroxyurea, suggesting a distinct mechanism of recombination.^{138,145} With the knock down of the molecules involved with homologous recombination, the frequency of rAAV2 gene targeting was found to decrease by approximately fivefold.⁴² These data, in conjunction with the extremely low rate of secondary mutations at targeted sites, strongly suggest that homologous recombination is the primary mechanism for targeted integration as distinct from random integration events.

The cell cycle has been found to determine the efficiency of gene targeting to a considerable extent.^{81,145} One study of gene targeting by rAAV revealed that every instance of gene targeting in a 97% stationary culture involved a cell that had undergone S-phase, as determined by BrdU analysis.¹⁴⁵ The likelihood of cells entering S-phase will therefore have a bearing on the types of cells that may be targeted *in vivo*. Intravenous delivery of a gene-targeting rAAV2 vector to neonate mice found that no gene targeting occurred in brain, lung, skeletal muscle, heart, or kidney.¹⁴⁶ Another experiment to study gene targeting using intramuscular delivery of rAAV2 in mice also failed to correct a mutant gene.⁸¹ However, gene targeting in the liver has been detected at low levels with rAAV2 and rAAV6.¹⁴⁶

CONCLUSIONS

To date, only some aspects of the transduction mechanisms of rAAV have been elucidated. Our present knowledge suggests that each sector of the transduction pathway depends, in part, upon cell type (including cell cycle), serotype, and vector dose. Therefore, in order to better tailor rAAV gene therapy treatments to specific diseases, transduction in each target tissue should be studied in a setting as close to its native physiology and morphology as possible. Given that the vast majority of transduction by rAAV is manifested by episomal concatamers of the vector genome, slowly dividing or postmitotic tissues will benefit most by *in vivo* rAAV gene delivery. However, even a low level of unintended or undesirable vector integration could result in adverse events. Unless random integration can be avoided, the situations under which rAAV is administered should be carefully considered.¹⁴⁷ Continued investigations into the mechanisms of rAAV transduction and genome integration are expected to enhance the specificity, efficiency, and safety of this tool for successful use in gene therapy.

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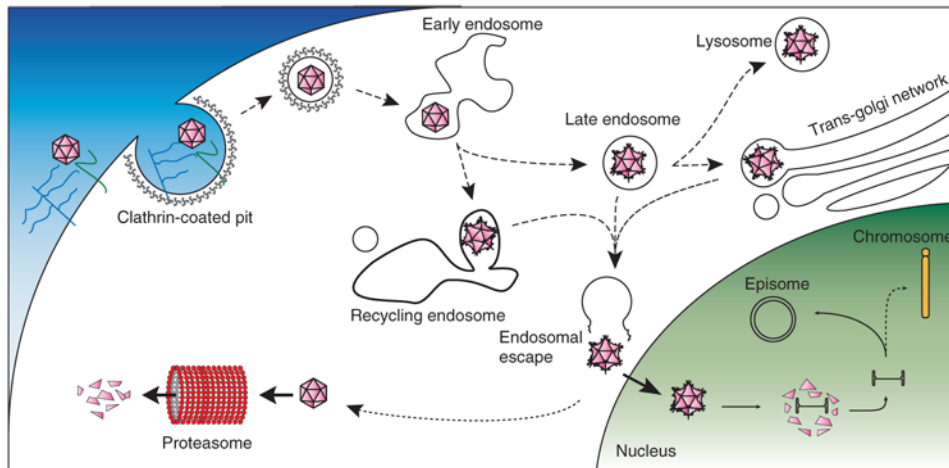


Figure 1. Cell entry and trafficking of recombinant adeno-associated virus (rAAV)

rAAV enters the cell through receptor-mediated endocytosis. Trafficking to the nucleus occurs through a number of possible pathways, some of which are represented here. The endosomal processing of virions results in externalization of nuclear-localization signals and a phospholipase domain on capsid proteins, allowing endosomal escape and nuclear targeting. Vector uncoating probably occurs in the nucleus, releasing the vector genome to form episomes or, rarely, to integrate into the host cell genome. As judged by the transduction enhancement caused by proteasome inhibitors, proteasomes are likely to be involved in vector degradation.

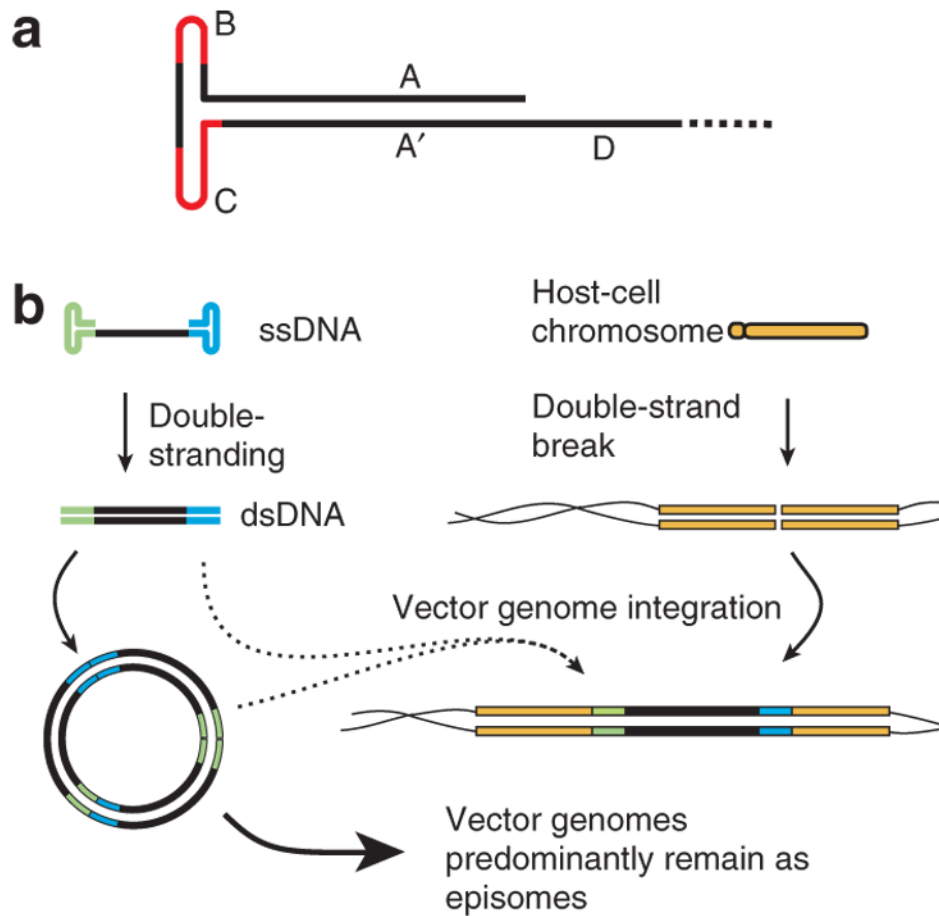


Figure 2. Fate of recombinant adeno-associated virus (rAAV) vector genomes

(a) Structure of an inverted terminal repeat (ITR) in the single-stranded AAV genome. Sequence elements of the ITR are labeled, and integration hotspots are red. (b) The single stranded rAAV genome becomes double-stranded through DNA synthesis and/or annealing. The double-stranded rAAV genome concatamerizes by head-to-tail, head-to-head, and tail-to-tail intermolecular ligation. The rAAV genome predominantly persists episomally. However, rare integration events do occur, most probably at chromosomal double-strand breaks. dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.