Gene Therapy: Recombinant Adeno-associated Virus Vectors

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Gene transfer using recombinant adeno-associated virus (rAAV) vectors shows great promise for human gene therapy. The broad host range, low level of immune response, and longevity of gene expression observed with these vectors in numerous disease paradigms has enabled the initiation of a number of clinical trials using this gene delivery system. This review presents an overview of the current developments in the field of AAV-mediated gene delivery. Such developments include the establishment of new production methods allowing the generation of high titer preparations, improved purification methods, the use of alternative AAV serotypes, and the generation of trans-splicing rAAV genomes. Together, these developments have improved results interpretation, host range, and the coding capacity of rAAV vectors. Furthermore, the recent identification of regions within the viral capsid that are amenable to modification has begun to address the issue of direct rAAV vector targeting, which could potentially allow targeted gene delivery to specific cell populations. The versatility shown by this vector has enabled new diseases to be realistically considered for therapeutic intervention and considerably broadened the scope of gene therapy.

Introduction

The human parvovirus, adeno-associated virus (AAV), has become extremely popular as a vector for the delivery of therapeutic genes in gene therapy paradigms. This small virus has many properties that have made it attractive as a delivery vehicle. Such properties include the potential for integration into the host genome [1], which may mediate long-term transgene expression, and because recombinant AAV (rAAV) vectors do not express any viral proteins, there is little immune cell mobilization or inflammatory effects observed towards rAAV-infected cells [2]. Furthermore, rAAV is not associated with any known human disease [3], has a broad host range, and is capable of infecting both mitotic and postmitotic cells [2,4••,5–7,8–15,16•,17•,18••–21••].

Here, we briefly review AAV biology, rAAV production methods, and previous gene transfer studies. Also, new developments in AAV technology are described, including the ability to increase vector-coding capacity, the use of alternative AAV serotypes, and the potential for direct rAAV vector targeting.

Adeno-associated Virus Structure and Replication

Adeno-associated virus has a single stranded DNA genome of 4680 bases [22] containing two open reading frames (ORFs) termed Rep and Cap, which are flanked by inverted terminal repeats (ITRs) of 145 bases (Fig. 1). There are six known serotypes of AAV, but the most extensively studied of these is AAV type 2 (AAV2); thus, all reference to genetic and structural characteristics will be based upon this serotype.

Adeno-associated virus is a "defective" member of the parvovirus family, meaning that AAV requires co-infection with a helper virus to replicate efficiently in host cells; such helper viruses include adenovirus (Ad) [23], herpes simplex virus 1 (HSV1) [24], and vaccinia virus [24]. There is evidence that certain stress-inducing factors, such as ultraviolet irradiation, hydroxyurea, heat shock, or several carcinogens [24–26], can also induce AAV replication, suggesting that cells possess the intrinsic capacity to support replication independent of helper virus function. The role of Ad in AAV replication is complex and has been shown to involve specific Ad genes including *E1A*, *E1B*, *E4 ORF6*, and *E2A* and *VA1 RNA*, []. AAV can be propagated as a lytic virus or maintained as a provirus integrated into the host genome. In the absence of helper virus, AAV enters a latent state in which the viral genome is integrated into the host genome at a specific locus on chromosome 19 []. The latent virus is stable for many cell cycles and can be rescued to enter the lytic phase upon subsequent helper virus infection.

The AAV virion is composed of three proteins: VP1, VP2, and VP3 [27]. The smallest capsid protein, VP3 (61 kd) is the most abundant within the virion, accounting for 90% of total viral protein. VP1 (87 kd) and VP2 (73 kd) are present at a 1:1 ratio and make up the remaining 10% of capsid protein. The capsid proteins are derived from the Cap region of the genome (Fig. 1). Three promoters have

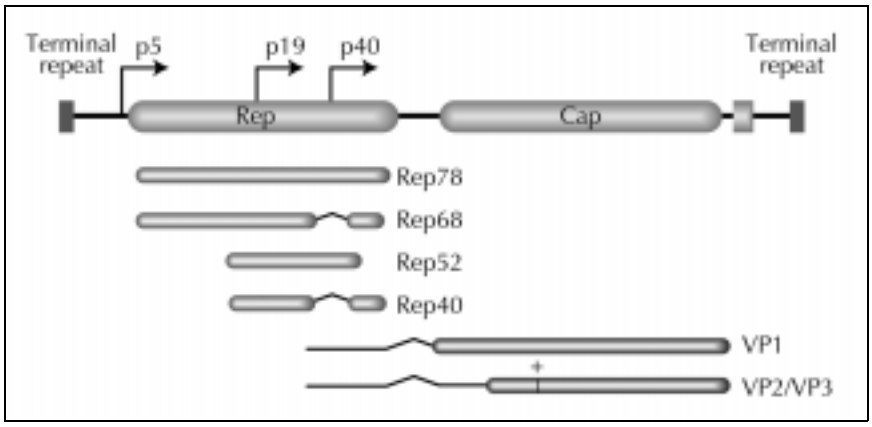

Figure 1. Schematic of adeno-associated virus (AAV) genome. **Top**: genetic arrangement of AAV genome, showing *Rep* and *Cap* genes, three promoters (*arrows*), terminal repeats (*black boxes*) and single polyadenylation signal (*grey box*). **Below**: AAV transcripts, the reading frames for each mRNA are shown. VP3 is synthesized from the same mRNA as VP2 using an ACG start codon (+).

been identified within the AAV2 genome; p5, p19 and p40 [27]; all Cap proteins are transcribed from the p40 promoter. Transcripts derived from these promoters share a common intron and poly adenylation signal.

There are at least four nonstructural proteins derived from the Rep ORF. These are designated according to their molecular weights; *Rep*78, *Rep*68, *Rep*52 and *Rep*40. *Rep*78 and *Rep*68 are transcribed from the p5 promoter, whereas *Rep*52 and *Rep*40 are transcribed from the p19 promoter. In vitro analysis of *Rep* function has revealed that *Rep*68/78 play multifactorial roles in AAV replication and integration including DNA binding [29], helicase activity [30], and site-specific and strand-specific endonuclease activity [30]. Moreover, *Rep*68/78 are essential for targeted integration of AAV DNA into the host genome through interaction of *Rep*68/78 with Rep-binding elements (RBEs) found within the ITRs and the chromosome 19 integration locus, designated AAVS1 [31]. As Rep proteins are essential for targeted integration into the AAVS1 locus, rAAV vectors that are devoid of *Rep* genes do not integrate site-specifically, but integrate randomly in a variety of cell types, including lung alveolar macrophages and postmitotic neurons [32].

Recombinant Adeno-associated Virus Production and Purification

The only viral genetic elements present within rAAV vectors are the ITRs. Furthermore, these 145 nt DNA sequences are the only *cis*-acting viral elements required for vector production. All other elements including, the *Rep/Cap* gene products, and helper virus proteins can be supplied *in trans*. Therefore, the present method for producing stocks of rAAV utilizes a three-component plasmid system [33•]: AAV plasmid vector containing the desired transgene flanked by AAV ITRs; AAV helper plasmid, which provides the necessary AAV capsid and replication proteins *in trans*; and Ad helper plasmid, which provides the necessary adenovirus proteins for efficient AAV genome replication and gene expression. By cotransfecting these three plasmids into human embryonic kidney (HEK) 293 cells, which supply the Ad *E1a* gene product, rescue, replication, and packaging of the transgene into AAV particles occurs (Fig. 2). The results of such a packaging scheme are exclusively, AAV particles carrying the recombinant DNA. Earlier production methods utilized adenovirus rather than Ad plasmid DNA. Thus early rAAV preparations contained significant amounts of contaminating Ad, which had to be physically removed or inactivated.

Although the triple transfection method of vector preparation is perhaps the most robust and versatile, it is unwieldy for large-scale vector production. Therefore, alternative strategies for rAAV production have been developed that include the use of hybrid vectors and packaging cell lines. In one instance a cell line stably transfected with the AAV *Rep/Cap* genes, is infected with two adenoviruses; one an *E1b* defective adenovirus, which induces *Rep* expression and supplies helper functions, and the second, a hybrid virus containing the rAAV vector genome cloned into the Ad *E1* region [34]. Alternatively, a recombinant replication-defective herpes simplex virus vector has been developed that carries both AAV *Rep* and *Cap* genes [35]. Infection of cell lines containing integrated rAAV genomes with this virus supplies both helper functions and wild-type AAV functions, obviating the need for plasmid transfection. Other advances in rAAV production have led to the development of helper cell lines that also obviate the need for DNA transfection. Hela cells have been engineered to contain both AAV *Rep* and *Cap* genes, and vector transgenes flanked by the AAV ITRs. Upon infection with helper virus (*eg*, Ad) these cells can produce greater than 10^4 viral particles/cell [36]. The identification of the primary AAV cellular attachment receptor has led to the establishment of ligand affinity matrix chromatography methods for rAAV purification [37]. These techniques obviate the need for laborious $CsCl_2$ gradients, which had previously been used to purify vector, and yield significantly more virus of greater purity than could be attained before.

Recombinant Adeno-associated Virus Infection

As with all viral vectors, AAV infection is reliant upon the expression of a specific cellular receptor that allows viral binding, attachment and entry into the target cell. In this regard, the cellular entry pathway for AAV2 has recently been characterized. Biochemical and genetic studies

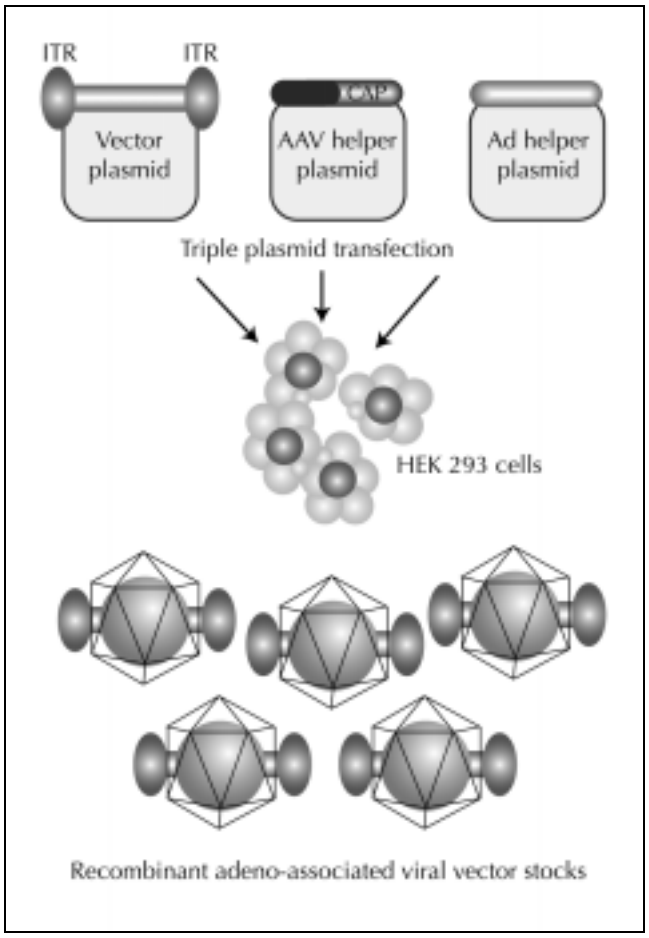

Figure 2. Recombinant AAV production. Transfection into human embryonic kidney (HEK) 293 cells using three plasmids containing the transgene of interest, *Rep* and *Cap* functions and essential adenoviral genes into HEK 293 cells. 103 particles/cell can be generated using this method. AAV—adeno-associated virus; Ad—adenovirus; ITR—inverted terminal repeat.

strongly support the involvement of the heparan sulfate proteoglycan (HSPG) as the primary attachment receptor for this virus [38]. Fibroblast growth factor receptor [39] and $\alpha_v\beta_5$ integrin [40], have also been implicated in either viral attachment or entry.

Recently, using fluorophore-conjugated AAV, the AAV cellular trafficking pathway has been elucidated [4••]. Similar to Ad, AAV enters cells via clathrin coated pits and becomes encapsulated into vesicles. Acidification of these endosomal vesicles allows the virus to escape and subsequently translocate to the nucleus [4••]. Interestingly, AAV accumulates at the nuclear membrane and although particles can be detected within the nucleus 2 hours after infection, the majority of virus remains outside the nucleus [4••]. This has suggested that nuclear transport may be rate-limiting for AAV infection of certain cell types. Indeed, a recent study looking at the infection of polarized airway epithelial cells suggests that one reason for the low AAV transduction efficiency in these cells is aberrant endosomal processing and nuclear trafficking of the viral particles [41]. Transduction of murine fibroblasts by rAAV2 based vectors may also be impeded by aberrant intracellular trafficking [42].

Before rAAV vectors can express their transgenes, the single-stranded vector genome must be converted into a double-stranded transcriptionally active form. Although adenovirus infection accelerates this conversion, second-strand synthesis can be a rate-limiting event for transgene expression from rAAV vectors [43]. As a result, maximal AAV-mediated gene expression is often delayed making rAAV vectors less advantageous for certain paradigms of gene transfer such as cancer. This delay, however, differs between cell and tissue types. For instance, in muscle, maximal gene expression is not observed until at least 1 month postinfection [44], whereas in neurons of the hippocampal region of the brain, maximal gene expression is observed within 1 week [45]. Moreover, in the brain, delay in maximal gene expression is dependent upon the brain region as well as the cell type being transduced. Infection of the striatal region of the brain with rAAV resulted in a delay of maximal gene expression of at least 6 weeks [46].

As stated previously, genomic conversion of AAV vector DNA and subsequent expression of the recombinant transgene is facilitated by adenovirus co-infection. It appears that this effect is mediated by the adenovirus *E4 ORF6* protein [41]. Further, there is evidence of an inhibitory cellular protein that binds to a single stranded region within the AAV ITRs ("D-sequence") and prevents viral second strand synthesis [47]. The activity of this protein, termed the single-stranded D-sequence binding protein (ssD-BP), is regulated by tyrosine phosphorylation. Inhibiting phosphorylation leads to a significant increase in rAAV transgene expression. Further studies have shown that the phosphorylation state of ssD-BP directly correlates with the ability of rAAV to transduce different cell types [48]. Recently, epidermal growth factor receptor (EGF-R) activation was shown to be coupled to the tyrosine phosphorylation of ssD-BP [49]. Such insights into the mechanisms of AAV-mediated gene transduction will ultimately lead to the increased effectiveness of these vectors for therapeutic gene delivery.

Immune Responses to Recombinant Adeno-associated Virus

It is clear that humoral and cell-mediated immune responses can limit the sustained expression from gene therapy vectors. Initial studies investigating the immune response against rAAV vectors in the muscle of mice showed only mild and transient inflammation [2]. However, neutralizing capsid antibodies were generated and could significantly reduce the efficacy of vector re-administration. Furthermore, secondary administration of rAAV into muscle resulted in a 25-fold increase in antibody titer [2]. Other studies have looked at cytotoxic T lymphocyte (CTL) and antigen-presenting cell (APC) responses to rAAV vectors. One study in mice showed that rAAV transduction did not lead to any CTL response or $CD4^+$ T-helper 1 activation, and

only a slight $CD4^+$ T-helper 2 IL-10 response was observed against the vector transgene [50]. Extensive investigation into rAAV directed muscle tissue transduction in mice has revealed that rAAV vectors avoid CTL responses by nonproductive transduction of APCs [51]. More recently, however, the cellular immune response to rAAV vectors has been shown to be dependent upon the route of vector administration. Intraperitoneal, intravenous, and subcutaneous administration of rAAV vectors expressing ovalbumin in mice has been shown to produce CTL responses against both transgene and vector, whereas intramuscular administration did not result in immune cell mobilization [52].

Latent infection with wt-AAV has been studied in rhesus macaques, a species that is a natural host for AAV. In this study three routes of administration were used; intranasal, intramuscular and intravenous. All routes, except intranasal administration resulted in humoral responses against AAV capsid proteins, evidenced by a four-fold increase in neutralizing antibody titer [5]. Further, only primates co-infected with wt-AAV and adenovirus developed CTL responses against AAV capsid proteins, contrary to previous studies performed in rodents.

The common immune response to rAAV vectors seems to be the initial humoral response against the viral capsid. The presence of neutralizing antibodies prevents re-administration of vector. Interestingly, one study has shown that over 80% of normal human subjects have anti-AAV antibodies and that 18% have neutralizing antibodies [6]. Because humans are natural hosts to AAV, the presence of neutralizing antibodies may impede efficient transduction during clinical trials when using AAV vectors. However, the use of alternative serotypes and selective modifications of AAV capsid proteins may overcome this obstacle.

Gene Transfer Using Recombinant Adeno-associated Virus

Recombinant AAV has been shown to infect a large variety of cell types both in vitro and in vivo. Skeletal muscle tissue has been shown to be highly receptive to rAAV infection and subsequent transgene expression. Initial studies expressing the marker gene, *LacZ*, showed that transgene expression could persist for up to 18 months in this tissue. In the same study rAAV vectors encoding erythropoietin (Epo) resulted in Epo secretion into the circulation, which persisted for 32 weeks, with a concomitant increase in erythrocytes for up to 40 weeks [42]. Such success has led to the expression of potentially therapeutic transgenes in skeletal muscle tissue, using rAAV vectors. Muscular dystrophy is one disorder with potential for treatment using rAAV vectors. Limb girdle muscular dystrophy (LGMD) is caused by mutations in the δ-sarcoglycan gene (SG). Expression of SG from rAAV in a hamster model for this disorder resulted in complete biochemical rescue [7]. More recently a single dose of rAAV vector expressing SG resulted in nearly complete recovery of physiological function with more than 97% recovery in muscle strength and substantial improvement of muscle histopathology [53].

Direct intramuscular injection of rAAV expressing human factor IX into mice resulted in 200 to 350 ng/mL of the factor IX in plasma, which persisted for up to 6 months [54]. Further, direct intramuscular injection of rAAV carrying the canine factor IX cDNA showed persistence of transgenes for up to 1 year in canines [55]. Subsequent clinical studies on patients with hemophilia B using rAAV vectors expressing human factor IX have shown promising results, and suggest that severe hemophilia B could potentially be converted to a milder form with rAAV therapy [56•].

Vascular endothelial and smooth muscle tissues have also proved receptive to rAAV. Initial studies using rAAV to direct expression of the marker gene *LacZ* demonstrated transducing frequencies of 90%, as measured using PCR in rat carotid arteries in vivo and showed no evidence of disrupted vessel architecture [57]. Further, primary cultures of rabbit, primate, and human smooth muscle cells have been shown to be efficiently transduced by rAAV vectors; in this same study intraluminal delivery of rAAV vector to carotid arteries of atherosclerotic cynomolgus monkeys resulted in efficient delivery of transgenes which could be enhanced by balloon injury [58]. Further studies in porcine myocardium in vivo showed that rAAV vectors are highly successful at transducing coronary vasculature with no apparent inflammation, resulting in transgene expression for up to 6 months [59]. Subsequent to these studies, antisense expression of angiotensin type I receptor (AT1-R), using rAAV in vivo has resulted in persistent reduction of AT1-R expression for up to 8 weeks. The reduction of the receptor was concomitant with a decrease in the angiotensin II-stimulated increase of intracellular calcium [60]. Such studies suggest that rAAV based gene transfer could prove useful in vascular disorders.

Cells of the central nervous system have also proven extremely receptive to rAAV based gene transfer. Neurons of the spinal cord have been shown to support gene expression from rAAV for up to 15 weeks [8]. Studies using AAV conjugated to a fluorphore demonstrated selective neuronal uptake in the hippocampus, with very little uptake in astrocytes [45]. Further investigation has revealed that the globus pallidal region of the brain is highly receptive to rAAV infection, and that certain regions of the brain may contain astrocytes that are amenable to rAAV infection [9].

Expression of potentially therapeutic transgenes from rAAV in the brain has led to the reversal of disease characteristics in numerous animal models. The expression of glial derived neurotrophic factor (GDNF) using rAAV injected into the substantia nigra resulted in the protection of neurons from the toxin 6-hydroxydodamine [10], suggesting rAAV based vectors may prove useful in the treatment of Parkinson's disease. More recently, infection with three rAAV vectors expressing tyrosine hydroxylase,

aromatic-L-amino acid decarboxylase, and GTP cyclohydrolase I, improved rotational behavior in a rodent model of Parkinson's disease. Such behavioral correction persisted for up to 12 months [11]. In a seizure model, antisense γ-aminobutyric acid A receptor expression from rAAV has been shown to increase seizure duration [12]. This study not only demonstrates the usefulness of rAAV vectors for antisense treatment, but also underlies the fact that rAAV infection does not detrimentally affect the intricate receptor interactions of neurons during synaptic signaling, an important point if disorders of the central nervous system are to be considered as targets for rAAV therapy.

Recent Developments

The success of rAAV as a gene vector system is due to the viruses' ability to readily infect cells expressing its native receptors. However, certain cell types, including lung epithelium are refractory for AAV infection even though they express the appropriate receptors. One reason for this low transduction efficiency is because of the polarity of these cells, resulting in the expression of HSPG mainly on the basal, nonexposed surface of these cells [13]. Other cell types, including astrocytes in the brain, are also not readily transduced by AAV2 [45]. Due to the refractive nature of some cell types to AAV2 infection, new vectors have been developed which are based upon different serotypes of AAV. AAV1, AAV3, AAV4, AAV5 and AAV6 have now been investigated for potential use as vectors for gene therapy [14,15,16•,17•,18••]. Studies using AAV4 showed that this serotype is capable of infecting a variety of cell types, and that the cellular receptor for AAV4 is distinct from that of AAV2 [14]. Also, AAV1 has been shown to be more efficient at muscle transduction than AAV2 [15]. Use of AAV5 has resulted in a 50-fold increase in airway epithelial transduction compared to AAV2 [16•]. In the brain both AAV4 and AAV5 have been shown to efficiently transduce AAV2 refractory cells including astrocytes [17•]. These studies have great importance to the development of AAV vectors for gene transfer, and subsequent therapy. The benefits of using alternative AAV serotypes include an increase in number of therapeutic targets, and the avoidance of neutralizing capsid antibodies on re-administration of rAAV vectors. Initial proof of principle of the benefits of sero-switching has been shown in lung epithelium, were transgene expression after AAV6 administration was not affected by previous AAV2 or AAV3 administration, suggesting cross-reacting capsid antibodies were not generated after the initial infection of vector [18••]. The ability of AAV serotypes to infect different cell types strongly suggests that the receptor targets for these serotypes are different from those already characterized for AAV2. It will be of interest to elucidate the cellular binding sites and subsequent intracellular trafficking mechanisms associated with other AAV serotypes.

Another method for expanding vector tropism is to engineer the AAV capsid to recognize alternative cellular receptors. This would not only allow rAAV to infect new cell types but it would also allow direct targeting to specific populations of cells, a concept that is essential for efficient and safe therapy. An early study inserted a 14 amino acid sequence containing an RGD motif, responsible for integrin binding into the AAV VP3 coding sequence. This resulted in the retargeting of the mutant rAAV vectors to the integrin receptor on receptor-bearing cell lines [19••]. More recently a comprehensive series of insertional mutations spanning the rAAV capsid was made. In this study 93 mutants were generated, at 59 different sites within the capsid gene. Most insertions gave rise to defective viruses, however, some were infectious. Two mutants containing serpin receptor binding peptide epitopes were shown to have altered tropism [20••]. Such knowledge of potential insertional sites within the AAV capsid proteins may eventually lead to the widespread retargeting of rAAV vectors to a plethora of cellular epitopes. Moreover, retargeting the viral particle may provide a way of improving processing by altering the entry pathway of the virus.

Further advances in rAAV technology have allowed the coding capacity of rAAV vectors to be dramatically increased. Initial concern about rAAV was that it could carry no more than 5 kb of genetic material. Allowing for promoter, transgene and untranslated messenger RNA sequences, many genetic constructs were in excess of rAAV capacity. Recent work has allowed the doubling of rAAV vector capacity up to 10 kb. During the process of AAV integration the viral genome undergoes concatemerization to form dimers. Heteroconcatemers of two distinct rAAV have been demonstrated in mouse muscle and liver in vivo. When two different rAAV vectors, one containing a promoter element, and the other containing a reporter gene construct without a promoter were co-administered to mice, gene expression was observed at 60% to 70% of that observed when both elements were placed in a single vector [21••]. Further studies have identified that this phenomenon is not isolated to separate promoter and gene vectors, but genes themselves can be separated and then rejoined by this concatemerization process [61]. However, recently a study showed that the AAV ITRs, which separate the expression constructs during the concatemerization process, possess internal enhancer activity [62]. In this study rAAV vectors containing the reporter gene GFP, without any promoter element, were able to express GFP in cells of the brain. The promoter activity was mapped to the viral ITRs. Because the AAV terminal repeats are an essential component of all rAAV vectors, this promoter activity may cause problems with split rAAV vectors. Furthermore, this activity may prevent tight transcriptional control when using cell-type–specific promoters or regulatory expression cassettes in the context of rAAV vectors. Nevertheless, the ability to increase vector capacity will provide an added advantage to rAAV vectors.

Conclusions

Adeno-associated virus vectors possess many qualities that make them attractive for a large portion of disorders being presently tackled by gene therapists. The ability of AAV to infect a large variety of cells allows this virus to be considered as a vector for many gene therapy paradigms. Further, the recent use of different AAV serotypes has increased the vector host range to encompass lung epithelium and astrocytes. More studies using different serotypes may identify further targets. Its integrative and non-immunogenic properties allow AAV to be considered for disorders requiring long-term genetic correction. Furthermore, rAAV can now be directly targeted to predetermined cell types using genetic capsid modifications, which will be crucial to a number of gene transfer strategies in vivo. Moreover, recent advances have potentially increased the coding capacity of rAAV vectors from 5 kb to 10 kb. These advances in vector technology cannot help but make a positive impact on the field of gene therapy. Now that rAAV can be grown to consistently high titers and purified efficiently using chromatographic methods clinical trials can be realized with the genuine hope that therapeutic benefits may follow in the near future.

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