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AAV-Mediated Gene Therapy for Research and Therapeutic Purposes

R. Jude Samulski[1] and Nicholas Muzyczka[2]

[1]Gene Therapy Center, University of North Carolina, Chapel Hill, North Carolina 27599; email: rjs@med.unc.edu

[2]Powell Gene Therapy Center, College of Medicine, University of Florida, Gainesville, Florida 32610; email: nmuzyczk@ufl.edu

Annu. Rev. Virol. 2014. 1:427–51

The *Annual Review of Virology* is online at virology.annualreviews.org

This article's doi:
10.1146/annurev-virology-031413-085355

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Keywords

viral vector, DNA transfer, parvovirus, gene therapy, transduction

Abstract

Adeno-associated virus (AAV) is a small, nonenveloped virus that was adapted 30 years ago for use as a gene transfer vehicle. It is capable of transducing a wide range of species and tissues in vivo with no evidence of toxicity, and it generates relatively mild innate and adaptive immune responses. We review the basic biology of AAV, the history of progress in AAV vector technology, and some of the clinical and research applications where AAV has shown success.

INTRODUCTION

The idea of using gene transfer to cure human disease emerged shortly after construction of the first restriction map of a viral genome, simian virus 40 (SV40). Many groups quickly realized that a mammalian virus such as SV40 could be used to study gene expression and to correct genetic defects. Several groups soon developed strategies for using SV40 as a DNA transfer vector (1, 2), but it became apparent that SV40 genomes persist in cell culture for only a limited period as episomes before they are diluted by cell division (3). This finding prompted a search for viral vectors that would provide long-term, persistent gene expression. Within a few years, proof of principle for retrovirus (4, 5), adenovirus (Ad) (6), adeno-associated virus (AAV) (7), and herpesvirus vectors (8) was published. These vectors have become standard research tools in the biological sciences, but the dream of curing human disease has proven to be more difficult than originally anticipated. This review focuses specifically on AAV vectors, which recently became the first vector system to be approved for clinical applications (9). We describe the key elements of AAV biology that affect vector production and cell tropism, describe recent advances in modifying AAV for specific purposes, and discuss the recent progress in clinical applications.

BIOLOGY OF AAV

Genome Structure

AAV is a small, nonenveloped virus that packages a single-stranded linear DNA genome, approximately 5 kb long (10, 11). A member of the family Parvoviridae, AAV was discovered in 1965 as a contaminant of Ad isolates (12). AAV has not been associated with any human or animal disease, even though most humans (>70%) are seropositive for one or more serotypes (13, 14). Both positive and negative DNA strands are packaged equally well, and infection can be initiated with particles containing either strand (15–17). The virus has a $T = 1$ icosahedral capsid, 25 nm in diameter, that is extraordinarily stable. It resists brief exposure to heat, acidic pH, and proteases. The viral genome consists of three open reading frames (orfs) that code for eight proteins expressed from three promoters (**Figure 1**) (11, 18). The mature capsid consists of the amino acid sequence of only one orf (*cap*) and the packaged DNA. Thus, recombinant AAV (rAAV) vectors present a very small target for the host immune system.

The coding regions of AAV are flanked by inverted terminal repeats (ITRs) that are 145 bases long and have a complex T-shaped structure (**Figure 2**). These repeats are the origins for DNA replication and serve as the primary packaging signal (19, 20). ITRs are the only *cis*-active sequences required for making rAAV vectors and the only AAV-encoded sequences present in AAV vectors (19, 21). Although the AAV ITRs have enhancer activity in the presence of Rep protein (22), they have minimal promoter or enhancer activity in the absence of Rep protein (23, 24). Thus, transgenes cloned into an AAV vector must be engineered with appropriate enhancer, promoter, poly(A), and splice signals to ensure correct gene expression.

DNA Replication

AAV cannot be propagated by itself. To establish a productive viral infection, AAV must be coinfected with a helper virus, and this provides a natural safety feature that helps prevent inappropriate spread of rAAV following clinical application (25). Ad is believed to be the natural helper virus in the wild because clinical isolates of Ad are frequently contaminated with AAV (12), but herpesvirus and baculovirus also can supply complete helper activity in cell culture (26, 27).

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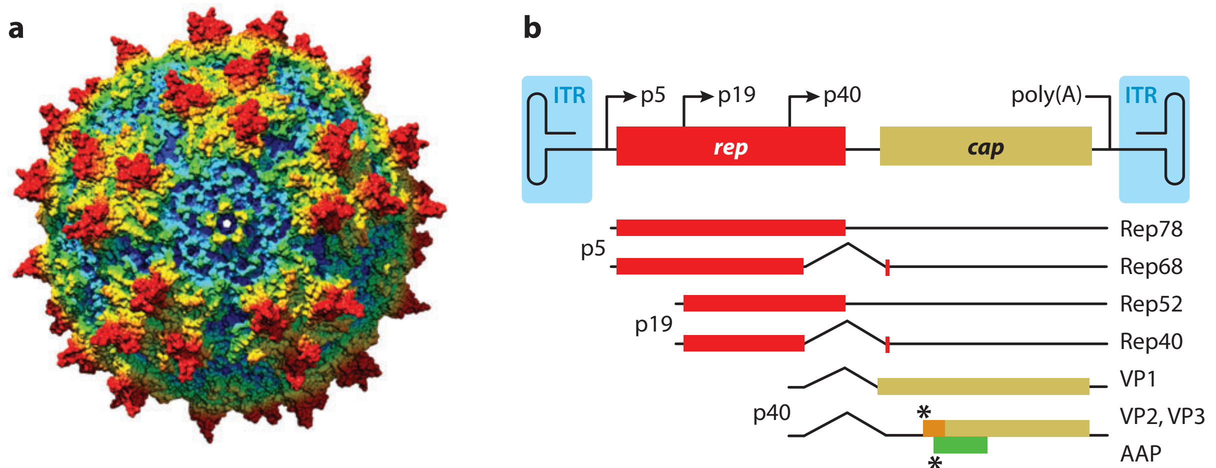

Figure 1

(*a*) The AAV capsid surface looking down the fivefold symmetry axis (note the fivefold pore in the center) is shown as a depth-cued space-filling model generated from the crystal structure of AAV2 (205) using Chimera. Surface amino acids are colored according to their relative distance from the center of the capsid, in the following order: blue (*closest*), cyan, green, yellow, red (*farthest*). (*b*) AAV genetic map. The ∼5-kb AAV genome contains three open reading frames (orfs) that code for functional proteins. The *rep* orf (*red*) codes for four Rep proteins (Rep78, Rep68, Rep52, and Rep40) that are synthesized from mRNAs initiated from the p5 and p19 promoters, each of which is either spliced or left intact. The two larger proteins (Rep78 and Rep68) have site-specific, single-strand endonuclease, DNA helicase, and ATPase activities that are required for AAV DNA replication (206, 207). The two smaller Rep proteins (Rep52 and Rep40) are required for packaging DNA into capsids (62) and retain only the helicase domain that is present in the larger Rep proteins (208). The p40 promoter initiates an mRNA that is alternatively spliced to make three capsid proteins from the *cap* orf (*yellow*). The two minor capsid proteins, VP2 and VP1, contain the same amino acid sequence that is present in VP3 but contain additional N-terminal sequences that are required for infection. The ratio of VP1, VP2, and VP3 in the capsid is approximately 1:1:10. The additional N-terminal sequences present in VP1 and VP2 contain nuclear localization signals and a phospholipase A2 activity (105, 110, 113, 209), both of which are required for infection. The spliced mRNA that codes for VP3 from a conventional AUG start codon (*yellow*) also codes for the minor VP2 protein, which has additional N-terminal residues (*orange*), from a weak upstream ACG start codon (*asterisk*) (210). In addition, the VP2/VP3 mRNA codes for an assembly-activating protein (AAP) (*green*) from a weak CTG start codon (*asterisk*) but in a different reading frame (18). AAP facilitates nuclear import of the major VP3 capsid protein and promotes assembly and maturation of the capsid, but AAP is not present in the mature capsid. Also shown are the 145-base (not to scale) T-shaped AAV inverted terminal repeats (ITRs) (*blue*).

AAV DNA is replicated by the so-called rolling hairpin mechanism (**Figure 2**), and replication has been completely reconstituted in vitro with purified components (10, 28). DNA replication requires the AAV-encoded Rep78 or Rep68; the cellular DNA polymerase δ complex and its accessory proteins, replication factor C (RFC) and proliferating cell nuclear antigen (PCNA); and minichromosome maintenance complex (MCM). MCM is the cellular DNA helicase used for unwinding chromosomes during cellular DNA replication. AAV DNA replication in crude cellular extracts also requires a single-stranded DNA–binding protein, either the cellular replication protein A (RPA) or a helper virus–encoded single-stranded DNA–binding protein (29, 30). The use of highly conserved cellular enzymes for AAV DNA replication may help explain the unusually broad host range of AAV. rAAV has been generated in human, bovine, and insect cells, and the vector has been shown to transduce a variety of preclinical animal models, including mouse, dog, pig, rabbit, horse and non-human primate.

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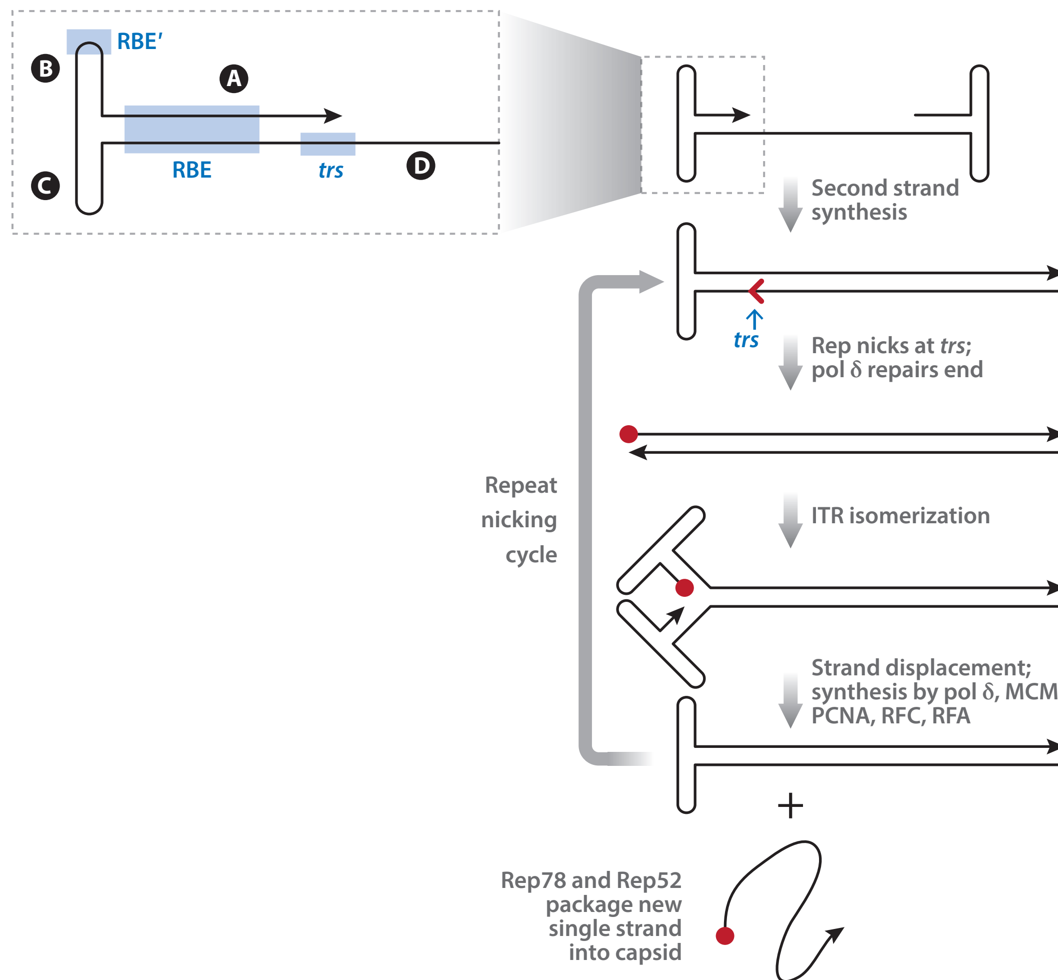

Figure 2

AAV DNA replication. The inverted terminal repeat (ITR) of AAV consists of two small palindromes (B and C) flanked by a larger palindrome (A) and an additional 20-base sequence (D) that is repeated at both ends (211). The ITR contains a 22-bp sequence [Rep-binding element (RBE)] that binds the AAV Rep78 and Rep68 proteins in a specific orientation (58, 212–215). If the ITR is in the palindromic (hairpinned) configuration, the Rep protein also contacts a 5-base sequence at the tip of one of the short palindromes (RBE′), which activates the Rep DNA helicase and strand-specific endonuclease activities (214, 216, 217). When AAV DNA is uncoated in the nucleus, the ITR of the incoming single-stranded genome snaps into a hairpin that provides a natural 3′-OH primer (*small arrow*) for the synthesis of the second strand. This produces a duplex molecule that has a covalently closed (hairpinned) end. The large Rep proteins then bind RBE and RBE′ within the hairpin, and the activated endonuclease cleaves one strand at a specific site within a 7-base recognition sequence called the terminal resolution site (*trs*) (*blue arrow*). This creates a new 3′-OH primer (*red arrowhead*) that is used to repair the ITR to form a normal blunt-ended duplex molecule. During cleavage, a molecule of Rep78 or Rep68 (*red circle*) is covalently attached to the 5′-end phosphate via a tyrosine-phosphate linkage. The ITR is then reconfigured into a double hairpin to produce a 3′-OH primer (*red arrowhead*) that directs strand displacement synthesis down the length of the genome using the cellular complexes pol δ, MCM, and their accessory proteins (28). This displaces a single strand, which is packaged, and reforms a duplex molecule that is covalently closed at one end, beginning a new cycle of nicking, repair, and strand displacement synthesis. Each time this cycle is repeated, a new single strand is generated for packaging. Because the two ends are identical, the process occurs equally well from both ends, generating both positive and negative strands for packaging. Abbreviations: MCM, minichromosome maintenance complex; PCNA, proliferating cell nuclear antigen; pol δ, polymerase δ; RFA, replication factor A; RFC, replication factor C.

Helper Virus Function

Expression of adenovirus DNA replication proteins [Ad DNA polymerase, Ad terminal protein, and Ad DNA-binding protein (DBP)] has little to no effect on AAV DNA replication (31–33), suggesting that in the case of Ad coinfection, AAV relies primarily on cellular replication proteins. Of the three Ad replication proteins, only the absence of DBP has an effect on the yield of AAV DNA (3-fold), and this may be due to its role in activating transcription from the AAV p5 promoter (31, 34). The Ad helper functions have been identified as Ad E1a, E1b, E4 orf6, DBP, and VA (viral associated) RNA. Both E1a and DBP act as transcriptional activators that induce the AAV p5 promoter (34–36). Rep78 in turn can activate the three AAV promoters in the presence of an Ad coinfection by as much as 450-fold (22, 37–40). E1a also induces S phase in host cells (41), which increases the level of the cellular DNA replication enzymes needed for DNA replication. Thus, coinfection with Ad and expression of E1a essentially act as a sensing switch to turn on AAV gene expression when the cellular environment is committed to DNA replication. In contrast, when Ad is not available, the p5 Rep proteins autorepress the p5 promoter (40), producing barely detectable levels of Rep protein and keeping the AAV genome silent in the latent state. The other Ad helper functions, E1b, E4 orf6, and VA RNA, perform various tasks that provide a window for AAV replication. These tasks include promoting second-strand synthesis of AAV; inhibiting p53-induced apoptosis; inhibiting the MRN complex, which would otherwise convert AAV genomes to concatemers; preventing entry into mitosis (thereby freezing cells in S phase); shutting off host cell translation; promoting AAV mRNA transport to the cytoplasm (42); and inhibiting the interferon-induced double-stranded RNA–activated protein kinase R (DAI/PKR) (41, 43–47).

With herpesvirus coinfection, the situation is different. Expression of both the herpes single-stranded DNA–binding protein, UL9, and the herpes helicase-primase complex, UL5/UL8/UL22, is required (48, 49). In addition, expression of the herpes DNA polymerase complex, UL30/UL42, stimulates but is not essential for AAV replication (50, 51). Thus, with herpes, the virus-encoded replication proteins appear to supply the primary single-stranded DNA–binding protein and may supply some helicase activity. Alternatively, their primary role may be to recruit AAV DNA to replication centers in the context of herpes-infected nuclei. In addition, three herpes regulatory proteins (ICP0, ICP4, ICP22) supply essential helper functions (49, 50), and these appear to be required for AAV *rep* gene expression. Finally, baculovirus has also been shown to provide complete helper function for AAV propagation, but the viral elements involved are not known.

Packaging

Newly synthesized AAV DNA is packaged into preassembled empty particles (52). The icosahedral AAV particle contains an 8.5-Å-diameter pore at the fivefold symmetry axis (**Figure 1**) that is believed to be the portal for DNA entry because mutations in amino acids at this pore affect packaging to various extents (53). Empty capsids also can bind to Rep protein complexes and Rep complexes can bind to each other (54–58). Thus, the packaging signal for AAV DNA may be the covalently attached large Rep protein present at the 5′ end of newly synthesized AAV DNA (**Figure 2**). This protein would serve to tag newly synthesized AAV DNA for packaging and form a bridge between the newly synthesized DNA molecule and the capsid. In support of this idea, Salvetti and colleagues (59–61) have shown that AAV genomes that have no ITRs also can be replicated and packaged by using the Rep-binding element (RBE) present in the p5 promoter. The p5 RBE is normally used for p5 promoter repression during viral latency and for activation of AAV transcription during productive infection. However, in the absence of the ITRs, the p5 RBE and

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a nearby cryptic *trs* site can support limited AAV DNA replication and packaging. This aberrant packaging of ITR-negative genomes appears to be due to the covalent linkage of Rep at the cryptic p5 *trs*; when the p5 RBE or the cryptic *trs* is deleted, both replication and packaging of the ITR negative genomes are abolished. Taken together, these findings suggest that the covalently bound Rep protein serves as the packaging signal. Finally, the small Rep proteins, Rep52 and Rep40, use their helicase activity as a motor for loading DNA into the capsid (62).

The First AAV Vectors

When work on the design of AAV vectors began, no genetic system was available. Because AAV could be grown only in the presence of a helper virus, there was no standard AAV plaque assay that could be used for isolating AAV viral clones. The problem was solved when Samulski et al. (63) discovered that a plasmid clone of wild-type AAV was infectious when transfected into human cells that had been infected with Ad. This discovery allowed investigators to delete AAV sequences, substitute them with foreign DNA, and grow rAAV stocks by complementing the rAAV genomes with plasmids that expressed the *rep* and *cap* genes (64, 65). Hermonat & Muzyczka (7) were the first to show long-term expression of a marker gene under the control of a foreign enhancer or promoter delivered via rAAV infection in cell culture. That same year, Tratschin et al. (66) showed that gene transfer was possible with AAV by demonstrating transient expression of a marker gene. These early vectors still retained *rep* function, but subsequently McLaughlin et al. (19) demonstrated that essentially all the AAV internal coding regions could be deleted and only the 145-bp terminal repeats were needed to ensure replication and packaging of the rAAV genome. *rep* and *cap* functions were supplied in *trans* by a plasmid deleted for the ITRs to prevent packaging of wild-type AAV. This was confirmed by Samulski et al. (21), and vectors containing only the AAV ITRs became the standard approach for AAV-mediated gene transfer that is used today.

PRODUCTION METHODS

Wild-type AAV normally generates more than 10^5 DNA-containing particles per cell, often called DNase-resistant particles (DRPs). However, the early DNA transfection systems produced only 100–1,000 DRPs per cell. This limited amount was presumably due to the inherent inefficiency of plasmid transfection and to the suboptimal complementation of *rep* and *cap* functions. In addition, helper virus functions were supplied by coinfection with wild-type Ad, thus producing mixed stocks of rAAV and wild-type Ad (19, 21). To remove Ad, early workers used CsCl density centrifugation to separate rAAV from Ad and differential sensitivity to heat to inactivate the contaminating Ad. Neither method was completely effective, and so early rAAV stocks frequently displayed toxicity due to the contaminating Ad components.

Plasmid Transfection Method

The first major breakthrough in AAV vector technology came when three groups independently cloned the Ad helper functions on a separate plasmid and eliminated the Ad replication and capsid genes, thereby eliminating Ad in the rAAV stocks. Two groups cloned the necessary Ad genes on a separate plasmid and propagated rAAV by a triple-plasmid transfection (**Figure 3**), in which each plasmid contained the rAAV genome, the *rep/cap* sequences, or the Ad helper genes (67, 68). By eliminating sequences that could be used for homologous recombination, these groups further reduced, but did not eliminate, wild-type-like, replication-competent AAV contamination. Grimm et al. (69) devised a double-plasmid transfection protocol in which one plasmid contained

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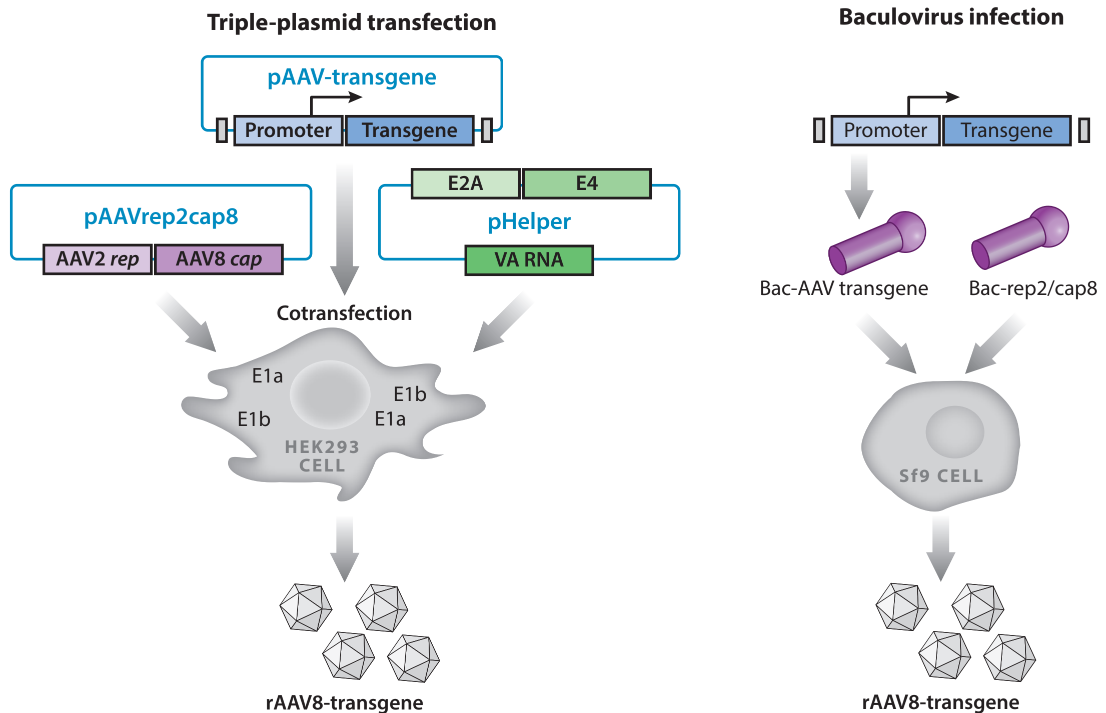

Figure 3

rAAV production methods. In the triple-plasmid method, HEK293 cells expressing adenovirus E1a and E1b are cotransfected with an adenovirus helper plasmid (pHelper), a *rep/cap* plasmid expressing AAV2 *rep* and AAV8 *cap* (pAAVrep2cap8), and the transgene plasmid carrying the rAAV-transgene cassette (pAAV-transgene). In the baculovirus approach, the rAAV-transgene cassette is built into a baculovirus, which is then used to infect Sf9 insect cells that are coinfected with a second baculovirus expressing *rep2* and *cap8* under control of baculovirus promoters. Both the baculovirus and plasmid transfection methods produce rAAV8 expressing the transgene, but the baculovirus method (and similar herpesvirus methods) typically produces 100 times more virus per cell and is more easily scaled to large volumes of cells.

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the rAAV sequences and the other contained the *rep/cap* and Ad genes. This group also replaced the p5 Rep promoter with an alternative mouse mammary tumor virus (MMTV) LTR promoter and eliminated the low-level replication and packaging of the ITR negative *rep/cap* sequences mentioned above.

Scalable Production Methods

The work of these three groups (67–69) produced the so-called double- and triple-plasmid transfection methods commonly used today for research-grade rAAV. However, they still relied on DNA transfection; thus, these methods were not suitable for the high-volume, scalable production necessary for clinical applications. Clark et al. (70) produced the first scalable rAAV production method by cloning a HeLa cell line that contained both the *rep/cap* genes and the rAAV genome integrated into the host's chromosomes. This producer cell line was stable and could be infected with wild-type Ad to generate mixed stocks of Ad and rAAV. In principle, the cell line could be expanded and grown in high-capacity fermenters. Many variations of the stable cell line coupled with helper virus infection or DNA transfection have been developed and reviewed (71–76).

The most promising scalable approaches currently in use involve the infection of cell lines with two (or three) viruses containing the *rep* (and/or *cap*) genes and the rAAV genome, respectively. The first approach uses a strain of herpes simplex 1 virus defective for an essential herpes gene (e.g., *ICP27*) that reduces the growth of herpes but is not essential for rAAV production (72, 77); the second approach uses baculovirus as the carrier (**Figure 3**) (76). These viruses are then used to coinfect a suitable cell line (e.g., BHK for herpesvirus or Sf9 for baculovirus). Many variants of both the herpesvirus and the baculovirus methods have been published (27, 51, 72, 76, 78–80). Both methods are scalable and generate wild-type levels of virus, $>10^5$ DRP per cell. Crude rAAV stocks are typically $>10^{11}$/mL and after purification can be concentrated to 10^{14}–10^{15} DRP/mL. However, some serotypes, notably AAV2, appear to aggregate at concentrations above 10^{13} DRP/mL.

Virus Purification

Because rAAV is a relatively simple, nonenveloped, and highly stable protein complex, all the standard high-throughput protein purification methods can be used for rAAV purification. Tangential flow filtration and standard ion exchange chromatography, as well as a variety of affinity chromatography approaches, have been used (51, 72, 76, 78–80). These include heparin and glycan columns that take advantage of the known interaction of different AAV serotypes with cell surface proteoglycans (81–83), and monoclonal antibody columns specific for one or more serotype (69, 84). rAAV preparations are a mixture of both empty and full capsids, and thus far only density gradient centrifugation (either CsCl or iodixanol) has been effective for removing the empty capsids from virus preparations. The ratio of empty capsids to full capsids varies with the production method used, ranging from higher than 20:1 (54, 85) to as low as 0.05:1 (86). Empty capsids presumably add to the antigenic signal when injected in vivo, which may become an important consideration when choosing the purification method for clinical applications. On the other hand, a recent study suggests that empties can be used as decoys to soak up circulating neutralizing antibodies and improve transduction (87).

For clinical applications, the virus stock is typically tested for adventitious agents and titered to establish the number of DRPs per milliliter and the number of infectious units per milliliter (iu/mL) (75, 81, 82, 88). The DRP/iu ratio is usually referred to as the particle-to-infectivity ratio and can vary over a wide range (2:1 to 10^5:1) depending on the serotype and tissue culture cell line used to measure infectivity. The particle-to-infectivity ratio is a useful measure of vector stock potency during virus production, but it has little predictive value for the potency of the virus stock in vivo, where the target tissue may have different virus receptors or different receptor density.

PERSISTENT GENE EXPRESSION IN VIVO

The second major breakthrough in AAV vector technology was made by several groups in 1996. Flotte and colleagues (89) used rAAV to transfer the cystic fibrosis transmembrane receptor (CFTR) gene to airway epithelia of primates and demonstrated persistent expression for 6 months. Similarly, injection of rAAV into mouse brain resulted in continuous expression of the transgene (90, 91). This suggested that expression could be long-lived, but the levels of expression were difficult to determine over time. However, Byrne and colleagues (92) and Samulski and colleagues (93) convincingly demonstrated that mice injected in vivo with rAAV expressing the erythropoietin gene and the β-galactosidase gene, respectively, produced persistent levels of gene expression that did not change over time. Such long-lasting levels of expression had never before been seen. Until

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then, most virus- and DNA-based vectors lost expression within a week to a few months after administration in vivo. Explanations for the loss of gene expression include immune clearance in the case of Ad vectors and epigenetic silencing in the case of retroviruses. In contrast, the Samulski group demonstrated that β-galactosidase was expressed more than a year and a half after intramuscular injection, and the Byrne group measured essentially constant levels of erythropoietin expression for six months after systemic infection. The following year, other groups demonstrated similar long-term, persistent gene expression after rAAV injection into the eye, brain, spinal cord, muscle, and liver (94–99). Data such as these suggest the exciting possibility that genetic diseases might be corrected with a single application of rAAV that would last a lifetime.

Cell Entry and Trafficking

AAV enters cells by binding to cell surface sugars present on proteoglycans (such as sialic acid, galactose, or heparan sulfate) and to cell surface receptors (e.g., fibroblast growth factor receptor or integrin), which have been reviewed (100, 101). Cell surface binding then triggers endosomal uptake through clathrin-coated vesicles and the CLIC/GEEC (clathrin-independent carriers/GPI-enriched) endocytic compartment pathway (100, 102–104). Following entry, AAV is found in virtually every cytoplasmic compartment of the cell. Within the first 2 h postinfection, most of the virus accumulates in a perinuclear location and undergoes a structural change when exposed to the acidic pH of the endosomal compartment (105, 106). Many groups have shown that exposure to acidic pH is absolutely essential for AAV infection, although its mechanism is still not clear (107–109). Among other things, exposure to acidic pH is necessary for inducing the extrusion of the N-terminal end of the minor capsid protein VP1 (VP1u) to the surface of the capsid (105). VP1u displays phospholipase A2 activity, which is thought to be involved in rupturing the endosome to allow the AAV capsid to enter the cytoplasm (110) or in promoting entry of the capsid into the nucleus (111). VP1u also contains nuclear localization signals as well as other ligands that presumably promote accumulation of virus at the nuclear membrane (105, 112, 113). Despite these uncertainties, it seems clear that the AAV capsid eventually emerges in the cytoplasm (105, 112) and enters the nucleus intact (105), where it uncoats its DNA. This is followed by second-strand DNA synthesis to form a duplex genome, which is capable of transcription and gene expression.

Second-Strand Synthesis

The synthesis of the second strand appears to be a major rate-limiting step for gene expression in vivo. It has been argued that duplex rAAV molecules are formed by annealing of positive and negative strands following in vivo infection (114). However, several groups have shown that virus particles containing only one rAAV strand are as infectious in vivo and in vitro as standard virus stocks that contain both strands (15–17). Many groups have shown that in vivo rAAV gene expression builds up over days to months after infection before it reaches a plateau (115). Evidence that the slow increase in gene expression is due to second-strand synthesis comes from studies by McCarty and colleagues (116, 117), who invented a method for packaging double-stranded (self-complementary) AAV DNA. This group discovered that if they inactivated one of the AAV ITRs so that it could no longer be processed by the Rep protein, the genome would replicate as an inverted dimer, which would self-anneal after viral uncoating. When these duplex genomes were used to infect animals or cell culture, expression of the transgene was immediate. Unfortunately, although self-complementary vectors were more efficient for transduction in vivo than single-stranded vectors were, they reduced the cloning capacity of AAV by half.

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Genomes Persist as Episomes

Studies of AAV and rAAV integration in cell culture led to the assertion, often made in the early literature, that wild-type AAV and rAAV achieved latency by integrating into host chromosomes. However, there is actually little support for this in vivo. In cell culture, wild-type AAV has a preference for integration into human chromosome 19 when Rep is expressed (118, 119). However, genomic studies of rAAV in vivo have shown that rAAV DNA molecules in muscle, heart, liver, brain, and lung are converted to circles and then persist predominantly as episomes containing multiple copies of the transgene cassette, usually in a head-to-tail configuration (120–127). The highest frequency of integration appears to occur in the liver, where approximately 1% of the cells integrated a copy of the rAAV genome. This was convincingly shown in mice that had undergone a partial hepatectomy. Upon hepatectomy, the affected mice lost 90% of the rAAV-directed gene expression, presumably due to the loss of rAAV episomes during hepatocyte cell division, but retained expression in hepatocyte clones that were the progeny of cells that integrated a stable copy of the rAAV genome (120). Low-level integration has also been demonstrated in skeletal and cardiac muscle (128) and brain (121). Taken together, these data suggest that rAAV is suitable for modifying the many nondividing somatic tissues of the body, such as eye, brain, and muscle, and less suitable for gene transfer to tissues that normally undergo cell division, such as hematopoietic stem cells. The fact that integration is a relatively rare event during AAV transduction in vivo reduces the chance of insertional mutagenesis and provides an additional margin of safety for AAV-mediated gene therapy.

Because rAAV exists primarily as concatemers formed by end-to-end joining, several groups have tested the possibility of circumventing the size restriction for rAAV vectors by placing the front and back halves of a transgene cassette into separate vectors. The two halves then recombine in vivo via homologous recombination or undergo *trans* splicing to reconstitute the full-length gene. This approach has been successfully demonstrated using animal models (129–131), but it is not yet clear whether the method is sufficiently robust for therapeutic purposes.

SEROTYPE REVOLUTION AND DESIGNER rAAV

The third major breakthrough in rAAV vector technology was the isolation and testing of new serotypes. For many years only five AAV serotypes were available, and most investigators used AAV2, the most widely studied serotype, for clinical applications. However, in 1998 Rutledge et al. (132) identified AAV6, which differs from AAV1 by only 14 amino acids but, nevertheless, had different in vivo properties. Gao et al. (101, 133) subsequently searched human and nonhuman primate tissues for new serotypes and identified over 100 new capsid variants. With one serotype isolated by Gao et al., AAV8, transduction in murine liver was 10- to 100-fold higher than with AAV2. Currently, 13 serotypes are widely available for packaging rAAV cassettes. Fortunately, the same cassette that was built with AAV2 ITRs can be packaged into any serotype capsid by merely exchanging the capsid-coding region in the helper plasmid or helper virus. This allows investigators to quickly test a variety of serotypes in preclinical animal models to determine which serotype is most efficient in their application. Often the best serotype in rodent models does not translate to humans, but investigators can test the same transgene cassette/serotype combination in mice and later in larger animal models to ensure that the optimum vector is used in human trials. The in vivo tissue tropisms of AAV1–13 have been reviewed elsewhere (100, 134, 135).

Many AAV serotypes have been crystallized, and their atomic structures have been determined (134). Capsid amino acid sequences are highly conserved in regions necessary for making contacts between monomer capsid proteins at the two-, three- and fivefold interfaces. The variations in

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capsid tissue tropism and neutralizing antibody binding are the result of changes in the so-called variable loop regions exposed on the capsid surface (**Figure 3**). The difference in tissue tropism can be huge (101, 135). Differences in tissue tropism often reflect differences in ligands on the capsid surface that bind different cell-specific receptors. However, tropism is a complex mixture that reflects receptor affinity, cell entry, trafficking efficiency, DNA uncoating, and, more recently, postnuclear gene expression (136).

To take advantage of the diversity of capsid architecture, three general approaches have been used to create new capsid variants. Using the rational design approach, Zhong et al. (137) modified surface tyrosines to phenylalanines after discovering that inhibiting tyrosine phosphorylation during capsid entry increased transduction. One of the new variants, which contained three Y-to-F substitutions, displayed significantly higher transduction frequency in some tissues (138). In another example of rational design, Bowles et al. (139) compared the surface amino acids of AAV1 and AAV2 to identify residues that might account for the increased muscle tropism of AAV1. They used a loop-swapping approach to design a capsid, AAV2.5, that contained five amino acid changes that gave AAV2 the superior muscle transduction seen with AAV1. This strategy has been used to identify a number of improved hybrid viruses and is reviewed elsewhere (135).

In a second approach, many groups have shown that short (8–30 amino acid) ligands can be inserted into a surface loop, amino acids 585–588, of AAV2 (140, 141). The insertions disrupt a heparan sulfate–binding motif (142, 143), thus detargeting AAV2, and decorate the capsid with 60 copies of a new ligand targeted to a specific cell type. These changes can significantly increase transduction (typically 10- to 100-fold) of the target organ. This approach has been expanded with the development of peptide display libraries, in which random peptide sequences are inserted into AAV2 and selected for viruses that target specific tissues (144, 145). In a similar approach, Warrington et al. (146) have shown that much longer ligands (up to 30 kDa) can be attached to the N-terminal end of VP2 without significantly affecting virus assembly or infectivity. This raises the interesting possibility of tagging AAV with single-chain monoclonal antibodies specific for a cell surface receptor.

Finally, in a third approach, several groups have constructed capsid libraries that consist of randomized capsid sequences in a single serotype or random recombinants of several different serotypes with complexities greater than 10^6 different capsid sequences per library (147–151). In a process called directed evolution (151), these libraries are then screened either in cell culture or in vivo to select capsids enriched for infecting a particular tissue or cell type (**Figure 4**) or capsids that no longer bind a neutralizing antibody. Often, several log improvement of infectivity in the target cell can be achieved with only a few amino acid changes.

In addition to capsid modifications that provide altered tissue tropism, many laboratories have shown that tissue-specific promoters retain their specificity in the context of AAV vectors and can be used to create a second layer of tissue-specific control of gene expression. For example, rod- and cone-specific promoters have been used to target photoreceptor cells in the eye (94, 152). An alternative approach has been the incorporation of microRNA targets into the transgene cassette to prevent AAV-directed gene expression in tissues where it might be harmful (153–157). In addition, several groups have successfully developed inducible promoter systems that in principle allow fine-tuning of gene expression (158–163).

CLINICAL TRIALS

Three general approaches are being used to treat diseases with rAAV. The first approach uses an organ as a depot to secrete a protein that is normally secreted into serum. Both muscle and liver have been used to secrete proteins such as alpha-1-antitrypsin (AAT) or factor IX following

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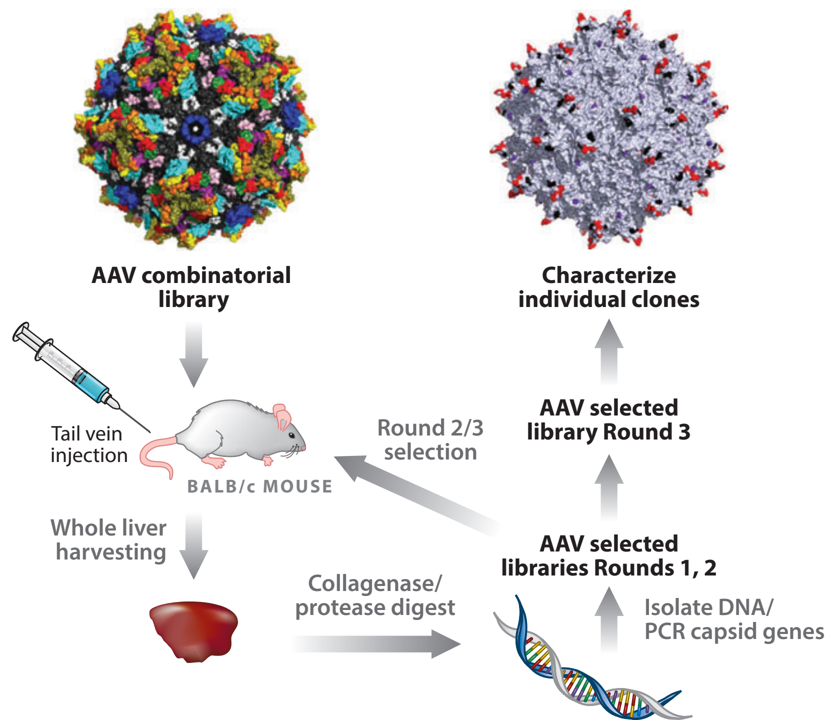

Figure 4

Directed evolution of novel AAV tropism. (*Left*) The AAV2 capsid contains nine variable surface loops (*various colors*) and conserved regions (*black*). Libraries of AAV, in which each library member contains randomly generated mutations in one or more variable regions, are injected into rodent or nonhuman primate models and the target organ is collected. (*Right*) Variants that successfully infect the target organs are PCR amplified to produce an enriched library, and the process is repeated to identify individual variants that are more efficient for transducing the target organ due to a handful of surface residue changes (*colored on gray background*).

intramuscular injection or intravenous injection (164, 165), which results primarily in liver transduction. In a second approach, systemic (intravenous) injection is used to treat diseases that affect all cells, notably lysosome storage diseases (166). In these cases, some of the recombinant protein is secreted and then endocytosed by distant cells, thus cross-correcting noninfected cells. Alternatively, the treated cells become a sink for removing metabolites that become toxic as they accumulate (167). A major challenge for systemic delivery has been identifying vectors that are capable of crossing the blood-brain barrier. The third approach is surgical injection into a specific, diseased organ. For example, many eye diseases are treated with either subretinal injection, which places virus in contact with the photoreceptor and retinal pigmented epithelial (rpe) layers of the eye, or intravitreal injection, which primarily affects retinal neurons (168). Cardiac diseases have been treated by injection into the heart (169). Similarly, some neurodegenerative diseases, such as Parkinson disease, that primarily affect a particular region of the brain (striatum) can in principle be treated by injection into the target region using stereotactic surgery (98, 170). In contrast, diseases that affect the whole brain, such as Alzheimer disease, are less amenable to sterotactic surgery. Injecting virus into brain parenchyma is similar to injecting virus into a bowl of gelatin, where the level of vector dispersion is dependent on the volume and rate of injection and the

affinity of the particular rAAV serotype for cell receptors at the site of injection. This awareness has led to the use of serotypes that show wider dispersion (e.g., AAV9) in brain (171, 172). In addition, for diseases in which the whole brain (Alzheimer disease) or the entire musculature (muscular dystrophy) must be corrected, physical methods to enhance virus dispersion have also been tested successfully. These methods include convection-enhanced delivery and mannitol in the brain (173) and isolated limb infusion in muscle (174).

Flotte and colleagues (175, 176) were the first to use rAAV in a clinical trial to correct cystic fibrosis, a genetic disease. The cystic fibrosis gene codes for a chloride ion channel whose loss leads to chronic lung infection, emphysema, and reduced life span. Relying on the low-level promoter activity of the AAV ITR, they inserted a promoterless CFTR gene into rAAV and applied the vector to nasal or lung airway epithelial cells. These trials demonstrated the safety of the vector and showed that transduction varied depending on the target tissue. Although CFTR expression could be demonstrated, the efficiency of the AAV2 vector used was too low to show clear therapeutic efficacy.

The first clinical success came when several groups investigated the use of rAAV for homozygous recessive *rpe65* deficiency. *rpe65* codes for a protein that regenerates 11-*cis* retinal in the retinal pigmented epithelial cell layer of the eye, and without it the patient is functionally blind in low light. In three independent phase 1 clinical trials, the rAAV-*rpe65* vector was injected subretinally into one eye of each patient (177–179). Significant recovery of vision was seen in some patients, and studies of gene expression in the portion of the eye that was treated showed virtually 100% correction in the photoreceptor cells that remained (180). The generally positive results with *rpe65* suggest that virtually any recessive loss-of-function genetic defect in the eye should be capable of correction. Studies are now underway for a variety of other genetic eye diseases, as well as diseases such as macular degeneration.

Several trials have also been conducted for factor IX deficiency (hemophilia B). This serum protein is an essential component of the blood clotting cascade, without which patients experience increased episodes of bleeding in response to mild trauma or spontaneous hemorrhage in joints and muscle. Earlier trials using rAAV2 in muscle or portal vein injections showed factor IX expression in some patients, which rapidly declined (181). The loss of expression was associated with a cytopathic T cell (CTL) response to AAV capsid protein but not the transgene. More recently, Nathwani et al. (165) switched to rAAV8 and used intravenous injection to deliver a codon-optimized, self-complementary factor IX cassette. In a phase 1 clinical trial, they showed dose-dependent and stable expression of therapeutic levels of factor IX in serum at middle and high vector doses. Expression was stable over 6 months of follow-up, and several patients no longer found it necessary to infuse factor IX protein. As in earlier hemophilia B trials, some patients appeared to mount an inflammatory response, as judged by increased levels of serum alanine aminotransferase. These patients recovered a normal enzyme profile after a short course of an immune modulator, prednisolone, and retained therapeutic levels of factor IX after immunosuppression was stopped.

Similar trials have been conducted for AAT deficiency (164). AAT is a protease inhibitor secreted by the liver that inhibits neutraphil elastase, a protease active in the lung. Without AAT, patients experience emphysema or chronic obstructive pulmonary disease. Injection of rAAV1-AAT into muscle produced wild-type AAT that was dose dependent and persisted for over a year with no loss of expression, but the serum concentration of AAT did not reach therapeutic levels. As in the hemophilia B trial, a CTL response to the capsid but not the transgene was detected; however, the CTL response did not affect AAT expression. The investigators suggested that CD4 regulatory T cells had been induced.

Several groups have developed potential therapies for neurodegenerative diseases such as Parkinson and Alzheimer that have shown limited success. In the case of Parkinson disease, a

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relatively small region of the brain, the substantia nigra pars compacta, releases dopamine to the striatum and controls a variety of brain functions. Progressive loss of nigral neurons leads to Parkinson symptoms. Various genes prevent or slow neurodegeneration or upregulate dopamine production. Because the striatum can be saturated with vector by stereotactic brain injection, various groups have tried to deliver these genes with rAAV vectors. Bartus et al. (170) have used rAAV2 to deliver neurturin, a neurotrophic factor, to striatal tissue in the hope of preventing neurodegeneration and increasing dopamine neuron synapses. They saw some evidence for improvement, but it was clear that most of their patients might have had too few nigral neurons to see a significant effect; ultimately, they did not reach their primary therapeutic end points. Christine et al. (182) overexpressed the final enzyme in the dopamine synthetic pathway, aromatic amino acid decarboxylase. They also saw some improvement clinically and clearly showed continuous expression of the gene over time. Encouraging results also have come from a study in which rAAV was used to treat Alzheimer disease by engineering expression of nerve growth factor in the hippocampus (183).

Perhaps the most difficult target diseases have been the muscular dystrophies and lysosomal storage diseases, which affect all cells in the body. To treat these diseases, vectors would have to be disseminated widely throughout the body and be able to cross the blood-brain barrier to treat brain and eye tissue. Many investigators have obtained proof of principle in preclinical animal models and in phase 1 trials (139, 184, 185), but the problem of disseminating rAAV systemically to all organs has not been solved. The hope is that a newer set of rAAV vectors that provide better control of viral tropism will solve these problems.

One group has succeeded in winning regulatory approval for an rAAV-based gene therapy designed to treat lipoprotein lipase deficiency. The lipase is normally present on a variety of cell surfaces, including muscle, and is involved in the metabolism of fat particles carried in blood. The European Commission approved in 2012 the use of uniQure's drug Glybera, an rAAV1-based vector for intramuscular injection (9). This is the first viral vector that has achieved regulatory approval in the West. For a complete list of rAAV clinical trials that have been completed or are ongoing, we refer the reader to **http://www.genetherapynet.com/clinical-trials.html**.

TOXICITY

To date, there has been no association between toxicity and AAV in clinical trials. However, two potential sources of toxicity have emerged that must be monitored in human trials. The first is the immune system. AAV elicits a mild, innate immune response due to the activation of Toll-like receptor 9 (TLR9) when rAAV infects antigen-presenting cells (186–188). TLR9 monitors unmethylated CpG residues in nucleic acids and activates the classical and alternative NF-κB pathways, which leads to the expression of proinflammatory cytokines and interferon response genes. This expression in turn generates a CTL response and a robust neutralizing antibody titer. The CTL response in an early factor IX clinical trial appeared to be against AAV capsid, either because the capsid is long lived in vivo or because contaminants of vector preparations included mispackaged capsid genes and empty capsids (59, 189); however, CTL responses to transgenes have also been documented (188). The widespread presence of memory B cells in response to some AAV serotypes is also believed to be responsible for the absence of expression following rAAV2–factor IX injection into some patients. Using rodent models, many groups have shown that little expression is seen following the second injection of the same rAAV vector when the vector is injected into peripheral organs. In contrast, repeat injection into the eye and brain, which are partially immunoprivileged, is often successful (190, 191). Several approaches are used

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to minimize immune responses to AAV-mediated gene transfer. The simplest is to treat patients transiently with immunosuppressive drugs to avoid a CTL response. This appears to have been successful in the human factor IX trial (165). The use of AAV serotypes that are less prevalent in the human population is also being investigated. In addition, efforts are underway to map neutralizing antibody epitopes on the serotypes that are currently being tested (192). This information is then used to eliminate these epitopes by mutagenesis. Alternatively, virus libraries are used to isolate escape variants that have lost epitopes for neutralizing antibodies (147).

The second potential source of toxicity is insertional mutagenesis and tumor induction. Sands and colleagues (193, 194) reported in a knockout mouse model that liver transduction with single-stranded rAAV expressing β-glucuronidase increased the chance of liver tumors. Examination of the tumors did not show a high correlation between integrated rAAV and tumors. In addition, other studies did not show an increased incidence of liver tumors after application of AAV vectors to liver in B6C3F1 mice, a mouse strain known to be liver-tumor prone (195, 196). Similarly, Wu et al. (197) showed that overexpression of *Tcf12*, a gene with unknown function, produced glioblastoma-like proliferation at the site of injection in rat brain, whereas other neuronal genes and a null vector did not. These examples suggest that overexpression of a gene in a limited number of cells may lead to tumor formation even if rAAV genomes do not integrate. Finally, McCarty and colleagues (198) examined tumor formation by self-complementary AAV vectors in a mouse liver model prone to hepatocyte tumor formation, C3H/Hej. They found that the frequency of tumors was elevated in mouse liver injected with either rAAV-GFP or a null vector containing only the enhancer or promoter. Taken together, this limited data set suggests that insertional mutagenesis may preclude targeting of some tissues, such as liver, and that each gene cassette may have to be monitored separately for potential oncogenic effects.

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AAV AS A RESEARCH TOOL

In addition to its success in treating human genetic diseases, rAAV has become an important research tool, and its impact is best seen in neurobiology. The first clear example came from injection of rAAV expressing α-synuclein into substantia nigra, the region of the brain that degenerates in Parkinson disease (199). Overexpression of α-synuclein in rat substantia nigra caused a progressive neurodegeneration that mimicked the course of Parkinson disease in humans and essentially produced a new animal model that could be used for identifying promising therapeutics. This approach can be used to create nonhuman primate and rodent models (200). The use of AAV to create transgenic animals also has been useful for functional genomic studies. Several groups have overexpressed or downregulated genes in the hippocampus or substantia nigra to determine their effect on learning and memory or neurodegeneration, respectively (201–203). Perhaps the most exciting development has been the recent pairing of AAV with channel rhodopsin expression in local brain regions (204). This pairing allows optical stimulation of specific neuronal populations (optogenetics), enabling researchers to study synaptic plasticity and connectivity.

CONCLUSION

It has taken 30 years to develop AAV vectors to a stage where it appears they might fulfill their promise. Scalable methods for virus production and purification are now available. The kinds of diseases amenable to intervention with vector technology are becoming clear. The hope remains the same: Gene therapy will provide a novel set of therapeutic reagents that will allow medicine to treat many diseases that were previously intractable.

DISCLOSURE STATEMENT

Both N.M. and R.J.S. hold patents related to AAV vector technology and are founders of companies that are developing commercial applications for AAV gene therapy.

ACKNOWLEDGMENTS

The authors thank Mavis Agbandje-McKenna, Yu-shan Tseng, and Sergei Zolotukhin for help in preparing the figures. This work was supported by National Institutes of Health grants (HL59412, NS69574, GM109524) and the Edward R. Koger endowment fund.

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