Gene Therapy

Adeno-associated Virus Vector

A majority of viral vectors currently used for gene therapy have evolved in nature as causative agents for various human illnesses, ranging from the common cold to cancer and AIDS. Interestingly, the wild-type adeno-associated virus (AAV), an alternative being investigated as a potential vector for gene therapy, is not associated with any disease in humans or animals.

As the name implies, AAV was initially identified in association with adenovirus, and in fact, the virus was thought to be a fragment of adenovirus. Work in subsequent years characterized the nature of AAV, as well as its genomic organization and life cycle.

Certain remarkable features of this virus, such as its non-pathogenicity, low immunogenicity, and ability for long-term transgene expression, have made it an attractive alternative for human gene therapy. This article reviews the biology and possible gene therapy applications of AAV vectors.

The AAV vectors are classified in the group Parvovirus (*parvus*, small). Although many serotypes have been identified, most of our understanding comes from the serotype 2 vector (AAV-2).

AAV is nonpathogenic, but it is capable of infecting both dividing and nondividing cells. Because AAV cannot replicate without a helper virus, it is also known as a dependovirus. In the absence of a helper virus, AAV-2 frequently integrates into a specific site of the human genome (19q13-qter).

The wild-type AAV genome is a single-stranded DNA molecule, consisting of two genes: *rep*, which codes for proteins that control viral replication, structural gene expression, and integration into the host genome, and *cap*, which codes for capsid structural proteins that encapsulate the viral DNA. These two genes produce four rep proteins and three capsid proteins.

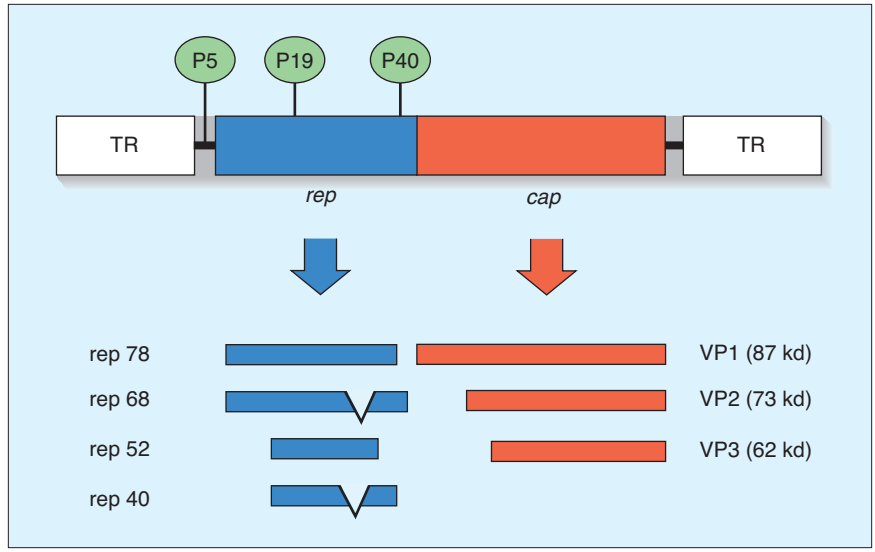
Three promoters at map units 5, 19, and 40 have been identified, of which promoters 5 and 19 transcribe rep proteins while promoter 40 transcribes cap proteins. Different-sized rep and cap proteins arise by alternate splicing, and overlapping coding regions in these proteins share the same amino acid sequences. At either end of the genome is a 145-basepair terminal repeat, vital for the rescue, replication, packaging, and integration of the viral genome.

In the last few years, other AAV serotypes, from 1 to 8, have been identified. Interestingly, there is significant variation in the capsid sequence among the serotypes.

Although not associated with any disease states, AAV-2 has the the ability to infect many human and nonhuman cells. It was previously believed that AAV infection occurs by nonspecific endocytosis, but the identification of cells resistant to AAV infection has led to the elucidation of molecular pathways of AAV entry into cells.

AAV-2 vectors use heparin sulfate proteoglycan as the primary receptor and fibroblast growth factor receptor type 1 and αVβ5 integrin as co-receptors. AAV 4 and 5 serotypes have been reported to use sialic acid for cellular binding.

On entry into cells, AAV is rapidly transported via endosomal traf-



Genomic organization of AAV-2. The transcripts from promoter P5 give rise to Rep78 and Rep68 by alternate splicing, whereas those from P19 give rise to Rep52 and Rep40 by similar mechanism. The three capsid proteins VP-1, VP-2, and VP-3 are synthesized using transcripts from the promoter P40. The terminal repeat (TR) sequences are necessary for viral replication, rescue, packaging, and integration.

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HUMAN DISEASES TREATED SUCCESSFULLY IN VIVO USING rAAV-BASED GENE THERAPY

Disorder	Target Tissue	Species
α_1-Antitrypsin deficiency	Muscle	Mouse
	Liver	Mouse
Anemia	Muscle	Monkey
	Muscle	Mouse
Alzheimer's disease	Brain	Rat
Cancer	Human cells (xenografts)	Rodents
	Tumor vasculature	Mouse
Cystic fibrosis	Lung	Human
	Lung	Monkey
	Lung	Rabbit
Fanconi's anemia	CD34+ cells	Human
Hemoglobinopathies	Erythroid progenitors	Human
	Erythroid progenitors	Mouse
Hemophilia B	Muscle	Dog
	Liver	Dog/mouse
Lactose intolerance	Gut	Rat
Lysosomal storage disease	Liver/heart	Mouse
Obesity/diabetes	Muscle	Mouse
Parkinson's disease	Brain	Monkey
	Brain	Rodents
Retinal degeneration	Retina	Mouse
Retinitis pigmentosa	Retina	Rat
Seizure	Brain	Rat

ficking to the cell nucleus, where the vector genome is released. Conversion of the single-stranded vector genome occurs, resulting in transcriptionally active double-stranded intermediates. This step is achieved either by second-strand synthesis of the single-stranded vector genome or by annealing of complementary sequences from vector particles containing positive or negative strands.

Several factors help determine the efficacy of the second-strand synthesis, including involvement of host cell polymerases and other growth factors. The double-stranded vector genome remains as an episome or integrates into host chromosomes as concatamers.

Cloning of the AAV-2 genome into a plasmid vector has facilitated a wide range of molecular manipulations that have elucidated several key events in AAV biology. When used as a vector, the *rep* and *cap* genes are replaced by a transgene and its associated regulatory sequences, including a promoter and polyadenylation signal. The total length of the recombinant genome cannot greatly exceed 4.7 kilobases, the length of the wild-type genome.

Production of the recombinant vector requires that rep and cap proteins are provided in *trans*, along with helper virus gene products such as adenovirus E1a, E1b, E2a, E4, and VA proteins.

Initial methods of recombinant AAV production involved cotransfection of an AAV helper plasmid (pAAV/Ad) along with an rAAV plasmid containing heterologous genes, flanked by AAV TRs, in 293, HeLa, or KB cells. These cells were subsequently infected with wild-type adenovirus. About 48 to 72 hours after the transfection-infection, the cells were lysed, yielding extracts containing rAAV that were heat inactivated at 56 °C to destroy contaminating adenovirus. DNase I digestion was used to remove unencapsulated and transfected plasmid DNA.

Further modifications in rAAV production and purification steps have helped to enhance utilization of rAAV in human clinical trials. Improvements have involved the generation of packaging cell lines, cloning of helper plasmids containing necessary adenoviral genes to eliminate any wild-type adenovirus in AAV preparations, gradient ultracentrifugation methods that allow isolation of rAAV fractions based on buoyant density, purification using affinity high-performance liquid chromatography, and large-scale culture using roller bottles and bioreactors. These refinements have resulted in high-titer rAAV yields of up to 10^{14} particles/ mL necessary for in vivo studies including human clinical trials.

The last decade has seen a steady increase in the application of rAAV vectors in preclinical and clinical gene therapy studies. Advantageous features of AAV, including genomic integration and

long-term transgene expression, prompted the use of AAV-based vector systems initially for the phenotypic correction of genetic or metabolic diseases.

However, increased understanding of the roles of key cellular factors and proteins involved in both genetic and nongenetic defects has led to AAV-based strategies for nongenetic diseases as well, including those caused by infectious and carcinogenic agents.

Such approaches, which involve sustained expression through gene transfer, seek to replace, augment, or block the expression of proteins toward a therapeutic goal. Examples include treatments to increase host immunity against infectious agents and tumors or to target tumor vasculature to control tumor growth and metastasis.

Several preclinical animal studies have shown remarkable efficacy of rAAV-mediated gene therapy in various genetic and nongenetic diseases. Although only a few human clinical trials have been initiated with rAAV vectors, the results observed in these studies are encouraging, particularly as related to the nontoxic and nonpathogenic nature of the therapy.

Despite the advantages of AAV vectors, certain characteristics, including the size of the transgene, time-lag before optimal transgene expression, infection efficiency, host immune response, and random integration, pose limitations for gene therapy using rAAV vectors. From recent studies, it is apparent that whereas some limitations can be overcome, a few remain as challenges.

The genomic content of rAAV is limited to approximately 4.7 kb, and hence, larger cDNA could not be packaged efficiently within AAV capsids. Recently, different groups have shown that heterodimerization of the AAV genome, containing splice signals, leads to efficient concatamerization and expression of genes larger than 5 kb after transduction.

Because AAV is a single-stranded DNA virus, efficient conversion of the single-stranded genome to the transcriptionally active double-stranded structure is a rate-limiting step. Strategies to package rAAV containing a double-stranded DNA genome significantly reduced the time required for transgene expression, although by this strategy, only half the wild-type genomic length could be packaged. With the heterodimerization strategy, it should be possible to overcome the packaging limitations of double-stranded DNA.

Variability in the infection rates of cell types and the abundance of AAV receptors in many cells or tissues are also concerns for targeted transduction of the vector. Targeted transduction using genetic and conjugate-based approaches indicate that it is possible to enrich tissue-specific transduction of AAV by using alternate cellular receptors for viral entry.

Furthermore, receptor-binding sites on the AAV-2 capsid have been identified which, upon genetic mutation, abolish binding of the vector to native receptors. However, these mutations do not compromise the packaging efficiency. Thus, development of AAV vectors using these mutant capsids should allow cell-specific transduction by genetic incorporation of target cell-specific ligands into these domains.

It is also known that almost 70% of the human population is seropositive for the AAV-2 vector, the most widely used serotype, which may limit its administration due to preexisting immunity. Advances using the principles of molecular genetics and immunology have overcome these issues, increasing the potential application of this vector system.

Studies in a primate model show that transient immunosuppression of the host permits readministration of rAAV. Also, the use of different AAV serotypes, which have high transduction efficiency yet differ in capsid sequence and immunogenic epitopes, may permit repeat administration.

Finally, in the absence of rep proteins, the rAAV vector integrates at random sites in human chromosomes. Although not reported in any preclinical rAAV gene therapy studies, in theory this possibility may pose concerns if random integration of the vector disrupts cellular genes affecting either normal cell function or initiating oncogenic events.

Despite the fact that AAV rep proteins mediate chromosome 19-specific integration, constitutive expression of rep is highly toxic to cells, and hence, co-delivery of a functional *rep* gene with a transgene is not preferred. Future strategies to provide rep protein or gene that are only transiently expressed under tightly regulated processes may overcome this deficiency.

Although gene therapy is presently encountering criticism, this powerful technology promises to overcome these limitations before becoming a conventional treatment modality for a variety of human diseases. With the remarkable safety profile and preclinical efficacy of rAAV vectors, it is becoming increasingly evident that AAV-based vectors will set a precedent in this field and prove to be a useful alternative for future molecular medicine.

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