

Adeno-Associated Viral Vectors

Subcloning of mammalian genes into prokaryotic plasmids or bacteriophages and genetic manipulation of retroviruses have emerged in the late 1970s as precursor tools to introduce foreign genes into human cells for the goal of human gene therapy. The principle of a gene transfer protocol is based on the administration of candidate coding sequences in a target rather than the final therapeutic gene products that generally exhibit short pharmacological half-lives. There has been since an outstanding identification of genes associated with diverse human pathologies and an expansion of the number of gene vectors available to express these disease markers for experimental and therapeutic purposes, allowing to develop a broad range of animal models of human disorders and to establish the first human clinical trials.

Consequently, gene transfer approaches have found many applications at preclinical and clinical levels, in particular for the study and treatment of tumors, cardiovascular diseases and angiogenesis, infectious and other acquired diseases (AIDS, viral hepatitis), genetic disorders (cystic fibrosis, beta-thalassemia, adenosine deaminase deficiency and severe combined immunodeficiency, mucopolysaccharidosis disorders, type-I glycogen storage diseases, X-linked chronic granulomatous disease), and various degenerative, chronic, inflammatory, and age-related diseases (Alzheimer's, Parkinson's, and Huntington's diseases, multiple and amyotrophic lateral sclerosis, arthritides). The strategies the most commonly adopted have employed gene replacement therapies or gene addition (growth and transcription factors, cytokines

and antagonists), the transfer of inhibitory nucleic acids to reduce undesired expression of endogenous or foreign genes, and the application of enhancers of metabolic pathways or of immune responses.

Functionality of a gene transfer approach necessitates first an evaluation in culture systems (*in vitro*) prior to translation in the recipient, by indirect transplantation of *ex vivo* genetically modified cells or by direct administration of the candidate treatment *in vivo* using a gene carrier. Although direct gene transfer approaches might be more advantageous as they do not necessitate to manipulate cells before reimplanting them in the patient, indirect strategies might allow for extensive testing of the modified cells before re-administration. Critical to the success of such approaches is the identification of a vector suitable for both efficient and stable expression of candidate genes within their targets, showing a rapid onset and without having detrimental effects (toxicity or immunogenicity). Several gene delivery systems currently employed are close to achieving these aims, providing transgenic expression in many cell types and tissues. They include methods based on the use of nonviral vectors (injection of naked DNA, gene gun, electroporation, ultrasound-facilitated and hydrodynamic gene transfer, cationic lipid and polymer delivery) and on vehicles derived from viruses, the principal of which being adenoviruses, retroviruses, and herpes simplex viruses. Although nonviral vectors are generally among the safest gene transfer methods available to date, they have several disadvantages as they mediate short-term

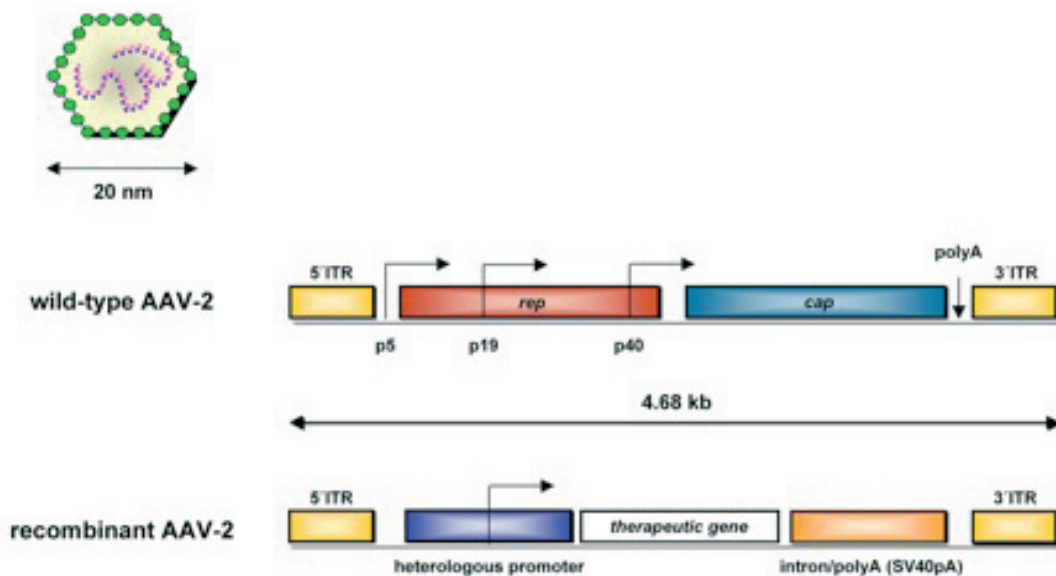


figure 1 AAV genomes

transgene expression and achieve rather low gene transfer efficiencies (1). In this regard, viruses may allow to generate gene carriers with increased potency, as they utilize natural entry pathways in human cells. However, the classical viral vectors listed above have many shortcomings, like the risk of insertional mutagenesis carried by integrating retroviruses, the strong immunogenicity of adenoviruses, and the cytotoxicity inherent to herpes simplex viruses.

Vectors based on a new class of virus, the adeno-associated virus (AAV), have emerged as favored gene vehicles. AAV is non-pathogenic, replication-defective small human parvovirus (25 nm in diameter) with a 4.7-kb single-stranded DNA (ssDNA) genome (2). Most recombinant AAV (rAAV) generated to date have been derived from the serotype 2 of the virus (AAV-2) (3), although other AAV have been cloned and partially characterized (twelve serotypes reported to date). In generating rAAV, both the viral rep (Rep40, Rep52, Rep68, and Rep78 proteins

for replication) and cap (VP1, VP2, and VP3 proteins for encapsidation) open reading frames can be deleted and only the two regulatory inverted terminal repeats (ITRs) are maintained (4) (Figure 1).

Permissivity to rAAV has been demonstrated in a broad range of cell types and tissues (muscle cells, hepatocytes, neurons, astrocytes, oligodendrocytes, retina, lymphocytes, macrophages, monocytes, vessel endothelial cells, keratinocytes, synovio-cytes, osteoblasts) (5-9) and in progenitor cells (10,11). These cells express on their surface the viral receptor, a ubiquitous cell membrane-associated heparan sulfate proteoglycan (HSPG) (12): Additional interactions with human fibroblast growth factor receptor 1 (13), integrins $\alpha 5/\alpha 5\beta 1$ (14), hepatocyte growth factor receptor (15), and platelet-derived growth factor receptor (16) have been also reported, depending on the cell type examined. Following receptor binding, rAAV particles are endocytosed into the cell and transported to the nucleus where

they are uncoated, with conversion of the genome to double-stranded DNA (dsDNA) intermediates (circular and linear) capable of expressing the transgenes (17). As some tissues can be refractory to transduction by naturally occurring vectors and knowing that intracellular trafficking of AAV depends on the concentrations of surface HSPG, the presence of co-receptors, and the biology of the viral serotype employed (17), large efforts have been made to bypass these rate-limiting steps in order to improve the efficacy of the vectors and to adapt their specificity to some targets by modifying the viral genome, specifically the genes coding for the capsid proteins. In this goal, and due to the recent availability of X-ray crystallography data of the structural AAV proteins (18), new rAAV have been artificially created by site-directed and insertional mutagenesis, peptide display libraries, chemical conjugation, and by generation of AAV libraries using DNA shuffling and error-prone PCR (19-21). Such manipulations have led to the production of mosaic and pseudotyped vectors (22-25) and to chimeric and hybrid constructs (adenovirus/AAV, herpes simplex virus/AAV) (26-28). Also, some tissues and cells difficult to transduce have been successfully targeted with the identification of new AAV serotypes that display new and preferential tropism. Most remarkably, the rate-limiting step of recombinant genome conversion of ssDNA into dsDNA has been circumvented through the use of self-complementary vectors (scAAV) which package an inverted repeat genome that can fold into dsDNA without the requirement for DNA synthesis

or base-pairing between multiple vector genomes (29). Another key issue for the use of rAAV as a gene transfer vehicle has long been the limited packaging capacity of this class of vector (about 5 kb). This problem has been partly solved by taking advantage of the ability of the virus to form head-to-tail (circular) DNA concatamers by intermolecular recombinations (30). Several groups have demonstrated the efficacy of simultaneously administering two vectors, each carrying one half of a gene, leading to concatamerization and expression of the entire gene by trans-splicing (31-33). Alternatively, the packaging capacity of rAAV has been expanded by incorporation of AAV in larger viruses (26,27) or by performing multiple transductions with separate vectors (34).

Notably, transgene expression via rAAV can be achieved both in proliferating and quiescent cells (35), in contrast to agents such as retroviruses that necessitate cell division. In human cells, the wild-type AAV genome integrates specifically within a short region of chromosome 19 (19p13-qter) named AAVS1 (36,37). Locus-specific integration requires only the Rep proteins and the ITRs (38). However, rAAV vectors deleted for the viral coding sequences integrate slowly and nonspecifically (39). rAAV-delivered transgenes thus persist in the targets as a mixture of stable episomes and genuine integrants, the relative proportions of which vary depending upon the cell type. In general, 99% of rAAV are maintained as episomal copies (40), indicating a very low risk of insertional mutagenesis compared with retroviral vectors. Remarkably, the episomal forms of

rAAV transgenes are also actively transcribed (41). Transgene expression via rAAV has been demonstrated over extended periods of time like in the mouse skeletal muscle (up to 1.5 years) (42) compared with adenoviral vectors that generally mediate only short-term expression. Yet, as the effects of rAAV delivery have been noted in some tissues only for one month (43), some groups have worked to overcome this problem by taking advantage of the property of the virus to integrate site-specifically in the human genome (44).

Besides, rAAV vectors are also more advantageous than those derived from adenoviruses or herpes simplex viruses, as they comparatively exhibit a diminished immunogenicity due to the complete removal of viral coding sequences in the recombinant genome. Indeed, administration of rAAV vectors does not significantly activate innate host immune responses (complement, cytokines, chemokines, monocytes and macrophages, natural killer cells, dendritic cells, granulocytes, polymorphonuclear leukocytes). Adaptive immune responses (humoral and cellular) have been demonstrated against the viral capsid and some transgene sequences (45), but the type, magnitude, and duration of which largely depend upon the vector dose, the route of administration, and the type and quantity of transgene produced. Pre-existing immunity to wild-type AAV in humans (96% of the population) is predominantly humoral, but with large variability between age groups and not all anti-AAV antibodies are neutralizing (about 32%). Only a minority of subjects demonstrate marginal lymphocyte proliferation in response to AAV proteins. Regarding the transgene itself,

certain combinations of vector/target do result in the stimulation of immune responses to the transgene product, in particular against those that are secreted. As a matter of fact, this property has been used for vaccination purposes (46,47). Strategies to overcome the immunity to AAV and to the transgenes are nonetheless under investigation by lowering the amounts of particles applied, by employing optimal routes of injection (intravenously, subcutaneously, or intramuscularly), by using alternative serotypes, and by modifying the capsid proteins or the promoter controlling transgene expression (low activity, regulatory, tissue- or disease-specific promoters). Novel, helper-free rAAV production methods have been also developed (48,49) to replace those based on the use of helper adenoviruses that provide in trans AAV rep and cap, carrying the risk of contamination by helper proteins and of inducing unwanted immune responses to rAAV transduction. Such methods, based on the transfection of packaging cells using multiple plasmid vectors have considerably decreased the risk of creating replication-competent AAV particles by recombination (50). These properties of rAAV make it a particularly attractive gene transfer system for applications in vivo. The benefits of employing this class of vector in clinical protocols are currently under active investigation for the treatment of cystic fibrosis (43,51,52), hemophilia (53-55), α 1 anti-trypsin deficiency (56), Duchenne muscular dystrophy (57), neurologic diseases (Canavan's, Batten's, Parkinson's, and Alzheimer's diseases) (58-61), rheumatoid arthritis (62), and various malignancies (prostate cancer, melanoma) (57).

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