AAV, adeno-associated virus
parvovirus

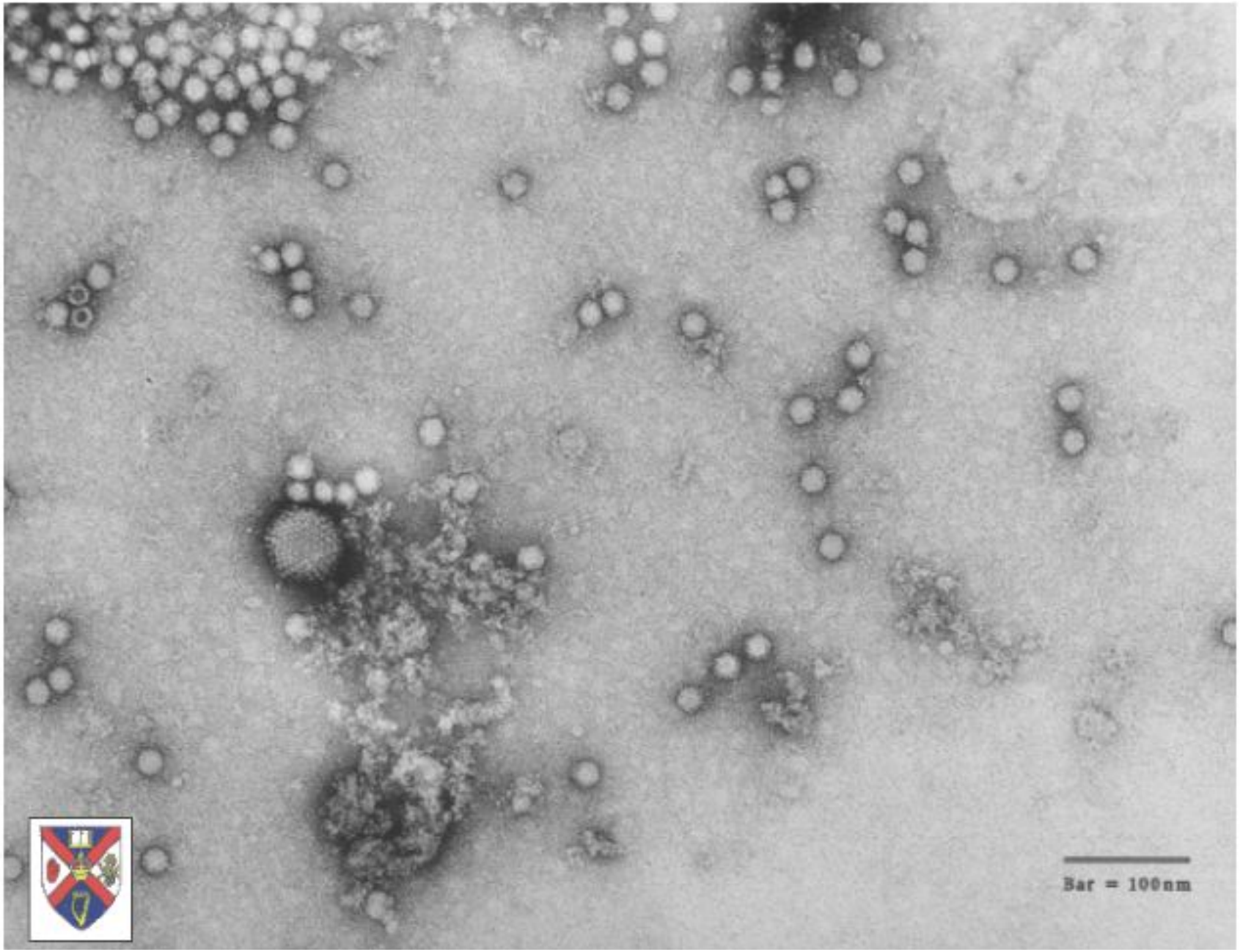
Its life cycle is dependent on the presence of a helper virus (adeno virus)

It is not pathogeneic

Its genome is small, well characterized and thus simple to manipulate

It may stably infect the host cell

AAV virions are small nonenveloped particles (20–25 nm)

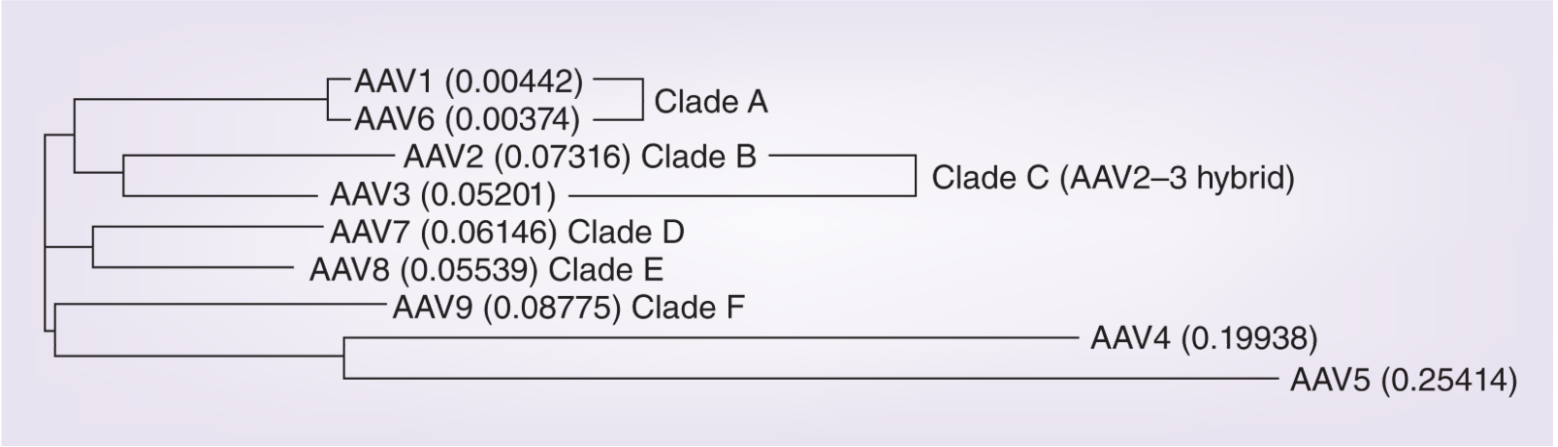


AAV, first discovered as a contaminant of Ad preprations

The first human adeno-associated virus (AAV) was discovered in 1965, as a contaminant of adenovirus (Ad) preparations (this is probably how it got its name). It is one of the smallest viruses with a non-enveloped capsid of approximately 22 nm.

Although 80-90% of adults are sero-positive with AAV2, infection has not been associated with any symptoms or disease.

AAV serotypes and virus phylogram



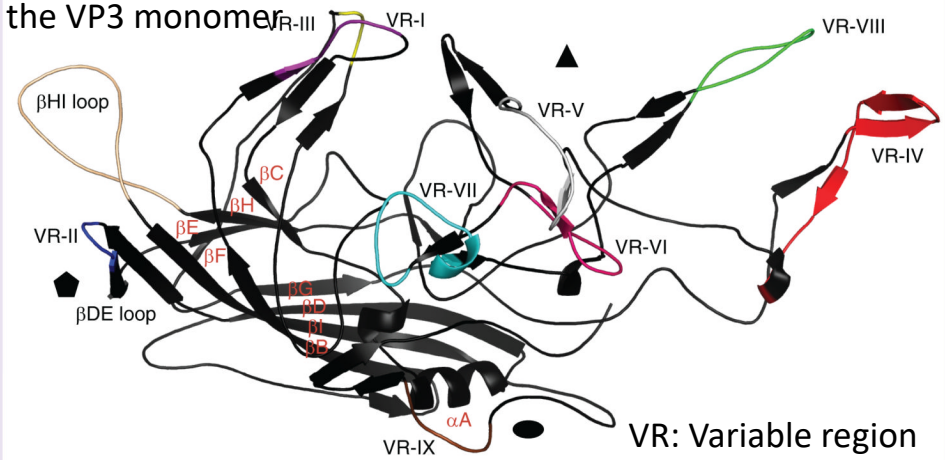
The AAV serotypes are separated into five clades (A–F) and two clonal isolates (AAV4, AAV5) based on VP1 amino acid sequence. ClustalW2 Phylogeny was used to generate the rooted phylogenetic tree. Distance values are shown for each serotype and indicate the number of substitutions as a proportion of the length of the alignment.

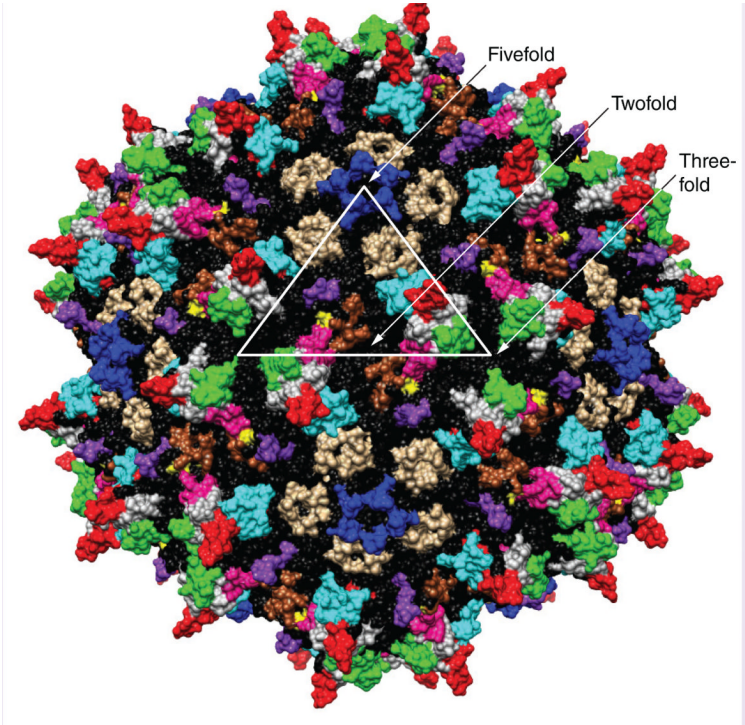
Adeno-associated virus capsid structure

icosahedral capsid consisting of 60 monomer; VP1 and VP2, 3 monomers; VP3, 54 monomers

The 9 variable regions (VR) of the VP3 monomer distinguish the different serotypes and the variable regions dictate the tropism of the virus

the VP3 monomer





An adeno-associated virus (AAV) VP3 monomer is shown with a conserved core region consisting of eight antiparallel β-sheets (βB–βI) and an α-helix (αA). Loop insertions between the β-sheets vary among the AAV serotypes. **Nine VRs are present on the capsid surface**, and are color coded and denoted with roman numerals (I: purple; II: blue; III: yellow; IV: red; V: gray; VI: hot pink; VII: cyan; VIII: green; IX: brown; βHI loop: tan).

Locations of the VRs and viral asymmetric unit (shown in white) on the surface of the AAV capsid. **The two-, three- and five-fold symmetry** axes make up the border of the viral asymmetric unit, which is defined as the smallest repeating unit on the **icosahedral capsid**, and 60 of these compose the AAV capsid. It is indicated by a triangle on the surface of the AAV capsid, encompassing the area from the fivefold pore down to the twofold symmetry axis and outwards to the threefold protrusions.

functional roles of adeno-associated virus variable regions

VR	AAV2 aa	Reported functional role(s)
VR-I	260–267	aa 263, 265, determinant of muscle transduction; A20 neutralization; 3C5 binding
VR-II	326–330	Transduction
VR- III	380–384	Transduction; A20 neutralization
VR-IV	449–467	aa 456–476, liver transduction efficiency determinant; aa 456–568, delayed blood clearance phenotype; 4E4 neutralization
VR-V	487–504	aa 498, 503, 504, effect on liver- and muscle-specific transduction; aa 456–568, delayed blood clearance phenotype; C37-B neutralization; 4E4 neutralization; 5H7 neutralization
VR-VI	522–538	aa 456–568, delayed blood clearance phenotype; aa 531, airway epithelium determinant
VR-VII	544–557	aa 456–568, delayed blood clearance phenotype; aa 550-568, liver transduction efficiency determinant; A20 neutralization; 3C5 neutralization
VR-VIII	580–592	aa 592, 595 reduced transduction efficiency in liver; aa 584, 598, airway epithelium determinant; aa 585–590, ability to traverse blood vasculature phenotype; aa 602, effect on liver- and muscle-specific transduction; aa 581–584 and 589–592, effect on liver and heart-specific transduction; ADK8 neutralization; C37-B neutralization; 5H7 neutralization
VR-IX	703–711	aa 699–735, determinant of heart tropism; determinant of improved melanoma tropism in AAV-1829; aa 706, mutants exhibited altered tropism; aa 705, 708, 716, determinant of muscle transduction; A20 neutralization; 3C5 binding

aa: Amino acid; AAV: Adeno-associated virus; VR: Variable region.

the variable regions are involved in the selection of the target cells thus regulate the virus tropism

Capsid homology among AAV serotypes 1 to 9

TABLE 1. Capsid homology among AAV serotypes 1 to 9

AAV	% Homology to AAV:								
	AAV-1	AAV-2	AAV-3	AAV-4	AAV-5	AAV-6	AAV-7	AAV-8	AAV-9
AAV-1	100								
AAV-2	83	100							
AAV-3	87	88	100						
AAV-4	63	60	63	100					
AAV-5	58	57	58	53	100				
AAV-6	99	83	87	63	58	100			
AAV-7	85	82	85	63	58	85	100		
AAV-8	84	83	86	63	58	84	88	100	
AAV-9	82	82	84	62	57	82	82	85	100

AAV Life Cycle

- AAV2 attachment is primary mediated by heparan sulphate proteoglycans,
- Internalization is aided by co-receptors, such as avb5 and FGFR1 etc.

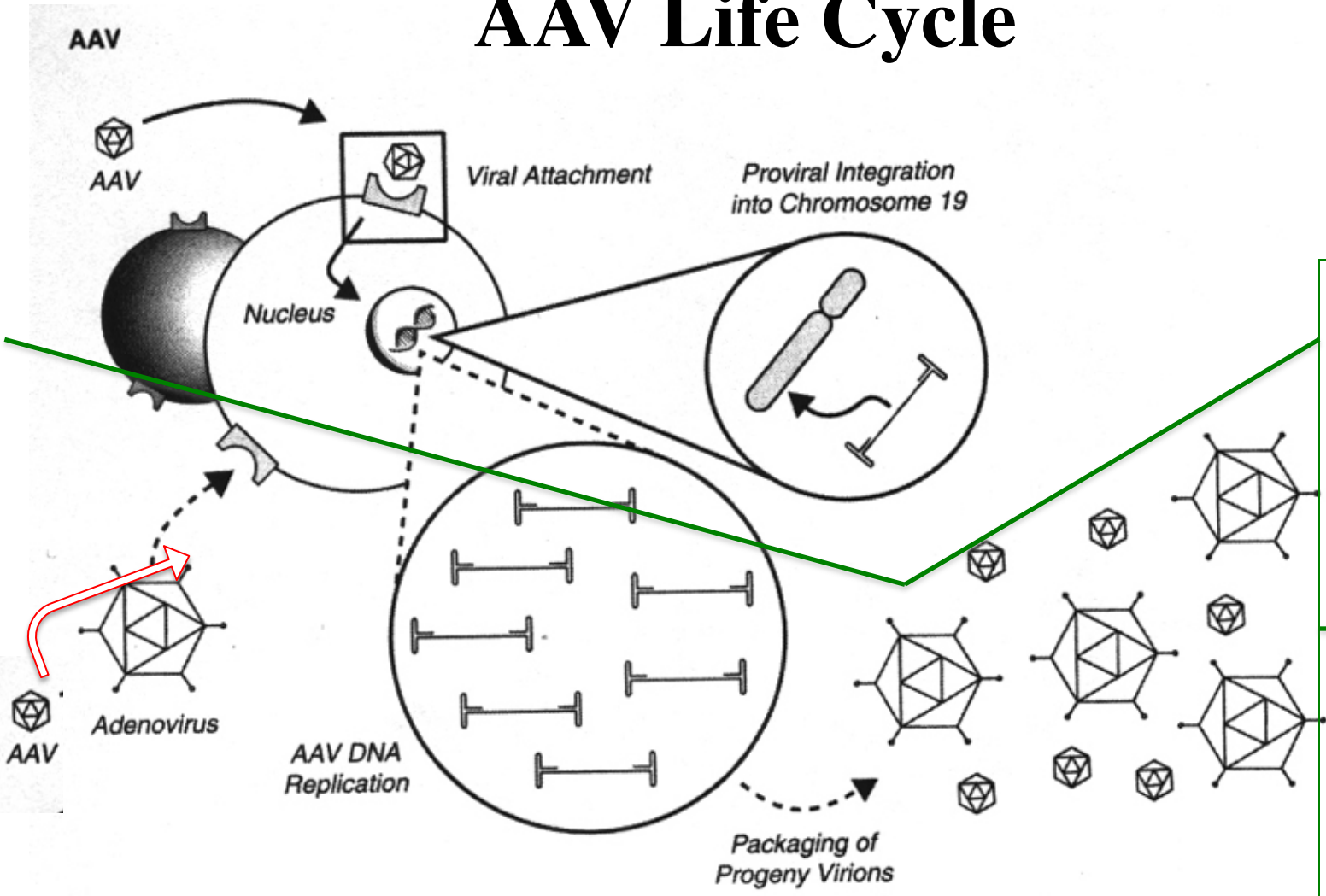
The use of ubiquitous heparan sulphate proteoglycans as docking sites explains in part the well-known broad tropism of this virus.

After entry into the host cell nucleus, AAV can follow either one of two distinct and interchangeable pathways of its life cycle: the lytic and the lysogenic.

The lytic pathway develops in cells infected with a helper virus such as adenovirus or herpes simplex virus (HSV),

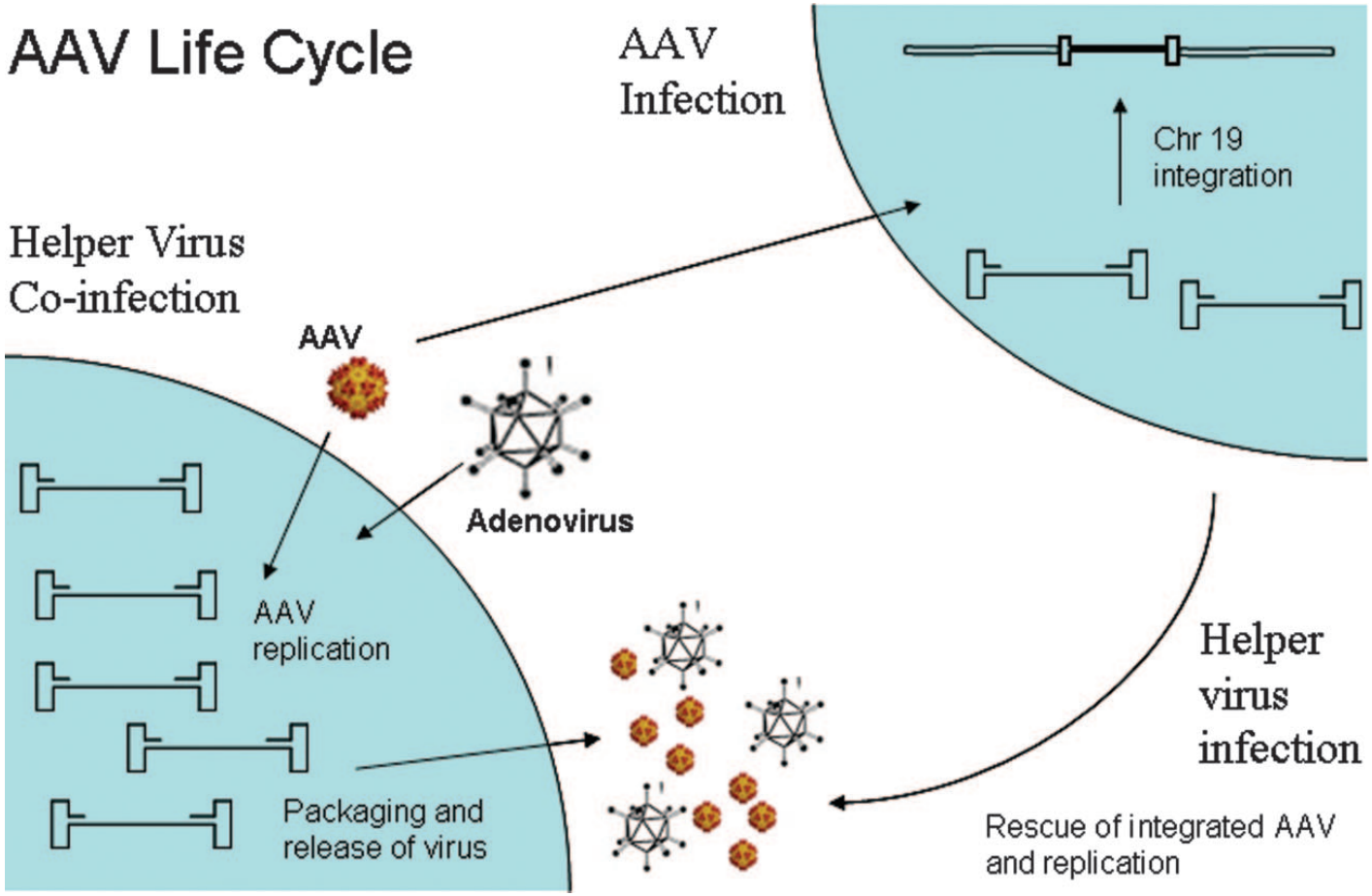
The lysogenic pathway is established in host cells in the absence of a helper virus

AAV Life Cycle



Lysogen: stable insertion of the proviral genome

Lytic: production of the progeny virions



AAV undergoes productive infection in the presence of adenovirus coinfection. This is characterized by genome replication, viral gene expression, and virion production. In the absence of adenovirus, AAV can establish latency by integrating into chromosome 19 (AAVS1). **The latent AAV genome can be rescued and replicated upon superinfection by adenovirus.** Both stages of AAV's life cycle are regulated by complex interactions between the AAV genome and AAV, adenoviral, and host proteins.

lytic *vs* lysogen cycle

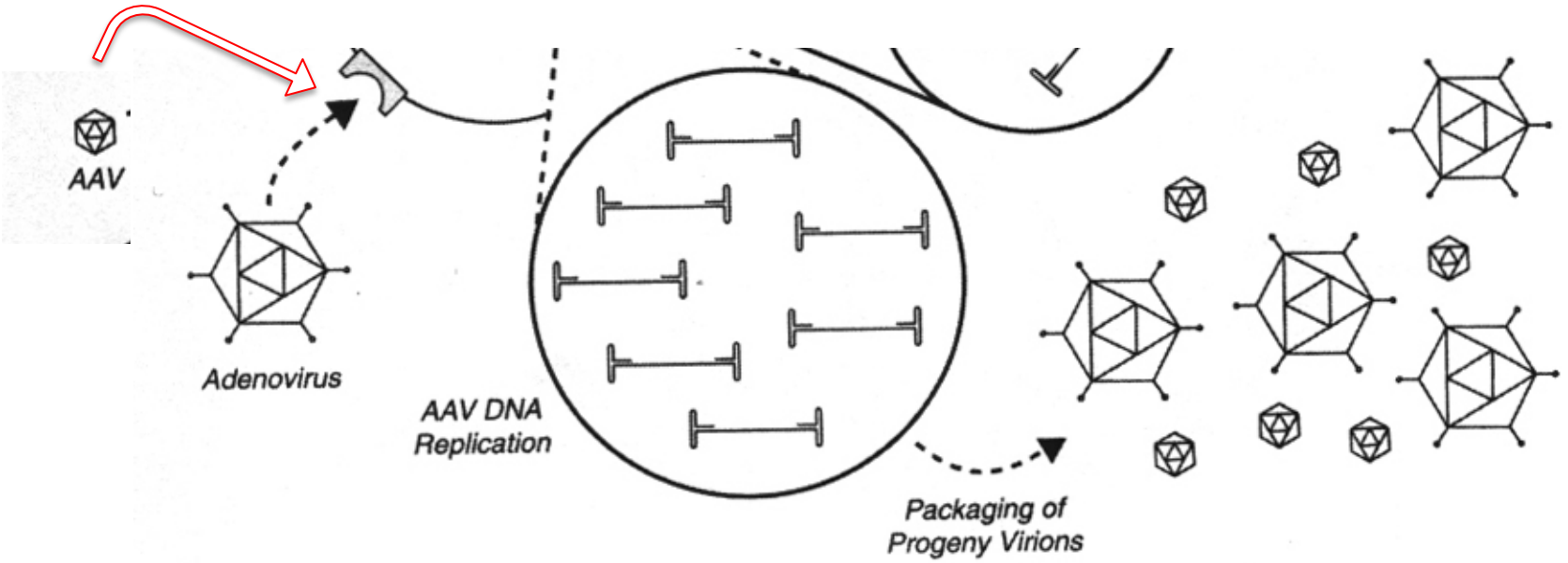
lytic
when the virions infect stressed cells:
infected with Ad or HSV
genotoxic agent (X γ UV radiation or chemicals such as MMS, hydroxyurea)
heat shock

lysogen
in most case, when cells are in physiological conditions the viral genome is integrated and the life cycle blocked

AAV production

Like all parvoviruses, the AAV genome is packaged as a **linear ssDNA molecule with palindromic inverted terminal** repeat (ITR) sequences forming dsDNA hairpin structures at each end.
These serve as replication origins during productive infection and as priming sites for host–cell DNA polymerase to begin synthesis.

AAV, unlike many of the autonomous parvoviruses, **packages either the plus or minus DNA strand with equal efficiency.**
While the conventional replication scheme of AAV requires *de novo* synthesis of the complementary DNA strand, there is **an alternative mechanism**, involving **the base pairing of complementary strands** from two infecting viruses containing DNA strands with opposite polarity.

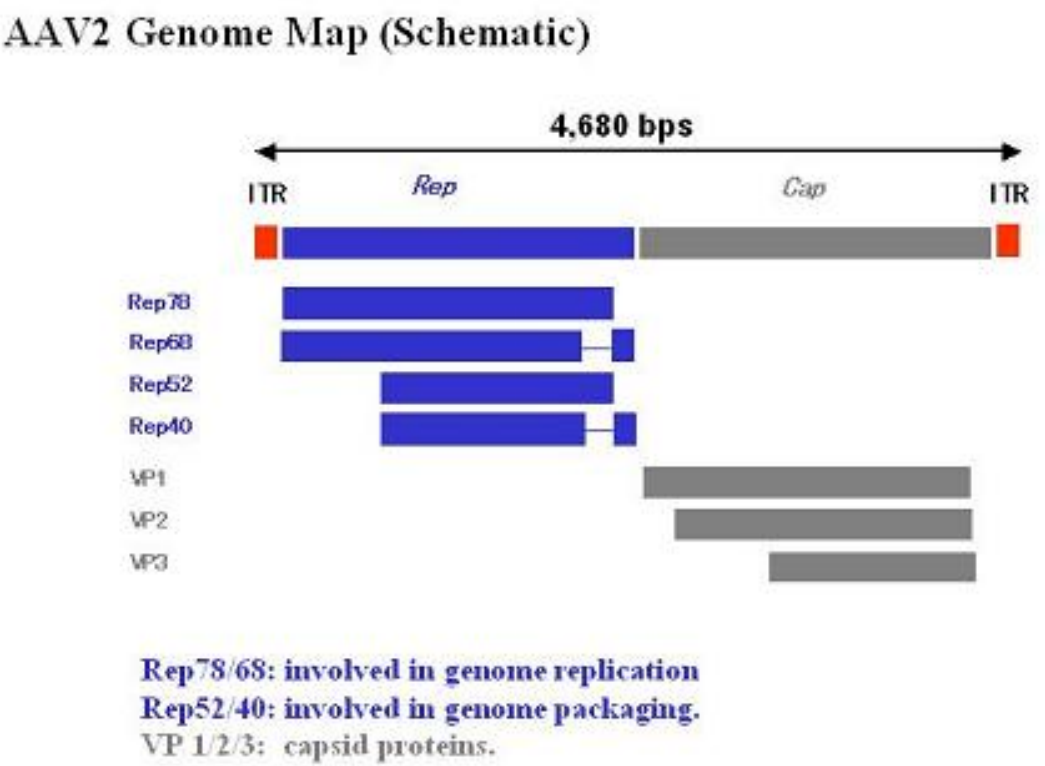


AAV genome

AAV has a linear single-stranded DNA (ssDNA) genome of approximately 4.7-kilobases (kb), with two 145 nucleotide-long inverted terminal repeats (ITR) at the termini. The **virus does not encode a polymerase** and therefore relies on cellular polymerases for genome replication. The ITRs flank the two viral genes, rep (replication) and cap (capsid), encoding non-structural and structural proteins, respectively.

Rep78/68, genome replication
Rep42/40, genome packaging

VP1 (virion protein 1, 87 kDa),
VP2 (virion protein 2, 72 kDa)
VP3, (virion protein , 62 kDa)
These capsid proteins assemble into a near-spherical protein shell of 60 subunits.



AAV ITR

The AAV genome is structurally characterized by 145-bp inverted terminal repeats (ITRs).

The **first 125 nucleotides of the ITR constitute a palindrome**, which folds upon itself to maximize base pairing and forms a T-shaped hairpin structure. The other **20 bases, called the D sequence, remain unpaired**.

The ITR is the origin of replication and serves as a primer for second-strand synthesis by DNA polymerase.

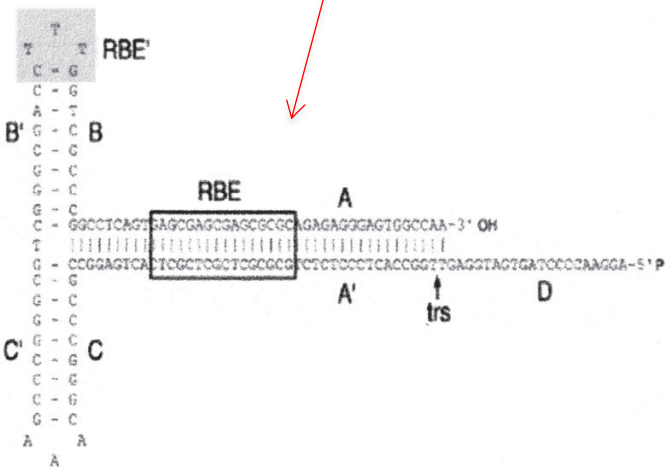
The double- stranded DNA intermediates are processed via a strand displacement mechanism, resulting in single-stranded DNA used for packaging and double-stranded DNA used for transcription.

Critical to the replication process are the Rep binding elements (RBEs) (RBE and RBE') and a **terminal resolution site (TRS)**, which is located within the ITR.

The ITR is also essential for AAV genome packaging, transcription, negative regulation under non permissive conditions, and site-specific integration.

ori of the AAV genome

RBE, Rep Binding Element
TRS, Terminal Resolution Site

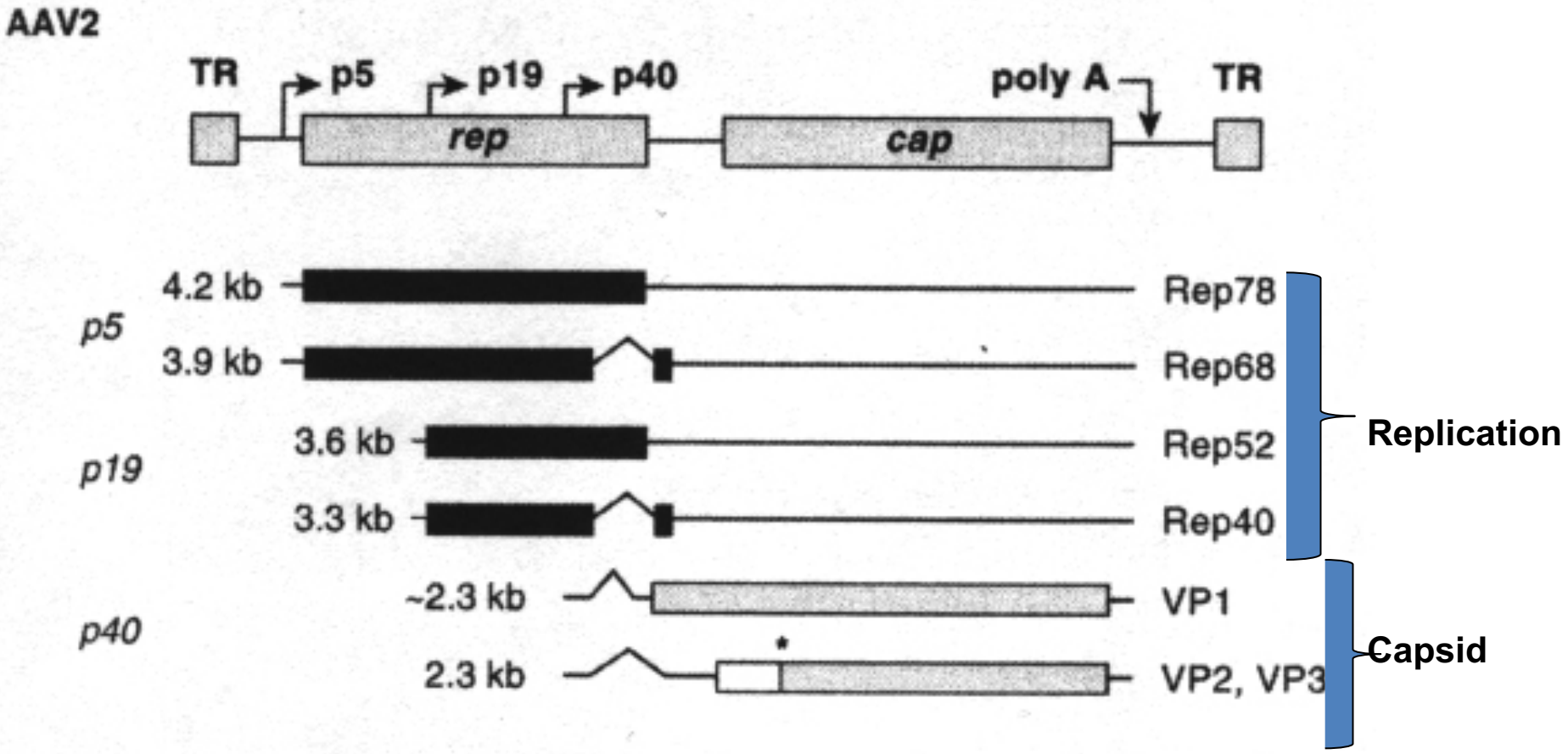


Secondary structure of an AAV-2 ITR showing the RBEs (RBE, GAGCGAGCGAGCGCGC; RBE', CTTG) and the TRS (GTTGG).

AAV carry a linear single-stranded DNA (ssDNA) genome, which is approximately 4.7 kb in size.

There are 2 viral ORFs, *rep* and *cap*, flanked by T-shaped ITRs. The ITRs are important for replication, packaging, and integration, and these are the only genetic elements from the virus that are retained in rAAV vectors.

The genome is translated into three mRNAs from three promoters p5, p19 and p 40;



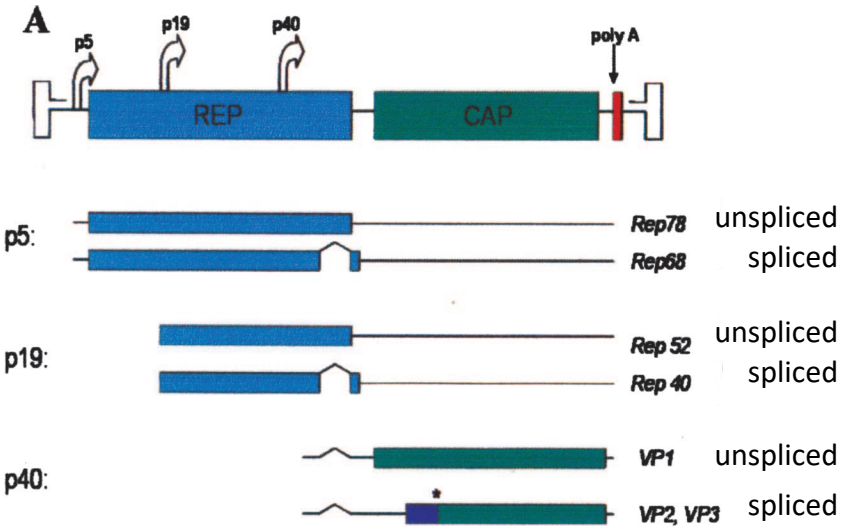
the Rep proteins

Rep78, produced from unspliced transcript
Rep68, produced from spliced transcript
Both are important regulatory proteins that act in trans in all phases of the AAV life cycle: required for **DNA replication**; they positively and negatively **regulate AAV gene expression** in the presence or absence of helper virus.

In addition, **Rep78 and 68 possess strand- and site-specific endonuclease activity** (nicking at the TRS) **and site-specific DNA binding activity** (binding at the RBE).

The smaller Rep proteins, **Rep52** and **Rep40**, produced from unspliced and spliced transcripts, respectively, are involved in the **accumulation of single-stranded viral DNA** used for packaging within AAV capsids.

All four Rep proteins possess helicase and ATPase activity.



Rep and Cap genes flanked by ITRs. The different Rep and Cap transcripts are produced from their respective promoters (P5, P19, and P40). The star indicates the alternative ACG codon used to produce VP3.

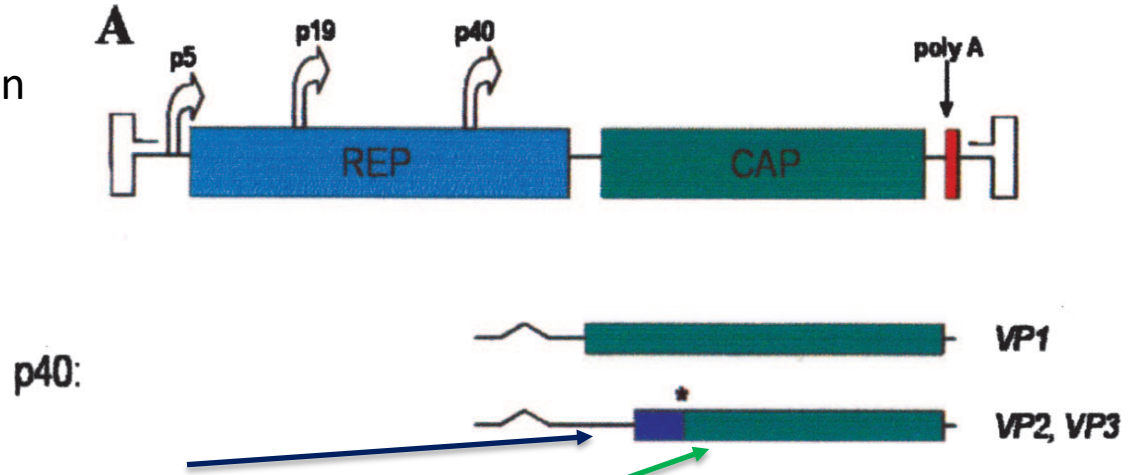
the Cap proteins

The Cap gene produces three viral capsid proteins (VP1, VP2, and VP3) using the P40 promoter.

Alternative splicing of the P40 transcript is used to produce the three viral proteins from two transcripts:
•the unspliced transcript produces VP1 (87 kDa), the biggest of the capsid proteins.
•the spliced transcript produces VP2 (72 kDa) and VP3 (62 kDa).

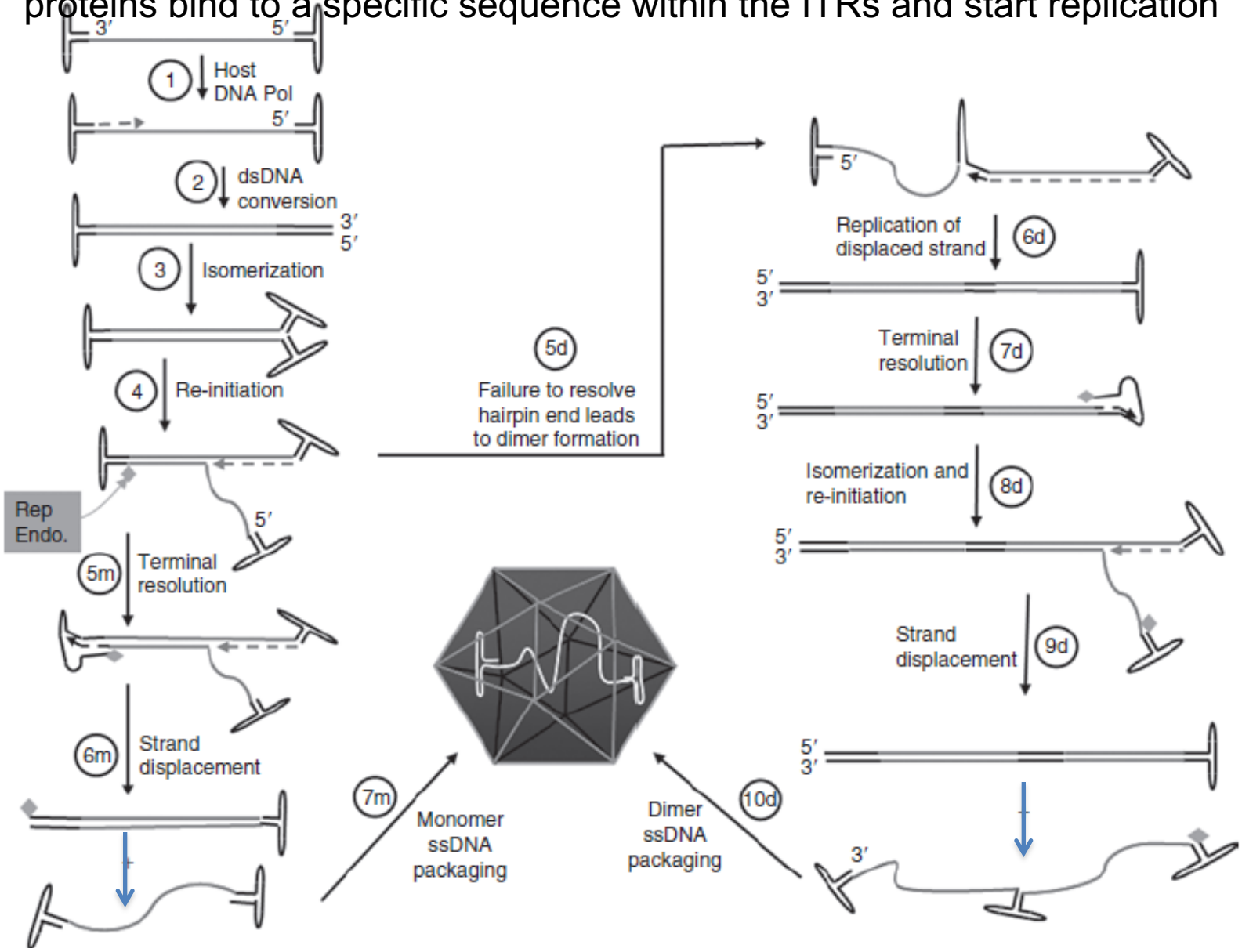
VP2 is produced using a nonconventional ACG start codon, whereas VP3 is produced using a down-stream conventional AUG codon (*). The AAV-2 capsid comprises 60 viral capsid proteins arranged into an icosahedral structure with symmetry equivalent to a triangulation number of 1. The capsid proteins (VP1, VP2, and VP3) are present in a 1:1:10 molar ratio.

molar ratio of the cap protein in the capsid
VP1:VP2:VP3=1:1:10



VP2 is transcrideb by a non conventional ACG start codon, while WP3 by a downstream conventional AUG

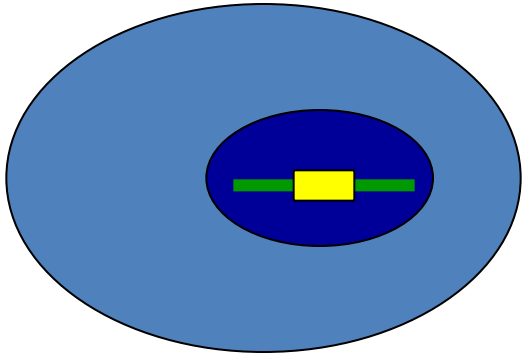
The self-complementary sequences in the ITRs fold back on themselves to form hairpin structures that contain the replication origins. The large viral Rep proteins bind to a specific sequence within the ITRs and start replication



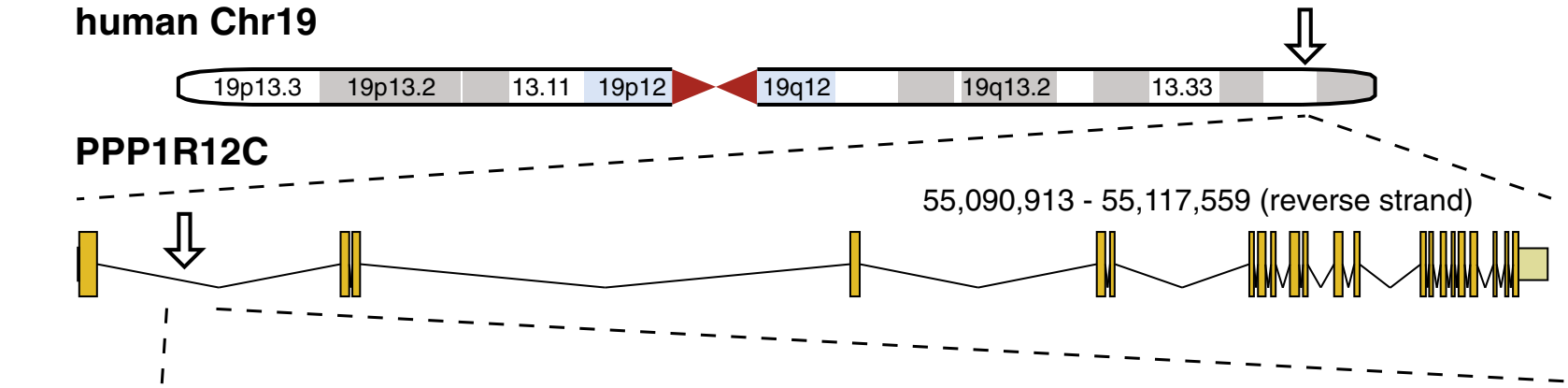
The lysogenic pathway

When AAV infects a human cell alone, its gene expression program is auto-repressed and latency is ensued by preferential integration of the virus genome into a region of roughly 2-kb on the long arm (19q13.3-qter) of human chromosome 19, designated AAVS1.

A 33-bp minimum AAVS1 sequence, which contains an RBE-like and a TRS-like sequence separated by 8 nucleotides, is necessary and sufficient to target AAV integration. The process of site-specific integration is not completely specific even under ideal conditions of Rep78 and Rep68 expression, with approximately **40 to 70% of integrants occurring in AAVS1**.



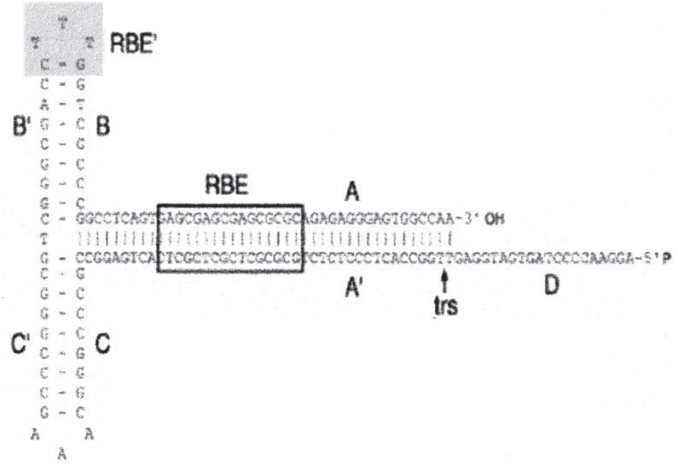
AAVS1

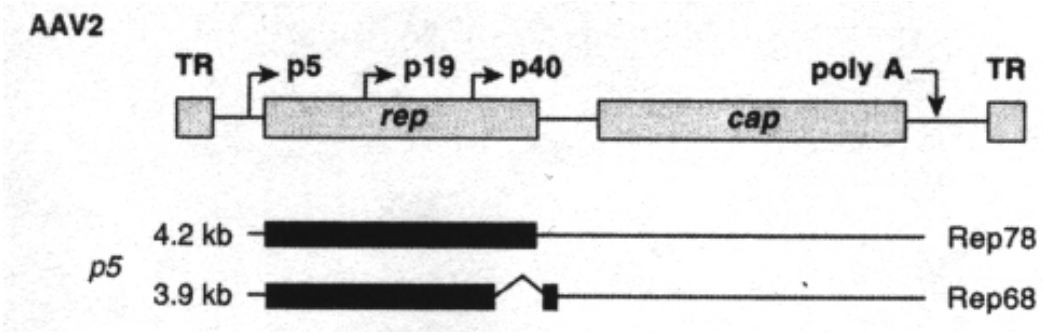


Originally described as a major hotspot for adeno-associated virus (AAV) integration, intron 1 of the protein phosphatase 1, regulatory subunit 12C (PPP1R12C) gene on human chromosome 19 is referred to the AAVS1 locus. This locus allows stable, long- term transgene expression in many cell types, including embryonic stem cells

Near many genes but apparently safe: The AAVS1 locus is near several muscle-specific genes, TNNT1 and TNNI3. The AAVS1 region itself is an up-stream part of the gene, **MBS85 (***myosin binding subunit* 85). The exact function of this gene is not clear, but its product has been shown to be involved in actin organization . Whether AAV integration into this site is suitable for human gene therapy applications remains to be evaluated. **Tissue culture experiments suggest that the AAVS1 locus is a safe integration site**.

The lysogenic pathway





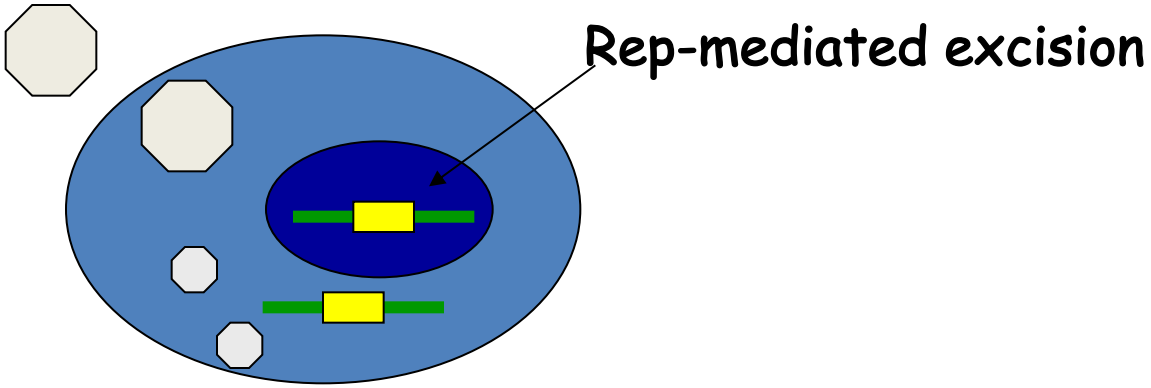
This site-specific integration involves the AAV ITRs and Rep proteins (Rep78, Rep68).

The AAV components that are required for integration are: the ITRs (in cis), Rep78 or Rep68 (in trans), and a 138-bp sequence termed the **integration efficiency element (IEE**), located within the **P5 promoter** in cis.

most of the provirus was integrated within the same sequence context at a specific region of human chromosome 19 (Ch19) (19q13.3-qter). Further, examination of cells with integrated rAAV revealed that **vectors containing the AAV Rep gene were mostly integrated within this specific region of Ch19**, while **those lacking Rep were integrated elsewhere, apparently at random.** The Southern blot results were corroborated in studies us- ing PCR and fluorescence in situ hybridization (37, 71). Metaphase spreads of IB3 cells revealed that AAV sequences associated with Ch19 accounted for up to 94% of the detected wtAAV provirus, but none of the rAAV sequences lacking the *rep* gene.

The lytic pathway

When a latently infected cell is super-infected with a helper virus, such adenovirus or herpes simplex virus, the AAV gene expression program is activated leading to the AAV Rep-mediated rescue (i.e., excision) of the provirus DNA from the host cell chromosome, followed by replication and packaging of the viral genome.
The adenoviral genes that provide helper functions regarding AAV gene expression have been identified and include **E1a, E1b, E2a, E4, and VA RNA**. Herpesvirus aids in AAV gene expression by providing viral DNA polymerase and helicase as well as the early functions necessary for transcription.



The induction of the lytic phase of the AAV life cycle from a stably integrated provirus can also occur in the absence of a helper virus, though with a lower efficiency, when the host cell is subjected to metabolic inhibitors, DNA damaging agents/genotoxic compounds

AAV tropism

AAV2 is probably the most widely used AAV serotype for *in vit*ro and *in vivo* gene delivery. It should be noticed that many other serotypes of AAV have been isolated, and every year there are some new serotypes being discovered.

Among them, AAV1, AAV2, AAV5, as well as AAV7, 8 and 9 etc, have been used for gene delivery studies. The question about which of these AAV serotypes is most appropriate or the best for a particular study model, remains open.

AAV serotypes and receptors

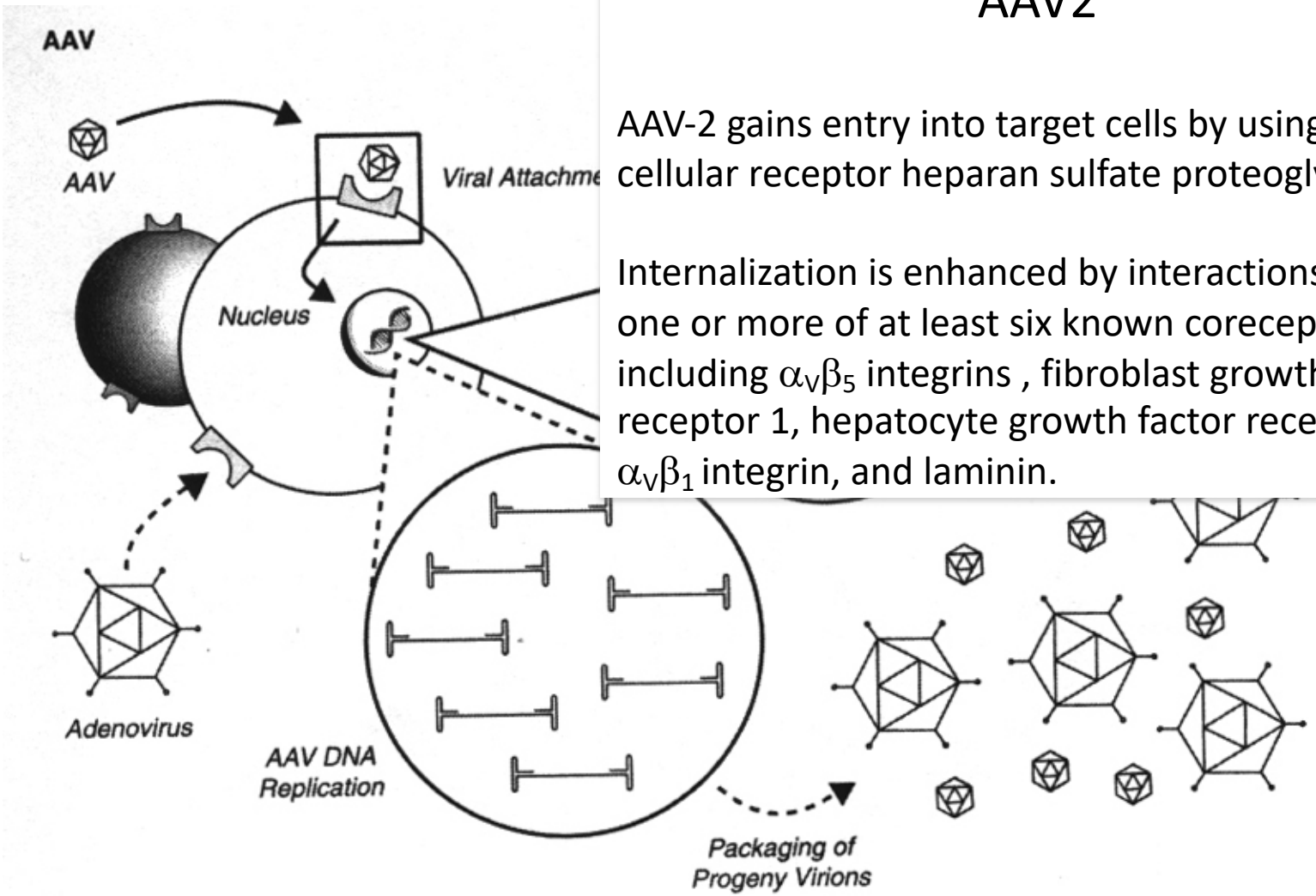
AAV vectors are based on the AAV2 serotype

serotype

Parvovirus	Recettore
AAV1	Acido sialico (legami α2-3-N e α2-6-N)
AAV2	Proteoglicani contenenti eparan-solfati (HSPG) Corecettori: integrina αvβ5, FGFR1, HGF-R
AAV3	Proteoglicani contenenti eparan-solfati (HSPG)
AAV4	Acido sialico (legami α2-3-O)
AAV5	Acido sialico (legami α2-3-O e α2-3-N) Recettore del PDGF (PDGFR)
AAV6	Acido sialico (legami α2-3-N e α2-6-N)
AAV7	Non noto
AAV8	Recettore della laminina (LamR)
AAV9	Non noto (LamR?)
Parvovirus B19	Antigene P dei globuli rossi
CPV (parvovirus canino)	Recettore della trasferrina Acido sialico (acido N-glicolil-neuraminico, NeuGC)
FPV (parvovirus della panleucopenia felina)	Recettore della trasferrina

The different serotypes of AAV utilize a variety of approaches for cell entry

- The primary attachment site for AAV2 is the ubiquitous heparan sulfate proteoglycan
- The fibroblast growth factor receptor 1 and integrin *αvβ5 have both been implicated as coreceptors*
- AAV4 and AAV5 use sialic acid



AAV2

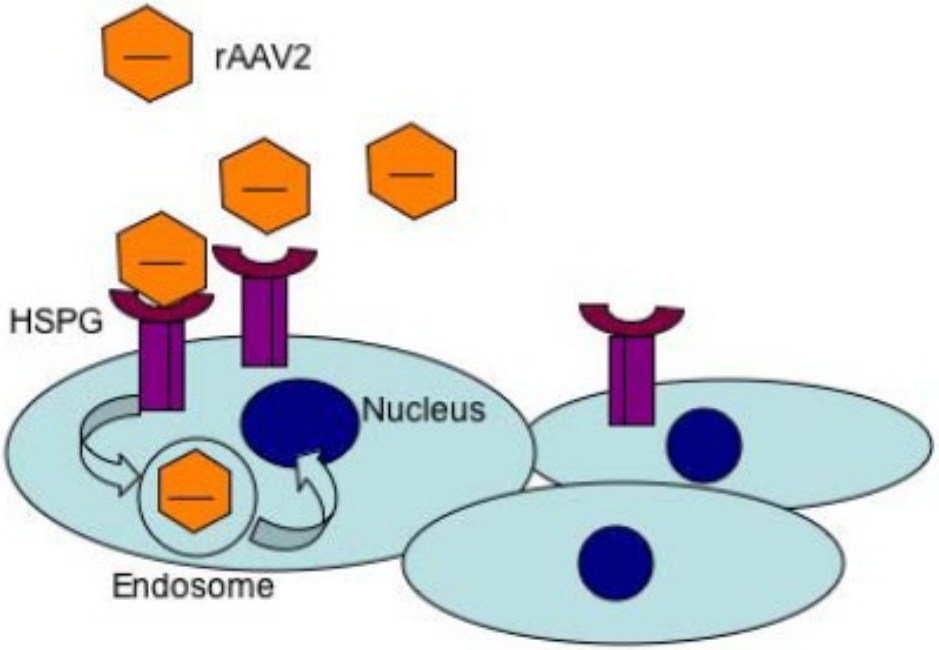
AAV-2 gains entry into target cells by using the cellular receptor heparan sulfate proteoglycan.

Internalization is enhanced by interactions with one or more of at least six known coreceptors including $\alpha_V\beta_5$ integrins , fibroblast growth factor receptor 1, hepatocyte growth factor receptor, $\alpha_V\beta_1$ integrin, and laminin.

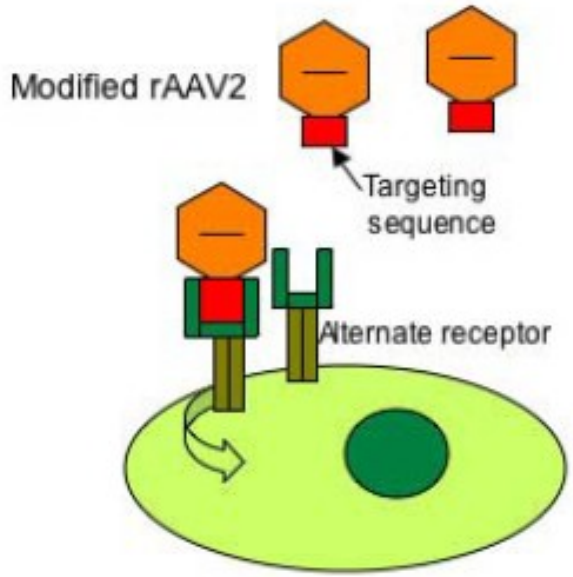
changing the AAV tropism

ure 1

Targeted rAAV binds alternate cell surface receptors



A.Normal rAAV2 binds host cells through HSPG



B.Modified rAAV2 containing targeting capsid sequences binds alternate cell surface receptors to expand viral tropism

changing the AAV tropism

The primary attachment receptor for AAV type-2 (AAV2) is heparan sulfate proteoglycan (HSPG). This receptor is widely expressed on many cell types, which creates a problem when delivering genes that promote cell death, as in anti-cancer gene therapy strategies. Conversely, cells that do not express HSPG cannot be transduced by AAV-2 based vectors. This can cause problems when gene delivery needs to be cell specific.
Targeting strategies have focused on either **1) attaching ligands**, such as bispecific antibodies, to the surface of intact vector particles, or **2) modifying the AAV capsid protein** itself to re-direct viral attachment and infection through an alternate receptor.

Genetic studies have shown that specific sites within the AAV2 capsid proteins can tolerate insertion of foreign peptide ligands. **The first evidence for HSPG-independent AAV2-mediated gene delivery was demonstrated by the insertion of a 15-amino acid peptide derived from the human luteinizing hormone into the AAV2 VP3 capsid protein (Shi et al., 2001).** This insertion had no effect on the packaging of recombinant AAV2 genome into viral particles, yet mediated **efficient binding of these particles to cell-surface luteinizing hormone receptor (LH-R) and infection of LH-R bearing cell lines**

Chimeric AAV variants

“containing capsid proteins that have been modified by domain or amino acid swapping between different serotypes”

Chimeric rAAVs are created either by directed evolution.

The directed evolution approach involves using either **error-prone PCR** or **DNA shuffling** to evolve the capsid proteins.
Error-prone PCR utilizes specific conditions to enhance the error rate of the polymerase to generate capsid variants with random mutations.
DNA shuffling uses restriction enzymes to digest the *cap* genes of selected sero-types. These fragments are then allowed to recombine, at random, into full-length capsid genes and then amplification of the variants occurs. Either method can be used to create a chimera, followed by a selective screening procedure with the preferred cell type in order to isolate mutants expressing the desired characteristics.

The rational capsid engineering uses structural and sequence information to design new chimeric viruses by transferring specific capsid residues or domains (such as VRs) from one serotype to another to confer a desired phenotype

Chimeras made by directed evolution

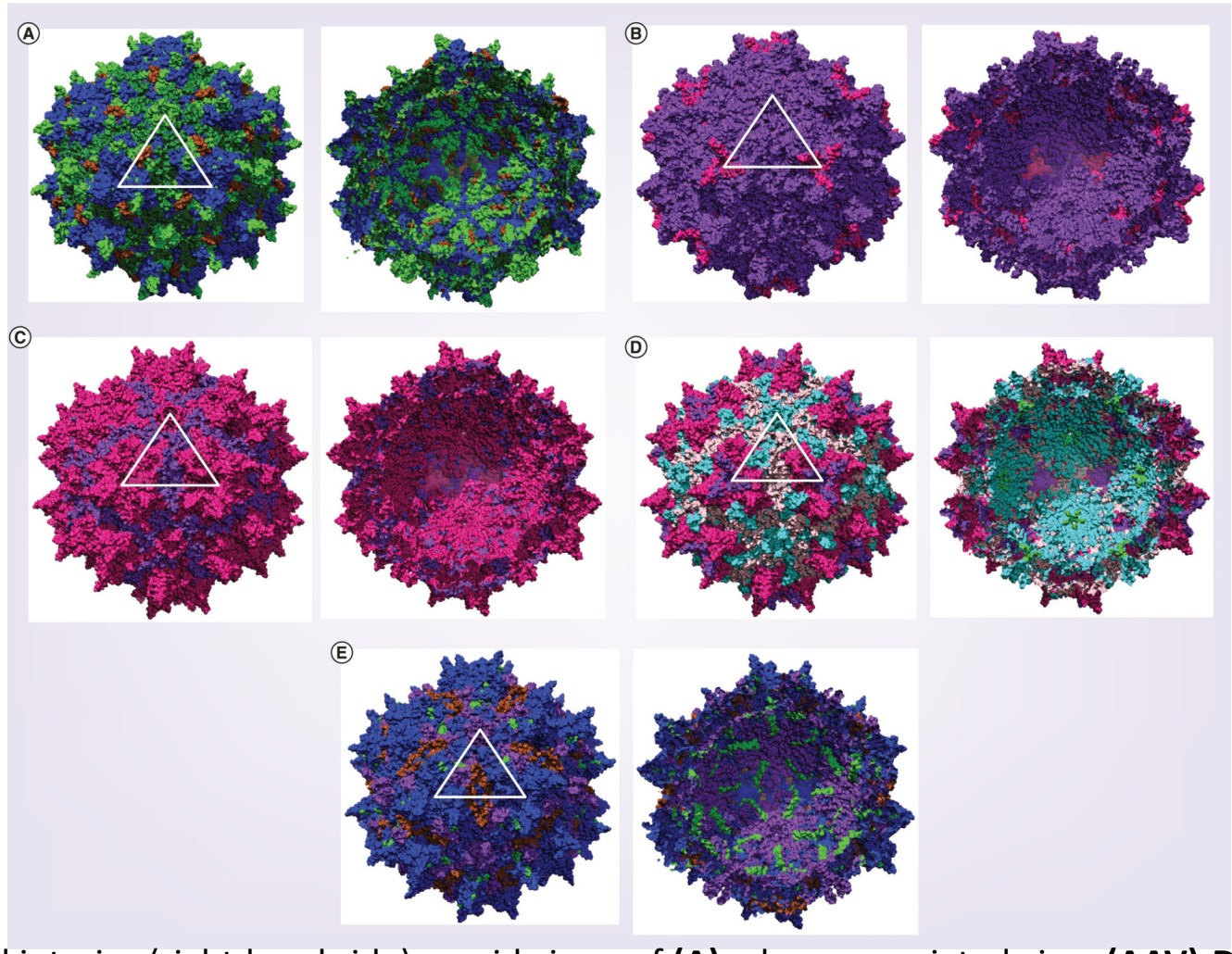
This chimera was created to have **enhanced tropism for liver and resistance to pre-existing neutralizing antibodies,** and was made using a two-steps approach.

First, the capsid genes of eight different AAVs (AAV2, AAV4, AAV5, AAV8 and AAV9, and animal isolates AAAV, BAAV and CAAV from avian, bovine and caprine species, respectively) were combined to create a shuffled AAV capsid library. The library was serially screened on human hepatoma cells (Huh-7 and HepG2), which naturally have a low permissiveness for AAV infection.
Second, the resulting clones underwent further selection in the presence of pooled human antisera (intravenous immunoglobulin), which have a neutralizing effect against many AAV serotypes, and then were amplified on the hepatoma cells for several more rounds of directed evolution.

Result: the AAV-DJ chimera **(Figure 5A)**, containing capsid VP contributions from AAV2, AAV8 and AAV9, has enhanced liver tropism *in vivo* in both naive mice and mice treated with intravenous immunoglobulin, an ability to evade known NAbs and can efficiently deliver the Factor IX transgene to liver tissue.

Models of adeno-associated virus chimeras created by **directed evolution**

The AAV-DJ chimera **(Figure 5A)**, containing capsid VP contributions from AAV2, AAV8 and AAV9



Exterior (left-hand side) and interior (right-hand side) capsid views of **(A)** adeno-associated virus **(AAV)-DJ**, **(B)** AAV-HAE1, **(C)** AAV-HAE2, **(D)** AAVM41 and **(E)** AAV-1829. Capsids are color coded by their contributing serotypes (and in lighter shades if there are multiple contributions from that serotype).
AAV1: purple; AAV2: blue; AAV3: yellow; AAV4: red; AAV5: gray; AAV6: hot pink; AAV7: cyan; AAV8: green; AAV9: brown. The viral asymmetric unit is shown in white .

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Daya S and Berns KI, 2008. Gene Therapy Using Adeno-Associated Virus Vectors. CLINICAL MICROBIOLOGY REVIEWS, Oct. 2008, p. 583–593.

HUMAN GENE THERAPY 25:3–11 (January 2014)

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Pioneer Perspective

Adenovirus:
The First Effective *In Vivo* Gene Delivery Vector

Ronald G. Crystal

Developing Recombinant AAV (General Principle)

AAV ITRs contain all cis-acting elements involved in genome rescue, replication and packaging,
AAV ITRs are segregated/separated from the viral encoding regions,
Rep and Cap gene regions encode trans acting factors

recombinant AAV vector design can follow the whole gene- removal or "gutless" rationale, as in the adenovirus system

- cis-acting viral DNA elements involved in genome amplification and packaging should be linked with the transgene of interest,
- the region(s) encoding the trans-acting viral factors**, rep and cap**, involved in genome replication and virion assembly may be provided in trans

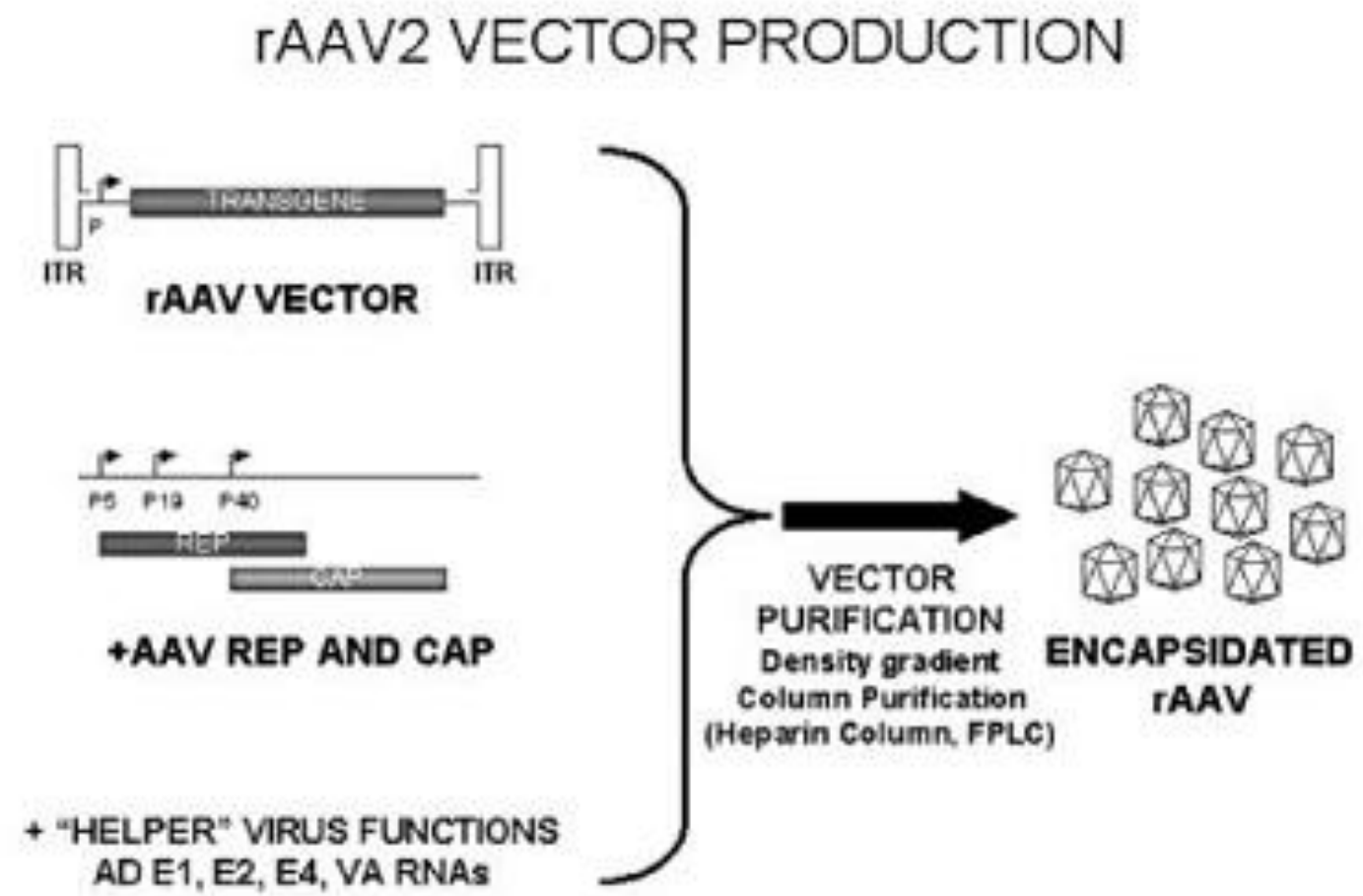
Helper functions

The adenovirus helper factors, such as E1A, E1B, E2A, E4ORF6 and VA, can be provided by either adenovirus infection or by transfecting a plasmid that provides these adenovirus helper factors, into the packaging cells

Given that HEK293 cells, a commonly used AAV production cells, already contains the E1A/E1b gene, so the helper factors need to be provided are E2A, E4ORF6 and VA.

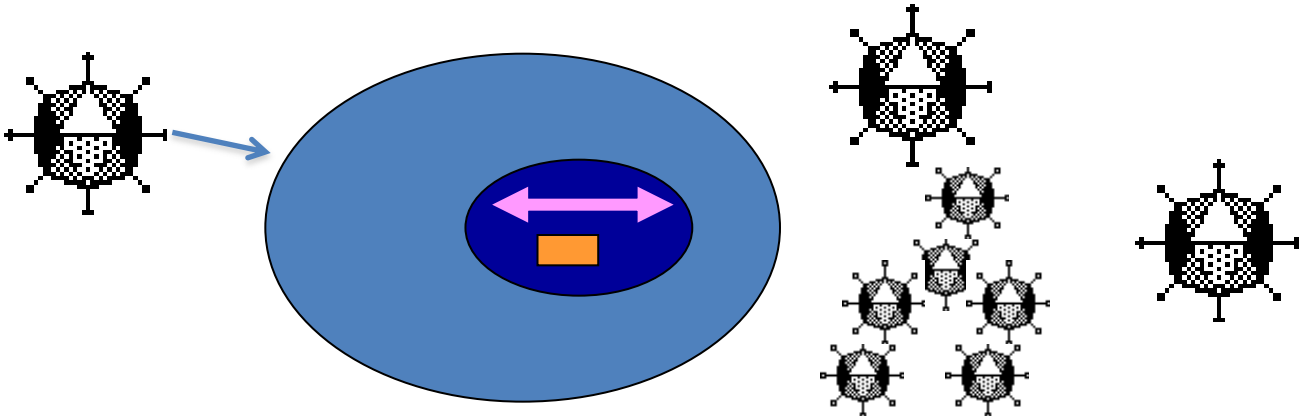
AAV production method 1

Method 1, the helper-free method, is based on the adenovirus-free transient transfection of all elements that are required for AAV production in host cells such as HEK293 cells



AAV production method 2

A second method relies on wild-type adenovirus infection of a cell line that stably transfected with the AAV rep/cap genes, as well as with the AAV vector DNA.

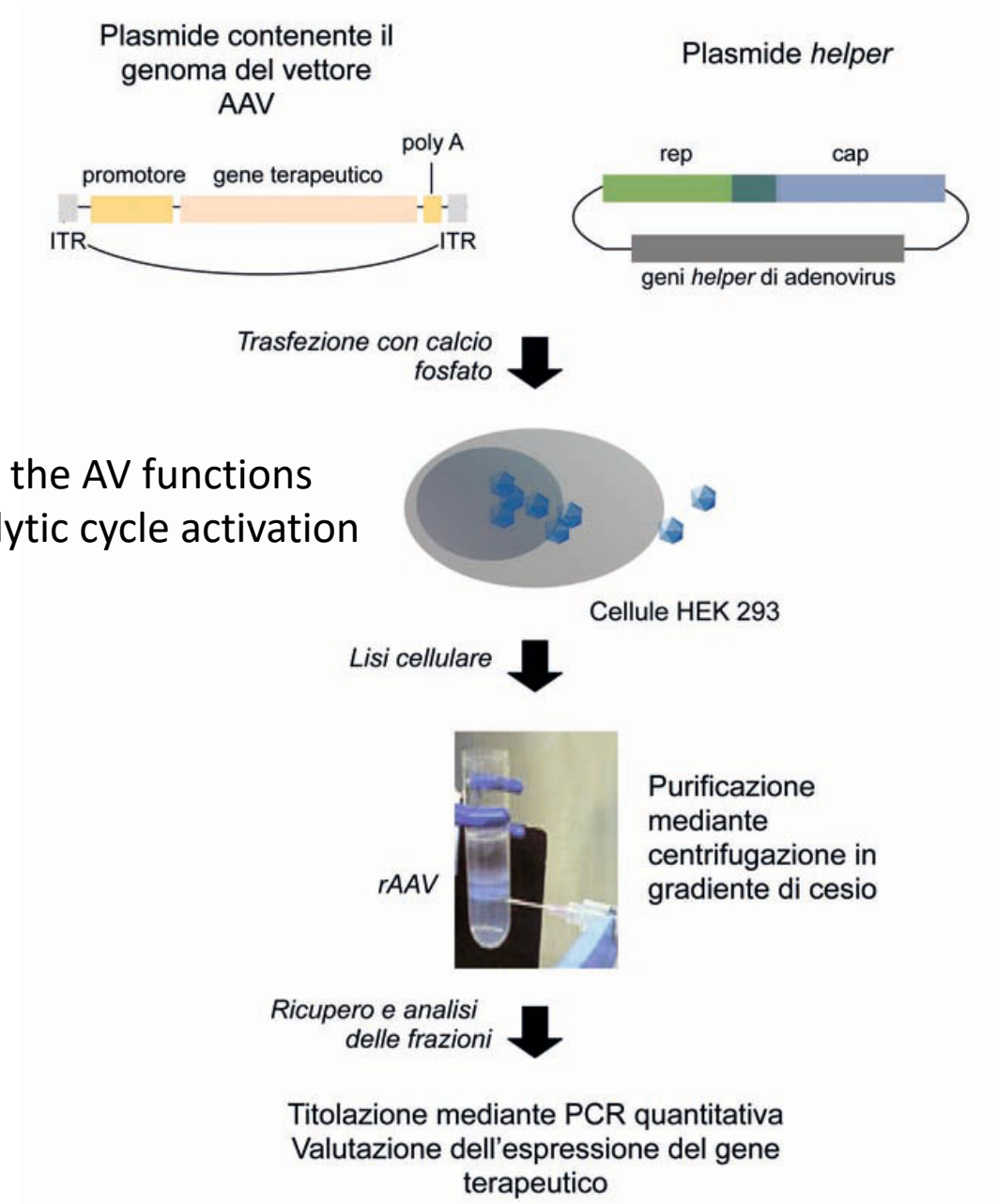


This method yields AAV as well as the helper adeno virus particles thus requiring the subsequent separation of AAV from the helper virus.

This can be done by CsCl2 gradient or cromatography, which separate on the basis of size differences

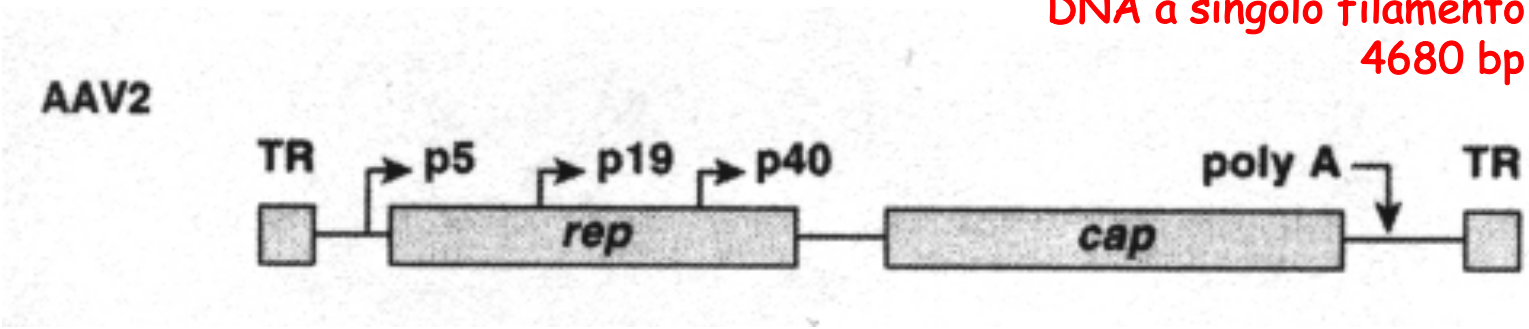
production of AAV vectors

HEK must contain the AV functions required for AAV lytic cycle activation



the AAV constructs

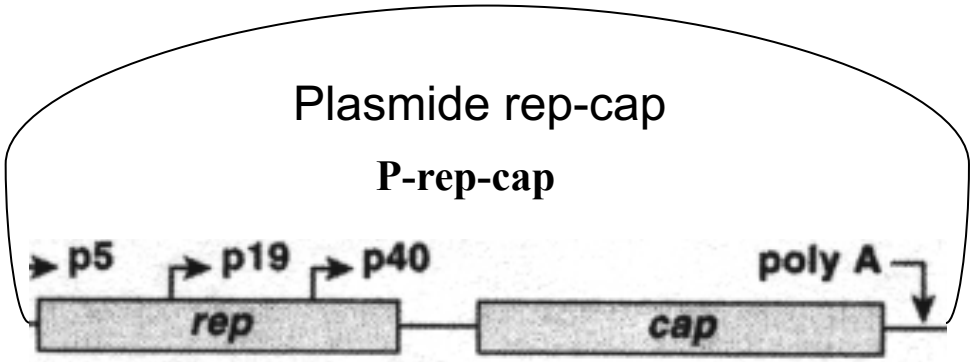
DNA a singolo filamento
4680 bp



Vettore AAV

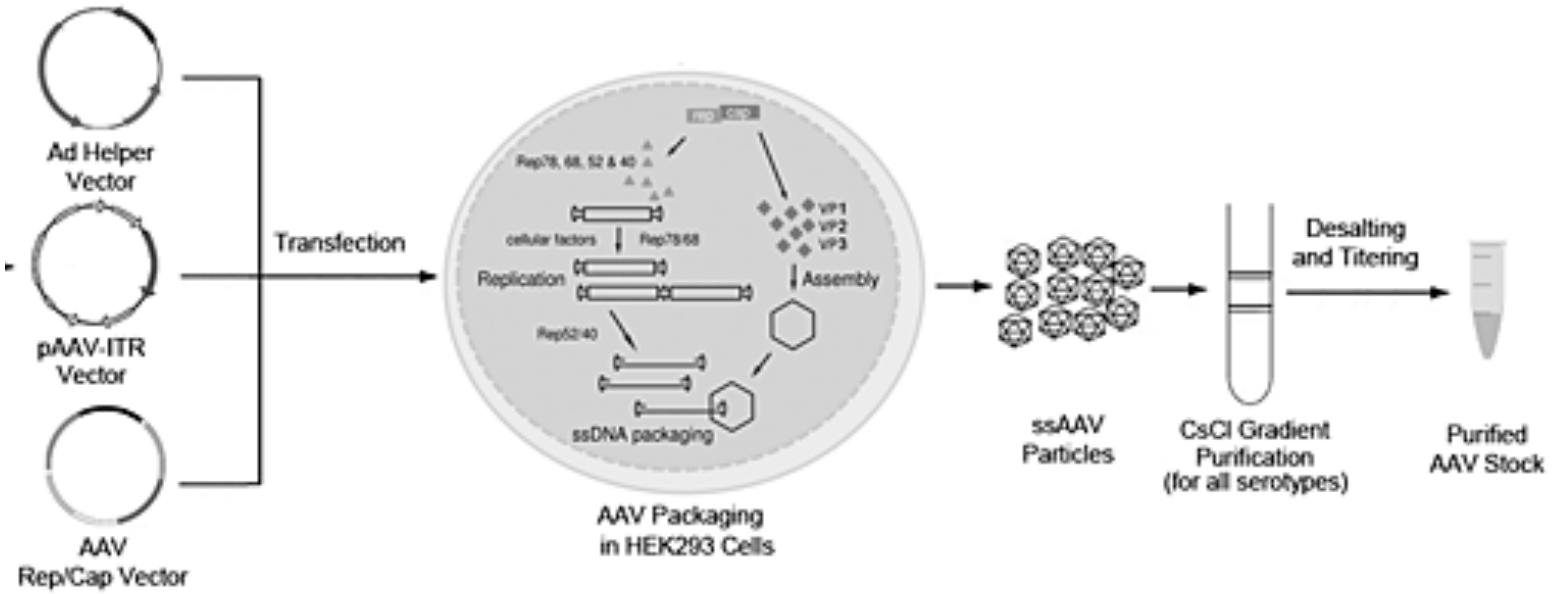
Trasfettati in cellule AD-infette

+



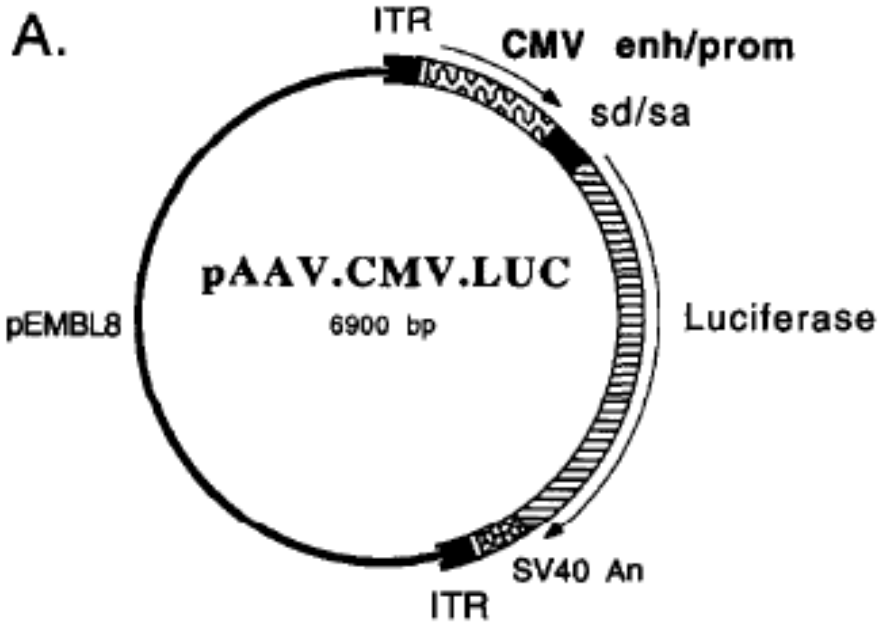
method 1

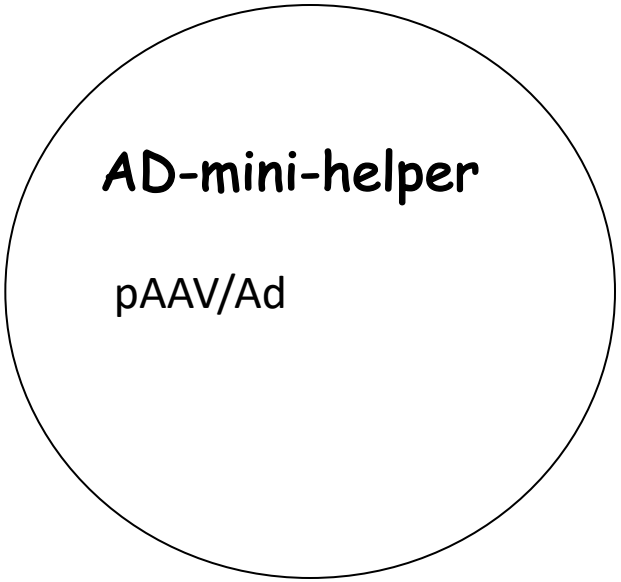
three constructs



Recombianant AAV production

Development of plasmid based systems in which transfection of mini-Ad plasmids into **293 cells (expressing AdE1 proteins**) supplies all the helper proteins (E2A, E4, and the VA genes) without production of infectious Ad. the AAV rep-cap plasmid should be also used.





pAAV + p-helper (E2A, E4, VA)

AAV only

In the absence of Rep proteins, ITR-flanked transgenes encoded within rAAV can form circular concatemers that persist as episomes in the nucleus of transduced cells. As the episomal DNA does not integrate into host genomes, it will eventually be diluted over time as the cell undergoes repeated rounds of replication. This will eventually result in the loss of the transgene and transgene expression, with the rate of transgene loss dependent on the turnover rate of the transduced cell.

AAVvectors based on the serotype 2 capsid have been the most commonly used for gene therapy studies and have demonstrated transduction in a large number of cell types and experimental model systems.

The vector can transduce nondividing cell types and has been used in muscle, retina, brain, liver, and lungs.

rate limiting steps for transduction and scAAV

1) conversion of the single-stranded (ssDNA) genome into the double-stranded DNA;
2) particle uncoating and/or transport to the nucleus;
3) dsDNA instability before integration

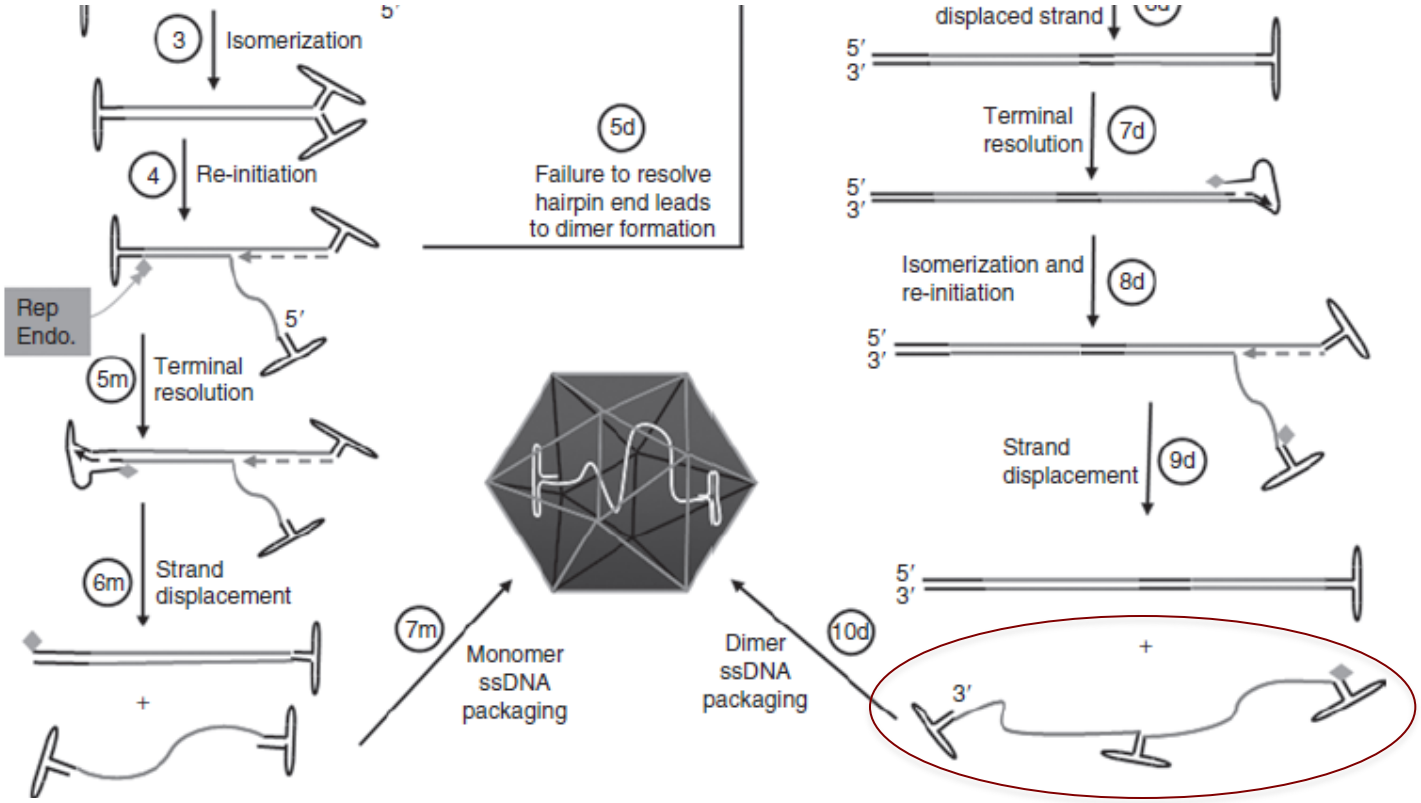
solutions

1) bypassed by the use of self complementary AAV (**scAAV**) which simply use dsDNA vectors
2) control the N pfu per cells (MOI moltiplicity of infection) in order to guarantee efficient integration; this may be cell specific
3) some treatments, such as DNA damaging factors or specific inhibitors of host cell factors, may enhance tranduction. However, their use in clinical settings is not feasible.

scAAV vectors

This can be achieved by taking advantage of the tendency to produce dimeric inverted repeat genomes during the AAV replication cycle. If these dimers are small enough, they can be packaged as a conventional AAV genomes.

Although complementary it is unlikely that the two halves of a scAAV genome are annealed into the virion shell[a]. Packaging depends on viral helicase activity suggesting that the DNA is unwound as it enters the capsid.



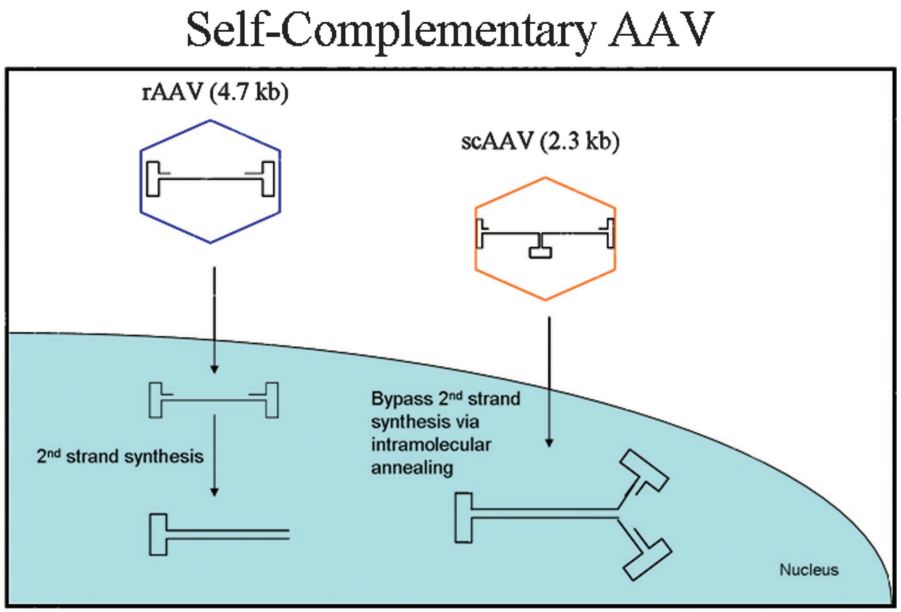
[a]Much of the ssDNA within the parvovirus virion is situated with the bases in contact with amino acid residues of the inner capsid shell, and the phosphate backbone sequestered toward the center, which would preclude substantial base pairing

scAAV vectors

dsAAV, can be made simply by reducing the vector construct size to ~2,500 base pair (bp) (2,200 bp unique transgene sequence plus two copies of the 145-bp ITR), such that the dimeric inverted repeat will be no larger than the normal AAV packaging capacity (~4,700 nucleotides).

In this case, the vector product is a mixture, containing virions with self-complementary genomes and virions that have packaged either one or two, monomeric ssDNA molecules. The proportion of dimeric to monomeric genomes varies widely between preps, from ~5 to >50% dimer.

In a study in which scAAV vector was made by this method, and contained only 3% dimeric genomes, a 600-fold increase in therapeutic efficacy over ssAAV vector was reported [1].

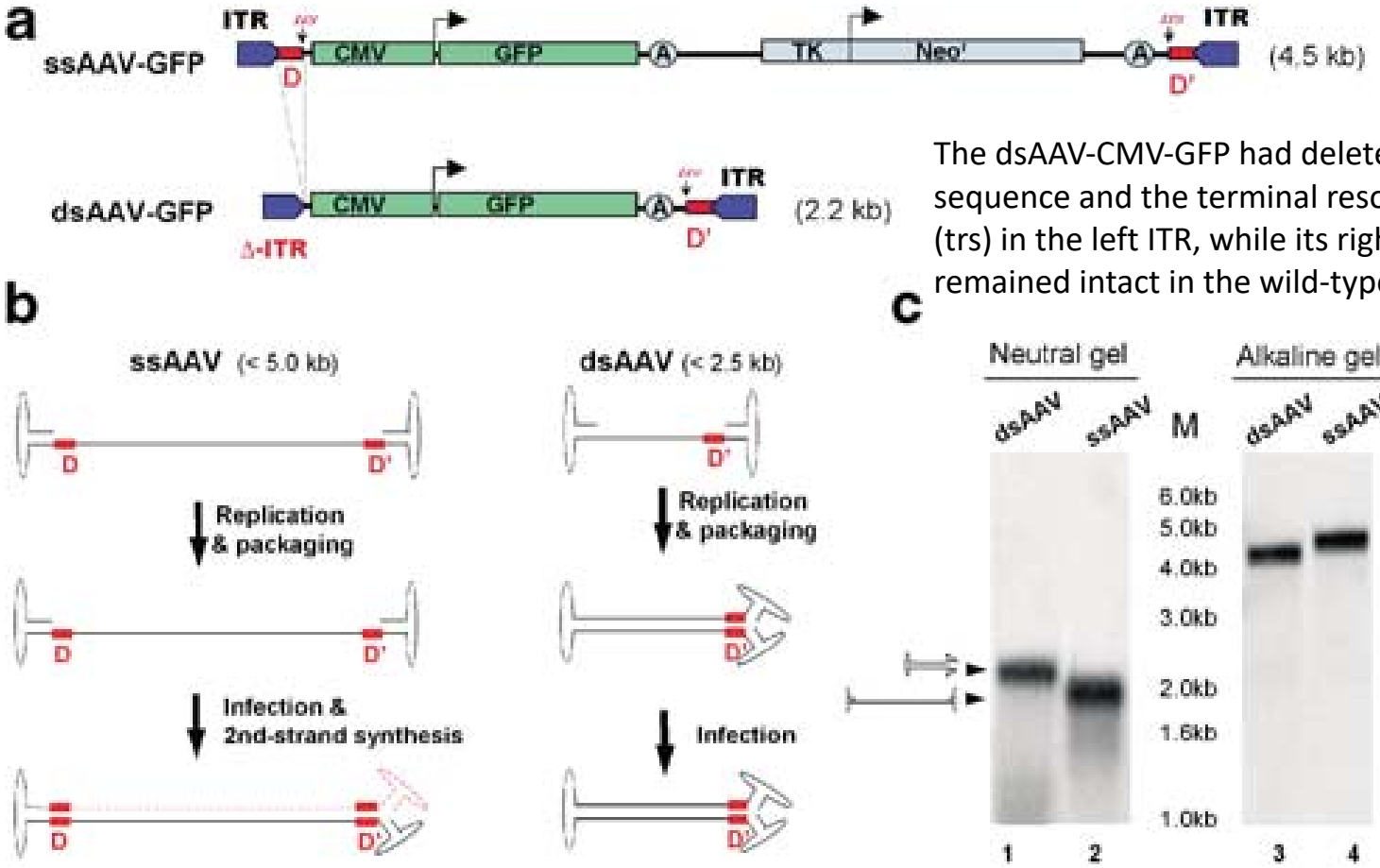


1) Koeberl, DD, Pinto, C, Sun, B, Li, S, Kozink, DM, Benjamin, DK Jr. et al. (2008). AAV vector-mediated reversal of hypoglycemia in canine and murine glycogen storage disease type Ia. Mol Ther 16: 665–672.

Construction and characterization of ssAAV and dsAAV vector

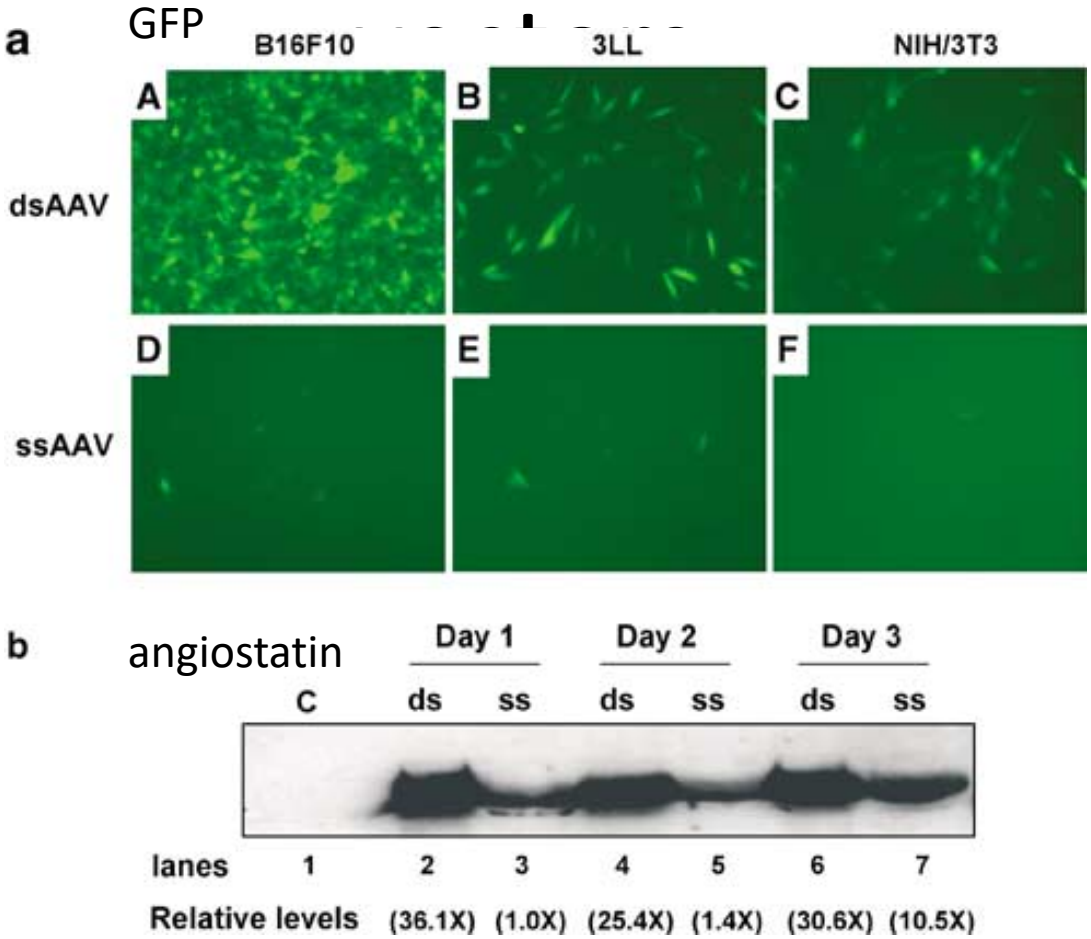
ssAAV-CMV-GFP vector (4.5kb) had two wild-type ITRs flanking the GFP and Neor gene expression cassettes.

The dsAAV-CMV-GFP had deleted the D-sequence and the terminal resolution site (trs) in the left ITR, while its right ITR remained intact in the wild-type form.



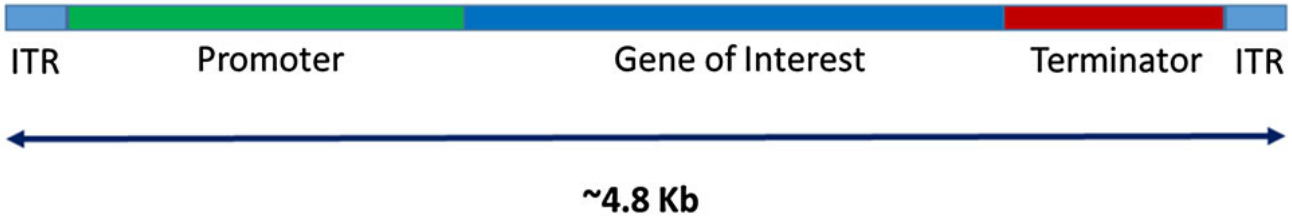
Diagrams of ssAAV and dsAAV viral DNA structures in the viral particles and in the host cells after infection. No conversion of ss- to dsDNA is required for the dsAAV after infection.

Comparison of transduction efficiencies by dsAAV and ssAAV



B16 F10, NIH3T3 and 3LL cells were infected with dsAAV-CMV-GFP or ssAAV-CMV-GFP vectors at 10 000 v.g./cell. Photos were taken 3 days after infection. (b**) Secretion of angiostatin** into the culture media from 293 cells after infection with dsAAV-CMV- angiostatin or ssAAV-CMV-angiostatin vectors at a dose of 500 v.g/cell.

AAV vectors



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The vector can transduce nondividing cell types and has been used in muscle, retina, brain, liver, and lungs.

How to chose the best AAV?

The choice of a particular AAV to use as a gene transfer vector is heavily reliant on several critically important criteria:

(1) which cell/tissue types are being targeted;
(2) the safety profile associated with the deliv- ered gene;
(3) the choice of systemic versus local delivery;
(4) the use of tissue-specific or constitutively active promoters.

AAV variant	Tissue tropism	Receptors	References
1	N/Sk	SA	[122–125]
2	Broad	HS, FGFR/HGFR, LR, a5b1	[126–130]
5	N, RPE, PR	SA, PDGFR	[123, 131–134]
6	Sk, Lg	SA, HS, EGFR	[48, 122, 135–137]
8	Lv, Sk, H, P	LR	[128, 138–144]
9	Lv, Sk, Lg	G, LR	[128, 140, 145]

AAV adeno-associated virus, *EGFR* epidermal growth factor receptor, *FGFR* fibroblast growth factor receptor, *G* galactose, *H* heart, *HGFR* hepatocyte growth factor receptor, *HS* heparan sulfate, *Lg* lung, *LR* laminin receptor, *Lv* liver, *N* neuronal, *P* pancreas, *PDGFR* platelet-derived growth factor receptor, *PR* photoreceptors, *RPE* retinal pigmented epithelia, *SA* sialic acid, *Sk* skeletal muscle

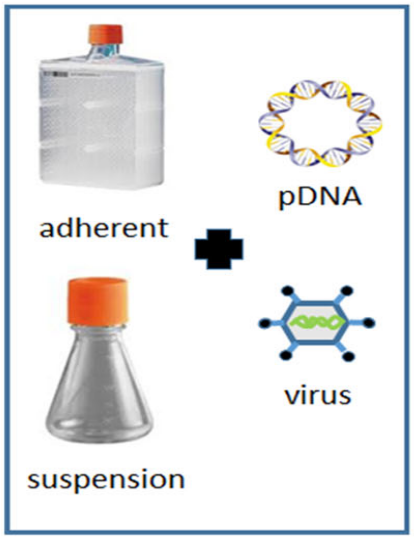
immunogenicity

In general, AAV has been shown to be less immunogenic than other viruses (i.e. adenovirus).

- However, the capsid proteins, as well as the nucleic acid sequence delivered, can trigger the various components of our immune system.
- Additionally, most people have already developed an immune response against the particular variants to which they had previously been exposed, resulting in a pre-existing adaptive response. This can include NAbs and T cells that could diminish the clinical efficacy of subsequent re-infections with AAV and/or the elimination of cells that have been transduced.

Large scale prepration and characterization

Cell Platform



Cell platform: HEK-293T, Sf9, or other suitable cell system can be grown on a small scale on 150 mm tissue culture-treated culture dish, hyperflasks, or shake flasks. Cells are then transfected with adenovirus helper virus, rep/cap, and ITR-transgene plasmids for 293T. Producer lines with integrated expression of rep/cap and ITR-transgene can be infected with adenovirus and grown to scale.

Scale-up

Scale-up: For larger-scale culture volumes, virus can be produced in roller bottles, continuous perfusion, or WAVE Bioreactor systems.

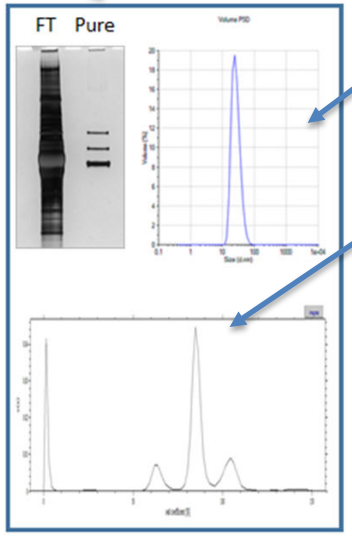
Large scale prepration and characterization

Purification/ Polishing

Purification/polishing: **Affinity or heparin chromatography** are optimal for isolation of virus from culture supernatants with or without cell pellet harvesting. **Benzonase/DNAse treatment** of eluted virus is required for removal of extraviral DNA contamination, followed by **anion-exchange chromatography** to fractionate 'empty' vs. 'full' AAV particles.

QC, quality control

QC/Release



Left, silver stain analysis of culture flow through (FT) next to affinity/ anion exchange purified AAV (pure). The three bands represent the viral capsid proteins VP1, VP2, and VP3.

Right, Dynamic light scattering analysis of purified AAV1 indicates a uniform particle distribution of approximately 25–30 nM.

Analytical ultracentrifugation can resolve the proportion of 'empty' vs, 'full' particles of purified material.

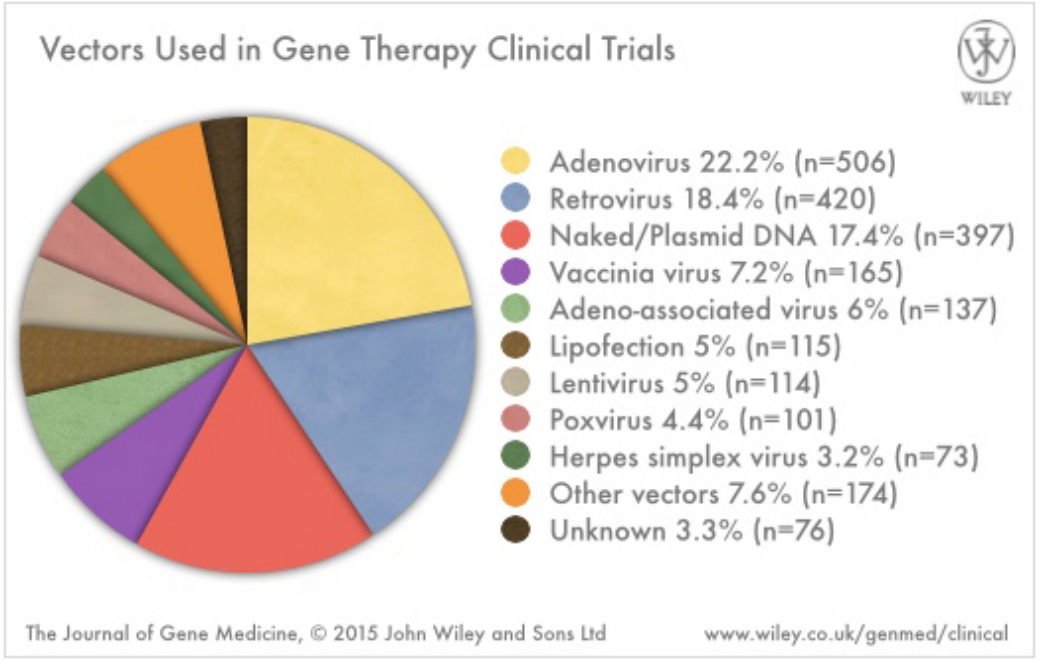
Additional assays include: PCR for determining titer in GC/mL, cryo or transmission electron microscopy for visual representation of purified particles, endotoxin testing, and other assays to evaluate the presence of residual host-cell protein contamination. AAV adeno-associated virus, FT flow-through, GC genome copies, rep/cap replication/cap- sid, QC quality control

Table 2 Current manufacturing platforms being employed to generate rAAV for clinical use

	Triple transfection (adherent)	Triple transfection (suspension)	Baculovirus-infected producer cell line	Herpes virus co-infection	Adenovirus-infected producer cell line
REP/CAP	Plasmid	Plasmid	Integrated in cell line	First rHSV	Integrated in cell line
ITR-transgene	Plasmid	Plasmid	BEV	Second rHSV	Integrated in cell line
Helper genes	Plasmid	Plasmid	BEV (same as above)	rHSVs (above)	Wt adenovirus
Cell line	HEK293 (adherent)	HEK293 (suspension)	Sf9 insect cells	BHK (suspension)	HeLa S3 (suspension)
Production system	CellFactory, roller, CellCube	Wave reactor (tens of liters)	200 L stirred tank reactor	10 L wave reactor	250 L stirred tank reactor
Efficiency of DNA delivery	++	+	+++	+++	+++
Scalability	−	++	+++	+++	+++
Yield (vector genomes/cell)	5×10^4 (AAV6) − 3.5×10^5 (AAV9)	9×10^4 (AAV4) − 2.1×10^5 (AAV2, AAV9)	8×10^2 (AAV12) − 5×10^5 (AAV3)	$7 \times 10^4 - 1 \times 10^5$	5×10^4
Safety concerns	None	None	None	Contaminating helper virus	Contaminating wild-type helper virus
Advantages	Quick to produce virus in small scale	Quick to produce virus in small scale	Added safety of insect cells and virus	No stable cell line required	Same helper virus for all production runs
	Helper virus-free AAV	Helper virus-free AAV	Efficient large-scale production	Efficient large-scale production	Efficient large-scale production
Challenges	Low scalability of triple transfection	Low scalability of triple transfection	Potentially low BEV stability	2 HSV helper viruses to produce	Stable producer cell line to produce for each project
				HSV sensitive to production conditions	
References	[146, 147]	[146, 148]	[87, 149, 150]	[151, 152]	[153]

AAV adeno-associated virus, *BEV* baculovirus expression vector, *HEK293* human embryonic kidney cell line, *rAAV* recombinant AAV, *REP/CAP* replication/capsid, *rHSV* recombinant herpes simplex virus type 1, *Sf9* Spodoptera frugiperda cell line

vectors in gene therapy trials 2015



Vector	Gene Therapy Clinical Trials	
	Number	%
Adeno-associated virus	**137**	**6.2**
Adenovirus	**480**	**21.7**

AAV in gene therapy clinical trials

Table 3 Selected examples of more than 50 clinical candidates employing rAAV

US trade name (generic name)	Company	Current status (US)	Molecular target	Major indication	Comments
Glybera® (alipogene tiparvovec)	uniQure	EMA approved, 11-2-12	LPL gene	LPL deficiency	AAV1; 20–40 or more shots to thigh muscle, depending on weight
Voretigene neparvovec (SPK-RPE65)	Spark Therapeutics	Phase III	RPE-specific protein (RPE65) gene	LCA (eye disease)	AAV2-hRPE65v2-101-based delivery of human RPE65 into the RPE (NCT00999609)
	MieraGTx UK II Ltd/Syne Qua Non Ltd/ UCL	Phase I/II	RPE-specific protein 65 kDa (RPE65) gene	LCA (eye disease)	AAV2/5 OPTIRPE65; ophthalmological (NCT02781480, NCT02946879)
rAAV2-CBSB-hRPE65	UPenn; NEI	Phase I/II	RPE-specific protein 65 kDa (RPE65) gene	LCA (eye disease)	rAAV2-CBSB carrying human RPE65 gene (NCT00481546)
rAAV2-hRPE65	HMO	Phase I	RPE-specific protein 65 kDa (RPE65) gene	LCA (eye disease)	rAAV2-hRPE65 delivery platform; ophthalmological (NCT02781480)
SPK-CHM	Spark Therapeutics	Phase I/II	Gene encoding defective/ missing REP-1	CHM (eye disease)	AAV2-hCHM for delivery to retina (NCT02341807)
CNGA3-ACHM	AGTC	Phase I	Achromatopsia CNGA3 gene	ACHM (blindness)	Ophthalmological conditions; subretinal injection (NCT02935517)
CNGB3-ACHM	AGTC	Phase I	Achromatopsia CNGB3 gene	ACHM (blindness)	rAAV2tYF-PR1.7-hCNGB3-delivered for ophthalmological conditions (NCT02599922)
scAAV2-P1ND4	NEI	Phase I	G11778A mutation in mitochondrial DNA	LHON (eye disease)	scAAV2-P1ND4v2 for gene therapy to correct G11778A mutation in mitochondrial DNA; (NCT02161380)
XLRS gene therapy	Biogen/AGTC	Phase I/II	Mutated XLRS gene	XLRS (eye disease)	rAAV2tYF-CB-hRS1 delivery platform; ophthalmological (NCT02416622)
BMN-270	Biomarin	Phase I/II	FVIII gene	Severe hemophilia A	(NCT02576795)
SB-525	Sangamo	Phase I/II	FVIII gene	Hemophilia A	Optimized AAV-cDNA hF8 construct (NCT03061201)
DTX101	Dimension Therapeutics	Phase I/II	FIX gene	Hemophilia B	AAVrh10 (NCT02618915)
SPK-9001 (SPK-FIX)	Spark Therapeutics/ Pfizer	Phase I/II	FIX19 variant gene	Hemophilia B	AAV8 expressing a codon-optimiZed, high-activity human factor IX variant (NCT02484092, NCT01620801)
AMT-060	uniQure/St. Jude's Hospital	Phase I/II	FIX gene	Hemophilia B	AAV5; 9 mo of sustained factor IX activity (NCT02396342)
SB-FIX	Sangamo	Phase I	FIX gene	Hemophilia B	AAV2/6 delivered ZFN technology to repair/replace FIX (NCT02695160)
scAAV2/8-LP1-hFIXco	St. Jude's Hospital/UCL	Phase I	FIX gene	Hemophilia B	AAV 2/8-LP1-hFIXco encoding FIX for hemophilia B (NCT00979238)
ADVM-043	Adverum	Phase I	AAT gene	AAT deficiency	AAVrh.10halpha1AT (NCT02168686)
AVXS-101	AveXis	Phase I	SMN gene	SMA	SC AAV9-SMN, which crosses BBB (NCT02122952)

Table 3 continued

US trade name (generic name)	Company	Current status (US)	Molecular target	Major indication	Comments
rAAVrh74.MCK. micro-Dystrophin	NICHD	Phase I	MicroDMD gene	DMD	rAAVrh74.MCK.micro-Dystrophin vector administered by IM route (NCT02376816)
LGMD2D	NCH	Phase I/II	α-Sarcoglycan gene	LGMD2D	SC AAVrh74.tMCK.hSGCA delivered systemically (NCT01976091)
rAAV1.CMV. huFollistatin344	NCH	Phase I	Follistatin gene	BMDSIBM	AAV1-based delivery of follistatin gene (FS344) to muscle to build muscle size and strength (NCT01519349)
rAAVrh74.MHCK7.DYSF.DV	NCH	Phase I	Dysferlin gene	Dysferlin deficiency	IM injection of rAAVrh.74.MHCK7.DYSF.DV gene vector to the EDB muscle (NCT02710500)
ART-102	Arthrogen	Phase I	NF-κB and IFN-β genes	RA	IA administration of AAV5.NF-kB. IFN-β in subjects with RA and active arthritis in the joint (NCT02727764)
Intracerebral gene therapy	INSERM	Phase I/II	ARSA gene	Metachromatic leukodystrophy	AAVrh.10 vector used to transfer cDNA encoding ARSA into the brain of children (NCT01801709)
CERE-110	Ceregene	Phase II	NGF gene	Alzheimer's disease	CERE-110 injected into the brain during a surgical procedure (NCT00876863)
CERE-120	Ceregene/ Sangamo	Phase I/II	Neurturin gene	Idiopathic Parkinson's disease	AAV engineered to carry the human gene for neurturin (NCT00985517)
AAV-hAADC	NIH	Phase I	AADC gene	GERT for AADC deficiency	AAV2-hAADC delivered to the SNc and VTA in children with AADC deficiency (NCT02852213)
AAV2CUhCLN2	Weill Cornell University	Phase I	TPP1	GERT for LINCL (form of Batten disease)	Direct CNS administration of AAV2 encoding human TPP1 cDNA (NCT00151216)
	Abeona Therapeutics	Phase I/II	SGSH gene	GERT for MPSIIIA (Sanfilippo A syndrome)	SC AAV9.U1a.hSGSH injected IV through a peripheral limb vein (NCT02716246)
SAF-301	Lysogene	Phase I/II	SGSH and SUMF1 genes	GERT for MPSIIIA (Sanfilippo A syndrome)	SAF-301 (AAV10-SGSH-SUMF1 cDNA) directly injected into both sides of the brain through six image-guided tracks, with two deposits per track, in a single neurosurgical session (NCT01474343)
DTX301	Dimension Therapeutics	Phase I	OTC gene	GERT to correct blood ammonia accumulation	AAV8-OTC-based delivery gene therapy to correct OTC deficiency (NCT02991144)

AAT α1 antitrypsin, *AAV* adeno-associated virus, *ACHM* achromatopsia, *AGTC* Applied Genetic Technologies Corporation, *ARSA* arylsulfatase A, *BBB* blood–brain barrier, *BMDSIBM* Becker muscular dystrophy sporadic inclusion body myositis, *cDNA* complementary DNA, *hAADC* human aromatic L-amino acid decarboxylase, *hCHM* human choroideremia, *CNG* cyclic nucleotide-gated, *CNGA3* alpha subunit of the cone photoreceptor CNG, *CNGB3* beta subunit of the cone photoreceptor CNG, *CNS* central nervous system, *DMD* Duchenne muscular dystrophy, *EDB* extensor digitorum brevis, *EMA* European Medicines Agency, *GERT* genetic enzyme replacement therapy, *FVIII* factor VIII, *FIX* factor IX, *HCV* hepatitis C virus, *HMO* Hadassah Medical Organization, *IA* intra-articular, *IFN* interferon, *IM* intramuscular, *IV* intravascular, *LCA* Leber congential amaurosis, *LGMD2D* limb girdle muscular dystrophy type 2D, *LHON* Leber's hereditary optic neuropathy, *LINCL* late infantile neuronal ceroid lipofuscinosis, *LPL* lipoprotein lipase, *MPS* mucopolysaccharidosis, *NCH* Nationwide Children's Hospital, *NCT* National Clinical Trial, *NEI* National Eye Institute, *NF-κB* nuclear factor-κB, *NGF* nerve growth factor, *NICHD* Eunice Kennedy Shriver National Institute of Child Health and Human Development, *NIH* National Institutes of Health, *OTC* ornithine transcarbamylase, *RA* rheumatoid arthritis, *rAAV* recombinant AAV, *REP-1* Rab escort protein-1, *RPE* retinal pigment epithelium, *SC* self-complementary, *SGSH* N-sulfoglucosamine sulfohydrolase, *shRNA* short hairpin RNA, *SMA* spinal muscular atrophy, *SMN* survival motor neuron, *SNC* substantia nigra pars compacta, *SUMF1* sulfatase modifying factor-1, *TPP1* lysosomal enzyme tripeptidyl peptidase 1, *UCL* University College, London, *UPenn* University of Pennsylvania, *VTA* ventral tegmental area, *XLRS* X-linked juvenile retinoschisis, *ZFN* zinc-finger nuclease

AAV CLINICAL TRIALS

TABLE 2. Clinical trials involving AAV vectors

Condition	Gene product(s)	Phase
CF	CFTR	I/II
Canavan's disease	Aspartoacylase	I
Parkinson's disease	GAD65, GAD65, AADC, neurturin	I
Alzheimer's disease	Beta nerve growth factor	I
Alpha-1-antitrypsin deficiency	AAT	I
Arthritis	TNFR:Fc	I
Leber congenital amaurosis	RPE65	I
Hemophilia B	Factor IX	I
Late infantile neuronal lipofuscinosis	CLN2	I
Muscular dystrophy	Minidystrophin, sarcoglycan	I
Heart failure	SERCA-2a	I
Prostate cancer	Granulocyte-macrophage colony-stimulating factory	I/II/III
Epilepsy	Neuropeptide Y	I

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AAV gene therapy: Hemophilia

The two common forms are hemophilia A and hemophilia B. Clotting requires a complex series of enzymatic reactions. Two of the required enzymes are factors VIII, the lack of which causes hemophilia A, and IX (hemophilia B).

The coding region factor IX and regulatory sequences could readily be encapsidated in the AAV vector.
Preclinical models: A factor IX AAV vector could be used to "cure" mice with hemophilia B and, more excitingly, also performed well in a canine model of hemophilia.

Clinical studies:
1.Initial phase I studies, performed by the intramuscular injection of an AAV-2 vector, could not rise serum factor IX concentration at therapeutic level.
2.Changing the vector tropism to the liver (hepatic artery) give rise to detectable transgenic factor IX in the serum for 4 to 9 weeks in the two subjects, at the highest dose ($2x10^{12}$ vector genomes/kg). More troublesome was a rise in liver transaminases in the serum, a sign of liver inflammation. Possibly at the highest dose, the multiplicity of infection (MOI) was sufficiently high that degradation products of the capsid were displayed on the surface of the transduced hepatocytes in sufficient quantity to induce the CTL response

The dose required to produce a detectable level of factor IX was also sufficient to induce a CTL response, which destroyed the cells expressing factor IX.

Possible solutions: 1) induce tolerance to AAV capside fragments; 2) development of a more efficient vector, which would enable a much lower MOI or dose so that the immune response would not be evoked.

rheumatoid arthritis

Rheumatoid arthritis is a disabling inflammatory disease in which the immune system reacts against the body's joint tissue. One way to counteract the effects of the cytokine tumor necrosis factor alpha (TNF-α) is the the use of the drug **adalimumab**.
Alternatively, AAV vector expressing a TNF inhibitor for an extended period of time, could be used.
Preclinical models: promising data were achieved in the animal model of disease.

Clinical studies: in the phase I clinical trial, one patient became extremely ill the day after the administration of the AAV vector and died within 4 days. Subsequent investigation established that the patient had died of an overwhelming *Histoplasmosis capsulatum* fungal infection. The patient had also been treated with adalimumab, one of whose side effects is known to be sepsis.
Whether this infection was not controlled because 1) of the adalimumab drug (the patient had also been treated with adalimumab) or 2) the TNF-a inhibitor expressed from the transgene, remain an open question.

Parkinson's disease

In Parkinson's disease, a loss of dopaminergic neurons leads to the loss of inhibitory gamma aminobutyric acid-sensitive input to the subthalamic nucleus.
Clinical study: 12 patients with advanced Parkinson's disease had an AAV vector carrying a transgene encoding glutamic acid decarboxylase injected into the subthalamic nucleus on one side.
Treatment: The patients received low, moderate, or high doses of the vector. The therapy was well tolerated, with no adverse effects attributable to gene therapy.

Therapeutic effects: The clinical impression was that motor activity on the treated side was improved significantly relative to the un- treated side regardless of dose. The observed improvement in motor activity persisted for at least 1 year.

conclusion from clinical trials

1. The first is that there has been relatively little toxicity that can be directly attributed to the AAV vector platform. Potential toxicity appears to arise from an inflammatory response involving cytotoxic T cells responding to fragments of the coat proteins from input vector. This seems to be dose-dependent.

2. Humoral immunity seems to play a role in some instances when the subsequent administration of a vector may be blocked, but toxicity per se has not been a significant observation. Here, the route of administration seems to be important; little humoral immunity has been noted when the pulmonary route is used

Raffronto fra diversi metodi di trasduzione utilizzati in terapia genica

	Adenovirus	**AAV**	**Retrovirus**	**Non virali**
Tropismo	Ampio	Ampio	Specifico	Vario
Dimensioni dell'inserto	<8 kb	<4.5 kb	5-6 kb	Illimitato
Integrazione nel genoma	No	Si	Si	Limitata
Divisione cellulare necessaria	No	No	Si	Preferibile
Espressione a lungo termine	No	Si	Si	No
Risposta immune	Elevata	Medio-bassa	Bassa	Bassa
Efficienza in vivo	Elevata	Elevata	Elevata	Bassa

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Pioneer Perspective

Adenovirus:
The First Effective *In Vivo* Gene Delivery Vector

Ronald G. Crystal