Herpes Simplex Virus Type 1/Adeno-Associated Virus Hybrid Vectors

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Abstract: Herpes simplex virus type 1 (HSV-1) amplicons can accommodate foreign DNA of any size up to 150 kbp and, therefore, allow extensive combinations of genetic elements. Genomic sequences as well as cDNA, large transcriptional regulatory sequences for cell type-specific expression, multiple transgenes, and genetic elements from other viruses to create hybrid vectors may be inserted in a modular fashion. Hybrid amplicons use genetic elements from HSV-1 that allow replication and packaging of the vector DNA into HSV-1 virions, and genetic elements from other viruses that either direct integration of transgene sequences into the host genome or allow episomal maintenance of the vector. Thus, the advantages of the HSV-1 amplicon system, including large transgene capacity, broad host range, strong nuclear localization, and availability of helper virus-free packaging systems are retained and combined with those of heterologous viral elements that confer genetic stability to the vector DNA. Adeno-associated virus (AAV) has the unique capability of integrating its genome into a specific site, designated AAVS1, on human chromosome 19. The AAV *rep* gene and the inverted terminal repeats (*ITRs*) that flank the AAV genome are sufficient for this process. HSV-1 amplicons have thus been designed that contain the *rep* gene and a transgene cassette flanked by AAV *ITR*s. These HSV/AAV hybrid vectors direct site-specific integration of transgene sequences into AAVS1 and support long-term transgene expression.

Keywords: HSV-1 amplicon vectors, adeno-associated virus, herpes simplex virus type 1, hybrid vectors.

HERPES SIMPLEX VIRUS TYPE 1 - BIOLOGICAL PROPERTIES

Herpes simplex virus type 1 (HSV-1) is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae,* genus *Simplexvirus*. It is a common human pathogen that causes infections of the orofacial mucosal surfaces and may rarely cause acute hepatitis, kerato-conjunctivitis or meningo-encephalitis.

The HSV-1 particle is composed of three different compartments, capsid, tegument, and envelope. The capsid proteins are involved in the formation and maturation of the icosahedral capsid [1] and packaging of the viral genome [2-6]. The tegument, which is located between capsid and envelope, is composed of viral proteins involved in transport of capsids to nuclear pores, attachment to the nuclear pore complex [7], release of the virus genome from the capsid into the nucleus [8], and remodeling the host cell environment to optimize replication [9-14]. The viral envelope is a lipid bilayer of host origin that contains 11 viral glycoproteins. These play important roles in viral attachment, entry, cell to cell spread, and egress [15, 16]. HSV-1 can enter the cells by receptor-mediated fusion between virus and cell membrane [17-20]. However, depending on the cell type and virus strain, HSV-1 can penetrate the host cell also by endocytosis [20-22] and phagocytosis [23]. In the cytoplasm, the capsid is transported to the nucleus *via* interactions with the minus-end-directed microtubule motor protein dynein [24-26] Capsids bind to the nuclear pore complex and release the DNA genome into the nucleus [7, 27, 28].

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The HSV-1 genome is a double-stranded DNA (dsDNA) of 152 kbp. It is organized in two segments, unique long (UL) and unique short (US), both of which are flanked by inverted repeats (see Fig. **1**). The essential *cis* elements for viral DNA replication and encapsidation include the origins of DNA replication, located in the UL (ori_L) and TR_S, (ori_S) regions [29, 30], and the packaging/cleavage signals (pac) that reside in the *a* sequences located at both termini of the genome as well as at the junction between the long and the short segments [31]. The viral genome circularizes after it reaches the nucleus [32] and serves as a template for DNA replication. However, there is also evidence that circularization is not required for replication [33]. The majority of the replicative intermediates are long concatemers that are thought to have been synthesized by a rolling-circle mechanism [34-36]. The concatemers are cleaved into unit-length genomes at the *pac* signals after filling pre-formed capsids [36, 37].

HSV-1 encodes approximately 89 genes [38], which are expressed in a cascade of three temporal phases: immediate-early, early, and late. The late genes can be subdivided in leaky-late (expression is not dependent on viral DNA synthesis) and true-late (expression depends on viral DNA synthesis) [31, 39, 40].

There are several hypotheses on the mechanisms of envelopment of the nucleocapsid. The generally accepted view suggests a two-step envelopment process in which the capsid acquires a primary envelope by budding at the inner nuclear membrane and then is de-enveloped by fusion with the outer nuclear membrane [41, 42]. The secondary envelope is acquired when the capsid buds into the Golgi or cytoplasmic vesicles [43-49]. The alternative pathways described include (i) budding at the inner nuclear membrane followed by intraluminal transport *via* ER and Golgi and (ii)

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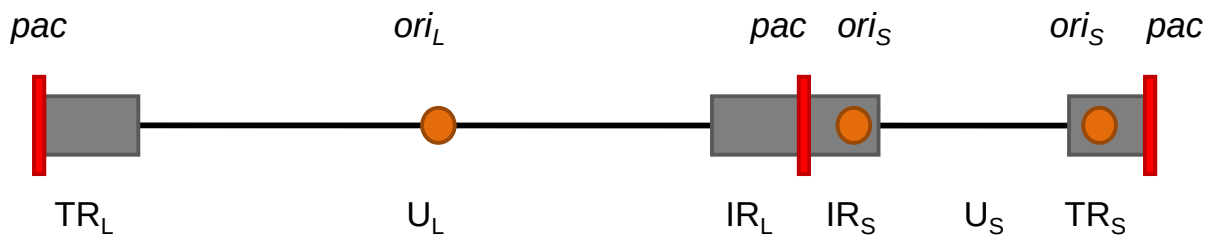

Fig. (1). Schematic representation of the HSV-1 genome. The HSV-1 genome is a linear double stranded DNA of approximately 152 kb in size, composed of two unique segments, U_L and U_S, which are flanked by inverted repeats, TR_L /IR_L and IR_S/TR_S. The minimal *cis* elements required for HSV-1 DNA replication and packaging include the origin of DNA replication, *ori,* and the cleavage/packaging, *pac,* signals.

exit *via* impaired nuclear pores and envelopment at the outer nuclear membrane or ER membrane [50-52]. Regardless of the mechanism of envelopment, mature virions seem to exit the cell by exocytosis *via* intraluminal transport to Golgi cisternae and formation of transport vacuoles [53-55].

An important aspect of HSV-1 biology is the capability of this virus to establish latent infections in sensory neurons of the trigeminal ganglia [56]. The latent HSV-1 genome is a circular, condensed episome, and viral gene expression is limited to the non-coding, latency-associated transcripts (LATs) [57]. Expression of LATs was demonstrated to increase the number of neurons in which latency is established [58] and to affect the efficiency of reactivation [59, 60]. Recent findings that LAT encodes several micro RNAs (miRNA) in HSV-1 infected cells corroborates with the proposed hypothesis that the exonic regions of LAT might function as primary miRNA precursors [61]. At least two of the identified miRNA precursors in latently infected neurons may facilitate the establishment and maintenance of viral latency by post-transcriptionally regulating viral gene expression [62-65].

Latent HSV-1 can periodically reactivate in response to a variety of stimuli, including fever, UV light, hormonal imbalance, malignancy or immune suppression, and enter a new lytic cycle, usually at the site of the primary infection. Recently, the requirement of ICP0 for viral DNA replication [66-68] and for exit from latency has been reconsidered, as *in vivo* studies showed that reactivation of HSV-1 genomes does not depend on viral DNA amplification [69] nor functional ICP0 [70]. Upon stress conditions, and in the absence of other viral proteins, VP16 was demonstrated to be activated [71], supporting the hypothesis that *de novo* expression of VP16 regulates entry into the lytic program in neurons. Repeated reactivation does not appear to kill the neurons, indicating that the extent of virus replication must be limited.

The understanding of the biological properties of HSV-1 and the molecular mechanisms of virus replication have allowed the design of specialized vector systems for somatic gene therapy, oncolytic virotherapy, and vaccination.

HSV-1 BASED VECTOR SYSTEMS

HSV-1 is an attractive vector for gene therapy due to its (i) large transgene capacity, (ii) high transduction efficiency and broad cell tropism that includes both dividing and non-dividing cells, and (iii) ability to establish latency while maintaining at least some transcriptional activity. However, as HSV-1 is a human pathogen, its use as a vector can result in host immune responses and cytopathogenic effects in patients, and possibly reactivation of and recombination with latent wild-type HSV-1. Taking these aspects into consideration, two different HSV-1-based vector systems, recombinant and amplicon, have been developed.

Initially, recombinant herpesviruses were constructed for functional studies of viral genes and development of vaccines. However, advances in site-specific modification of the viral genome facilitated the use of HSV-1 as a gene transfer vehicle [72]. Different approaches for the construction of recombinant HSV-1 vectors are based on the target tissue and purpose of gene delivery e.g. replication-conditional recombinant HSV-1 vectors are suitable for therapeutic treatment of tumors; replication-defective recombinant HSV-1 vectors are applied for gene replacement therapy [73]. Although some preclinical studies show promising results, several obstacles have to be overcome: (i) replication-defective mutants of HSV-1 can cause cytopathic effects in primary cultures of neuronal cells and inflammatory responses in neural tissue *in vivo*; (ii) most viral and nonviral promoters are silenced after injection into the brain. Therefore, the main focus in the development of new HSV-1-based vectors has been directed at achieving nontoxic, long-term gene expression in neurons.

The second type of HSV-1-based vector system, the HSV-1 amplicon vector, originated about three decades ago. Spaete and Frenkel analyzed the nature of defective virus genomes generated during the passage of standard HSV-1 stocks at high multiplicity of infection [74, 75]. Their investigations revealed that an *ori* and a *pac* signal were the only two *cis*-acting sequences required for the replication and packaging of defective virus genomes in the presence of *trans*-acting HSV-1 helpervirus (Fig. **2A**). The word amplicon was used to delineate the fact that multiple copies of a DNA sequence of interest can be amplified in a head-to-tail arrangement in concatemeric defective virus genomes and packaged into HSV virions [76]. HSV-1 amplicon vectors share similar structural and immunological properties with the wild type HSV-1 particle, which can trigger cell signaling and cellular responses that may have a transient impact on cell homeostasis or gene expression. However, the lack of virus genes and protein synthesis reduces the risk of reactivation, complementation and recombination with latent or resident HSV-1 genomes.

HSV-1 amplicon vectors have been used to infect efficiently a number of different cell types, including

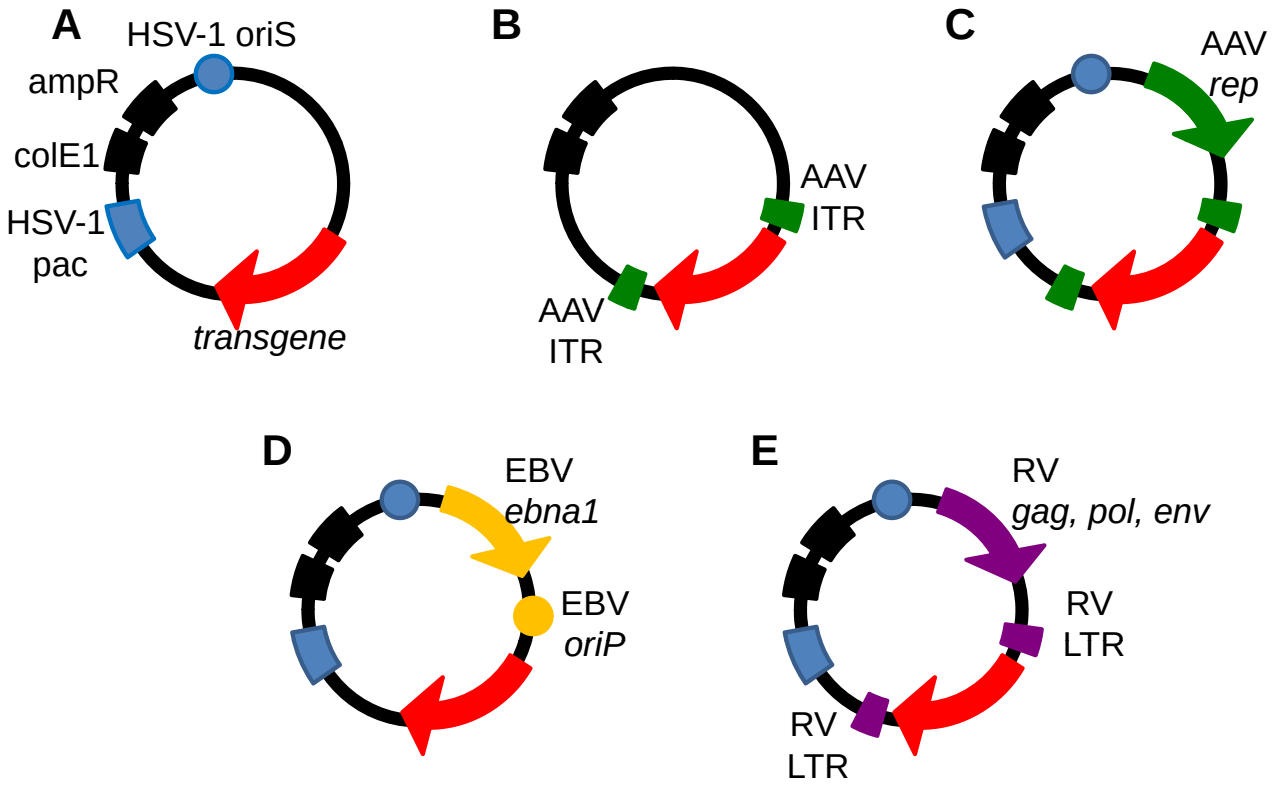

Fig. (2). Viral vectors. A) HSV-1 amplicon. The HSV-1 amplicon contains three types of genetic elements: i) sequences from bacteria (colE1 and ampR) that allow plasmid propagation in *E. coli*; ii) sequences from HSV-1, in particular an origin of DNA replication (*ori)* and a DNA packaging/cleavage signal (*pac*), which allow replication and packaging of the amplicon DNA into HSV-1 particles in the presence of HSV-1 helper functions in mammalian cells; and iii) a transgene cassette with the gene of interest. **B) Recombinant AAV vector.** Recombinant AAV vectors are bacterial plasmids that contain the AAV ITRs flanking a transgene of interest. Replication of the ITR cassette and packaging into AAV particles is achieved by supplying helpervirus functions and the *rep* and *cap* genes in *cis* or *trans* but outside the ITR cassette. **C) HSV/AAV hybrid amplicon.** In addition to the HSV-1 amplicon elements, HSV/AAV hybrid amplicon vectors contain the AAV *rep* gene and a transgene of interest flanked by AAV ITRs. **D) HSV/EBV hybrid amplicon.** In addition to the HSV-1 amplicon elements, HSV/EBV hybrid amplicon vectors contain the EBV origin of DNA replication (oriP) and the gene encoding EBNA-1 which together can support episomal retention and segregation of the vector in dividing cells. **E) HSV/RV hybrid amplicon.** In addition to the HSV-1 amplicon elements, HSV/RV hybrid amplicon vectors contain the retrovirus (MoMLV) *gag*, *pol*, and *env* genes, and the RV LTRs flanking a transgene of interest.

epidermal cells and dendritic cells in the skin [77, 78], some cell types in the cochlea [79, 80], hepatocytes [81], skeletal muscle [82], neurons [83], glioblastoma, and other tumor cells [84-86]. Despite the promising features of the HSV-1 amplicon vector as a gene delivery system, further developments concerning vector production, stability of transgene expression, and interaction with target cells are essential. Recently, the presence of bacterial sequences in the amplicon genome was shown to be responsible for the formation of inactive chromatin, leading to a rapid transgene silencing [87]. Strategies to increase the stability of transgene expression included the use of: (i) cell type-specific promoters [88, 89] and (ii) genetic elements from other viruses that confer genetic stability, such as integration of the transgene into host chromosomes [90, 91] or conversion of the amplicon genome into a replication-competent extrachromosomal element [92-95].

One of these strategies, the combination of genetic elements from HSV-1 amplicons and adeno-associated virus (AAV) to achieve site-specific integration of the transgene into the host genome and long-term transgene expression is described in detail below, after a short introduction into AAV biology.

ADENO-ASSOCIATED VIRUS – BIOLOGICAL PROPERTIES

Adeno-associated virus (AAV) belongs to the genus *Dependovirus* within the subfamily *Parvovirinae,* family *Parvoviridae* [96]. Different AAV serotypes have been identified that can infect a broad range of species; about 11 serotypes and more than 100 variants of AAV infect primates. Based on serological studies, AAV serotypes 2, 3, and 5 most probably have a human origin [97, 98], while AAV4 appears to have originated in monkeys [99]. AAV6 shares some genomic similarities with AAV2 and AAV1, raising the hypothesis that a recombination event could have occurred *in vivo* or in cell culture [100, 101]. The AAV serotypes 1 to 6 were isolated as contaminants in laboratory adenovirus stocks, while AAV 7, 8, 9, 10, and 11 were isolated as DNA molecules using a "signature PCR", a

screening-based strategy [102]. Despite being widespread among species and infecting different tissues, AAV infections have not been associated with any pathology. Primate AAV serotypes share significant sequence similarities, and the occurrence of cross-reaction of neutralizing antibodies may be species specific or depend on tissue type or route of administration [101, 103, 104].

The genome of AAV is a linear single stranded DNA of 4.7 kb, and either the positive or negative strand can be packaged with equal efficiency. The genome is flanked by inverted terminal repeats (*ITR*) of 145 bp, containing a palindromic sequence that forms a hairpin as a T-shaped secondary structure. The Rep binding site (RBS) and the terminal resolution site (TRS) are regions within the *ITR*s that play important roles in the replication and packaging of the AAV genome [105] (Fig. **3A**). Two open reading frames (ORFs), Rep and Cap, are responsible for encoding overlapping proteins through alternative splicing (Fig. **3B**). The Rep proteins, Rep78/68, and Rep52/40 are transcribed from two different promoters, p5 and p19, respectively, and are involved in DNA replication, transcription, and chromosomal integration. The p5 promoter contains a TATA box, a RBS, a TRS, the Yin Yang 1 (YY1) binding site, and a downstream sequence that can form a hairpin structure. The RBS is involved in Rep-mediated promoter regulation activity [106, 107], thus in the absence of helper functions small amounts of Rep are expressed that bind to the p5 promoter inhibiting transcription [108, 109]. The regulatory activity of Rep seems to be involved in the maintenance of a constant ratio of Rep and Cap proteins during infection in order to keep the balance between AAV genome replication and packaging. The Cap ORF encodes three overlapping proteins, VP1, VP2 and VP3, from a single promoter, p40. These structural proteins compose the AAV icosahedral capsid whose diameter ranges from 18 to 26 nm [110].

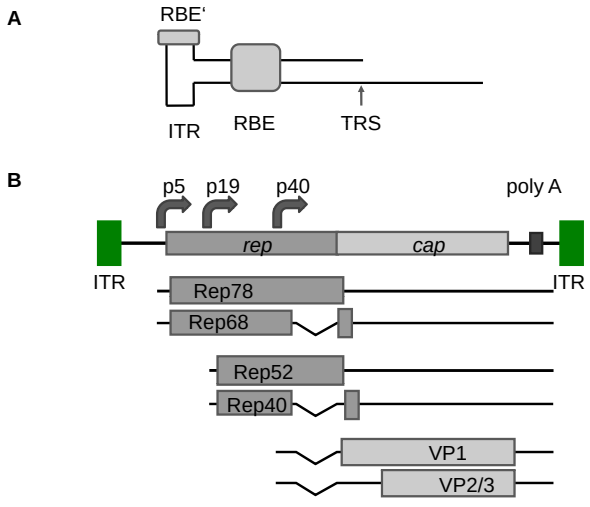

Fig. (3). Schematic map of the wild type AAV genome. A) Secondary structure formed by the inverted terminal repeat, ITR. Depicted are the Rep binding sites, RBEs, and the terminal resolution site, TRS. **B**) The AAV genome expresses two clusters of genes, *rep* and *cap*, from three different promoters, p5, p19, p40, by alternative splicing.

The replication of the AAV genome is based on a "rolling hairpin model". The hairpin structure at the *ITR* acts as a primer that converts the DNA into a double-stranded template, and together with the essential *cis*-acting elements RBS and TRS, and helpervirus functions, the replication and transcription of the AAV genome is initiated [111-113]. The Rep78/68 proteins play major roles in the replication process due to DNA-binding, endonuclease, and helicase activities. After binding to the RBS, Rep induces a site- and strand-specific nick at the TRS, creating a new genome end allowing the reinitiation of the synthesis and formation of a monomer extended form that can be packaged [114]. If the hairpin structure in the monomer turnaround form is not resolved before reinitiation on the other genome end, continued synthesis leads to double stranded dimer molecules, in which two genomes in the inverted orientations (head-to-head or tail-to-tail) are covalently joined by a single *ITR* [115]. Interestingly, the RBS and TRS signals located within the p5 promoter sequence have been demonstrated to act together as an alternative origin of DNA replication in the presence of adenovirus [116, 117] or HSV-1 helpervirus functions [108]. Replication from a plasmid cloned p5 replication origin led to the accumulation of large, head-to-tail linked concatameric replication products, which could readily be packaged into HSV-1 virions if the HSV-1 packaging/cleavage signal was included on the plasmid [108]. These findings indicate that the AAV p5 replication origin could substitute for the HSV-1 origin of DNA replication on HSV/AAV hybrid vectors (see below). AAV is a replication defective virus as it depends on a helper virus, such as adenoviruses [118], a herpesvirus [119-121], or vaccinia virus [122] for productive replication (Fig. **4**). Helper viruses are also responsible for inducing a cell cycle arrest in late S or G2 phase, as in the case of adenoviruses [123] or for down regulating host cell functions as in the case of HSV-1 as the helpervirus [124, 125].

Many studies have assessed the different elements from the helperviruses required for AAV replication. A model has been proposed where the HSV-1 helicase/primase proteins constitute a scaffold that recruits ICP8, Rep and cellular replication (RPA) proteins to the self-primed AAV DNA into replication compartments [126-129]. The HSV-1 polymerase complex is preferentially used for AAV replication instead of the cellular machinery [113, 130]. Interestingly, an inhibitory effect of Rep78/68 proteins has been described on HSV-1 replication [131, 132], suggesting a regulatory effect of AAV over HSV-1, thereby limiting expression of HSV-1 early genes [113, 129].

In the absence of helpervirus, the AAV genome can integrate into a specific site termed AAVS1, on chromosome 19q13.3-qter of human cells [133-136] (Fig. **4**). The integration is mediated by Rep78/68 and *ITR*s through a nonhomologous deletion/insertion recombination event [134, 136-143]. Also, an integration efficiency element (IEE) has been identified within the p5 promoter of AAV [144], and more specifically a 16-bp RBE was shown to be sufficient for AAV genome integration [145]. Rescue of the integrated AAV genome is possible by superinfection with helper virus [130]. Although HSV-1 ICP0 seems to contribute to the activation of the *rep* gene from latent AAV genomes [146],

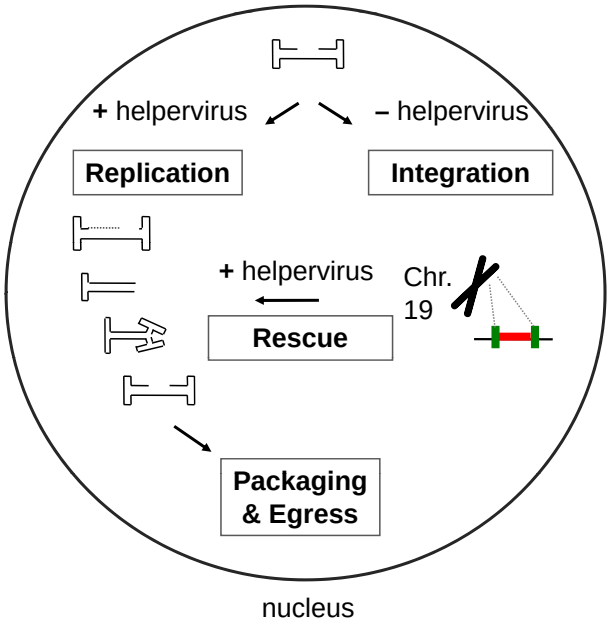

Fig. (4). The life cycle of AAV. Co-infection of AAV and helpervirus, adenovirus or HSV-1, leads to viral gene expression, viral DNA replication, and production of progeny virus. In the absence of helpervirus, the genome of AAV can integrate into a specific site on human chromosome 19. In the presence of helpervirus, integrated AAV genomes are rescued and enter the lytic replication cycle.

it is not sufficient to induce *rep* synthesis [130]. Some studies have demonstrated the autonomous replication of AAV under special conditions [147-149], however, the efficiency of replication is significantly lower than in presence of helpervirus functions.

AAV can infect different tissues and bind to unique cellular receptors, which can account for a serotype-specific tissue tropism. Several cellular receptors used by AAV for cell entry have been identified, including heparan sulfate [150], fibroblast growth factor receptor [151], and integrin alphaVbeta5 [152]. The initial steps of AAV infection, attachment to cellular glycosaminoglycan receptors and interactions with coreceptors seem to define the intracellular trafficking pathway of the capsid. Upon entry, AAV capsids are endocytosed *via* clathrin-coated pits [153], a process that requires dynamitin [154], and transported through both late and recycling endosomes. Trafficking in recycling endosomes appears to be favorable for efficient transgene expression [155]. The process of uncoating is still not well characterized [153, 156], however, AAV appears to enter the nucleus through a mechanism independent of the nuclear pore complex [157].

AAV BASED VECTORS

The broad cell tropism, lack of pathogenicity, and stable long-term gene expression make AAV an attractive vector for gene therapy [158]. AAV2 was the first AAV isolate to be developed into a recombinant vector for transgene delivery as it has been shown to infect a broad range of cell types in animal models [159], showing high efficiency in most of the tumor cells tested [160]. Recombinant AAV vectors are constructed by replacing the Rep and Cap ORFs with a transgene of interest flanked by the *ITR*s. Rep, Cap and helper functions can be supplied *in trans* in order to allow replication of the transgene in the host cell [135] (Fig. **2B**). Different methods of delivering helper functions have evolved, from co-infection with Ad or HSV-1 [161, 162], to plasmid-based protocols [163], and stable-expression by cell lines [164-166]. The development of a baculovirus based vector production method in insect (SF9) cells has also shown promising results [167, 168].

Recombinant AAV vectors have been tested in preclinical studies for a variety of diseases such as hemophilia, α-1 anti-trypsin deficiency, cystic fibrosis, Duchenne muscular dystrophy, rheumatoid arthritis, prostate and melanoma cancer, Canavan disease [169], Alzheimer's, and Parkinson's [170].

Recombinant AAV have shown efficient transduction of different regions of the brain, and are currently used in several clinical trials for neurological disorders [171-173]. Increased interest in designing AAV vectors for the treatment of neurodegenerative diseases that require gene delivery to broad areas or very local and specific areas of the brain are now the focus of many studies [174]. AAV2 has been the most widely used AAV serotype for gene delivery to the CNS, transducing almost exclusively neurons in different brain structures [175-178], and supporting long-term transgene expression in the CNS [179-181] as well as in the dorsal root ganglia [182]. AAV2 has shown higher transduction efficiency in glioblastoma *in vitro* and *in vivo* when compared to serotypes 4 and 5 [183]. However, other studies have demonstrated a higher distribution and transduction in the CNS when using rAAV serotypes 1 and 5 [175, 184, 185]. The different AAV serotypes have been exploited on their ability to efficiently transduce distinct regions of the brain due to different cellular tropisms [174, 183, 186].

Immune Response to rAAV Vectors

The brain has been thought to be an immune privileged, compartmentalized organ that lacks an adaptive immune response. Some studies have suggested that viral vectors induce little immunogenicity, especially when injected once in the parenchyma of naïve animals [104, 187, 188], or that the presence of antibodies to both capsid proteins and transgene products seems not to correlate with reduction in transgene expression [187, 188]. Further studies with pre-immunized animals, however, established that circulating neutralizing antibodies can affect intracerebral rAAV-mediated transduction, and even suggested that the adaptive arm of the immune system can be primed by intracerebral rAAV2 administration [104].

Immune responses in the absence of expression of AAV genes have also been observed in naïve animals in a dose-dependent manner [104]. This has been suggested to occur due to the slow process of AAV capsid uncoating, thus allowing antigen presentation of processed capsid peptides by MHC-I, or by an immune reaction specific to the transgene [189, 190].

Improvements on rAAV Vectors

Despite the explicit advantages of rAAV as a vector for gene therapy, improvements in the regulation of transgene expression need to be achieved in order to confer safety.

Much research is focused on efforts to limit vector spread, in order to achieve specific tissue or organ delivery, or to enable the transduction of tissues that are refractory to naturally occurring AAV vectors. Engineering of AAV vectors for altered tropism, enhanced transduction efficiency, and evasion of antibody neutralization includes manipulation of the AAV capsid by insertion of peptide ligands, conjugate-based targeting, and presentation of large protein ligands on the AAV capsid [191]. The diversity of AAV serotypes brings the possibility to evade preexisting immunity by engineering hybrid or pseudotyped AAV vectors derived from different serotypes [192-194].

Another strategy that focuses on the transduction efficiency is the improvement on the second-strand synthesis step during AAV replication (Fig. **5**). The development of self-complementary AAV (scAAV) vectors relies on the packaging of an inverted repeat genome that can fold into dsDNA without the requirement for DNA synthesis or base-pairing between multiple vector genomes, thereby bypassing the rate-limiting second-strand DNA synthesis [195]. The scAAV vectors displayed enhanced transduction in comparison with conventional AAV vectors in some tissues and cancer cells but their efficiency still depends on tissue, cell type, and route of administration [196, 197].

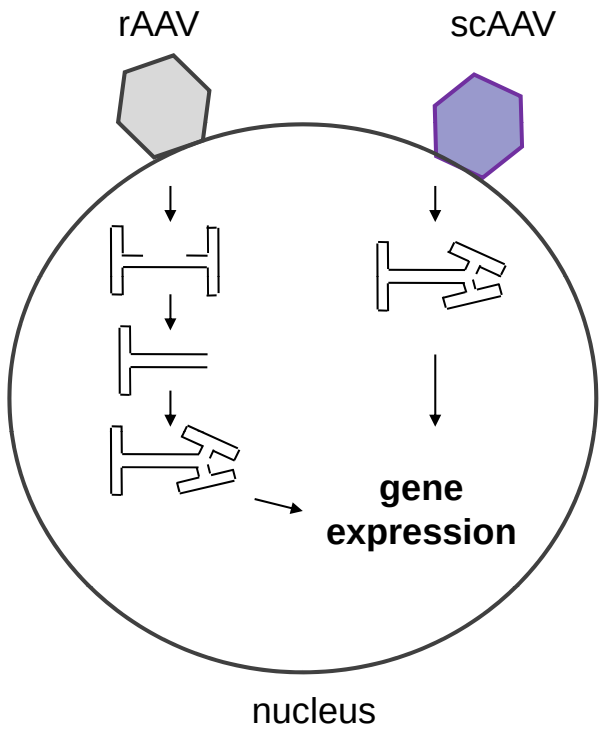

Fig. (5). Comparison between self-complementary AAV (scAAV) and rAAV vectors. scAAV delivers a dimeric inverted repeated DNA molecule thereby bypassing the rate-limiting second-strand DNA synthesis of rAAV.

Modifications on purification protocols using chromatography techniques have also contributed to increased yields of rAAV and to considerable elimination of contaminating infectious helper viruses [198-201].

HSV/AAV HYBRID VECTORS

Rationale on the Construction of Hybrid Vectors

Hybrid gene transfer vectors are designed to combine advantageous properties of different viruses to enhance efficiency of transgene delivery, vector stability and long-term transgene expression, while maintaining high safety standards [133, 202]. For example, the instability of HSV-1 amplicon vector delivered transgene DNA and transient transgene expression can be overcome by introducing genetic elements that allow the amplicon DNA to be maintained as an episome or to integrate into the host cell genome [203]. The maintenance of the DNA as a replicating episome with chromosome-segregating capability can be achieved by using oriP and the EBNA-1 gene from Epstein-Barr virus (EBV) [204]. Alternatively, viral elements such as AAV *ITR* and *rep* [91, 178], or retrovirus components [205] can be used to allow HSV-1 amplicon vector delivered transgenes to integrate into the cell genome. HSV-1 based hybrid vectors have also been constructed to facilitate the production of rAAV vectors.

Hybrid Vectors for the Production of rAAV Vectors

The efficiency of rAAV production for routine clinical use is a major concern, as most of the systems used for rAAV production rely on transfection protocols, thereby limiting scale-up procedures [206-209]. Replication defective rHSV-1 vectors lacking specific genes (e.g. ICP4, ICP27), which have been developed in order to reduce pathogenicity and cytotoxic effects in vector infected cells, can also be used as helper viruses for the production of rAAV vectors. Specifically, the ability of rHSV-1 that lack the ICP27 gene to efficiently act as a helper virus for rAAV production has been demonstrated [210] ; rAAV production in the absence of ICP27 appeared to be even enhanced. This may be due to the role of ICP27 in regulating transcription and translation of viral and cellular genes, for instance in the inhibition of splicing of host and AAV transcripts, which reduces synthesis of Rep and Cap proteins. The use of replication defective rHSV-1 to deliver AAV *rep* and cap has also been explored and is a very promising approach as it generates higher yields of rAAV with no detectable helpervirus contamination. Moreover, when allied to infection of a cell line that provides the rAAV template to be packaged, transfection steps can be avoided entirely for the production of rAAV [161, 211]. A protocol with a single infection step can also be accomplished by inserting an AAV *ITR*-flanked transgene (rAAV genome) cassette into the genome of the rHSV-1 helpervirus [210].

HSV/AAV Hybrid Vectors for Site-Specific Integration into AAVS1

Over the past 2 decades, the development of improved HSV-1 amplicon packaging systems, in particular the development of helper virus-free packaging systems, has greatly reduced toxicity and immunogenicity, but has had little effect on the stability of amplicon-mediated transgene expression [81, 212-214]. On the other hand, classical rAAV vectors have a small transgene capacity (~4.6 kb) and, due to the replacement of the *rep* and *cap* genes by transgenic sequences, do not conserve the potential of the parent virus for site-specific integration.

HSV/AAV hybrid amplicon vectors have been developed to overcome these limitations. In addition to the standard HSV-1 amplicon elements, HSV/AAV hybrid vectors incorporate the AAV *rep* gene and a transgene cassette that is flanked by AAV *ITRs* (Fig. **2C**). By placing the *rep* gene outside of the *ITR* cassette, it is not expected to integrate into the host genome. Loss of *rep* after integration of the *ITR* cassette eliminates a potential source of toxicity and the risk of rescue/excision of integrated *ITR* cassettes if the cell is infected by a helpervirus. Because HSV/AAV hybrid vectors can be packaged into HSV-1 virions, they conserve the high efficiency of gene transfer, the large transgene capacity, and the availability of helper virus-free packaging systems. However, after delivery into the host cell nucleus, the vector has the potential to act like AAV with *rep*-mediated site-specific integration of the *ITR*-flanked transgene cassette into the AAVS1 sequence of human chromosome 19 [91].

The initial study on HSV/AAV hybrid vectors demonstrated that these vectors can be packaged into HSV-1 virions by using either helper virus-dependent or helper virus-free packaging systems [81, 215]. Hybrid vectors supported transgene retention and expression significantly longer than standard amplicons [215]. Although the possibility of transgene integration had not been specifically addressed in that study, the percentage of cells expressing the transgene was consistently higher with hybrid vectors that contained the *rep* gene than with those without *rep*, or with standard amplicons.

Two other studies have specifically addressed the question whether HSV/AAV hybrid vectors mediate genomic integration, both randomly or site-specifically at the AAVS1 site on human chromosome 19 [91, 178]. Heister and colleagues constructed HSV/AAV hybrid vectors that contained enhanced green fluorescent protein (EGFP) reporter gene flanked by the AAV *ITRs* and AAV *rep*. Replication assays demonstrated that both the AAV elements and the HSV-1 elements were functional in the context of the hybrid vector, as shown by the presence of replication intermediates of the *ITR*-flanked transgene cassette and high molecular-weight concatemeric products of replication from the HSV-1 origin of DNA replication. Such hybrid vectors could be packaged into HSV-1 virions, although the *rep* sequences incurred a drastic (20 to 2,000-fold) reduction in titers. Site-specific integration at AAVS1 was directly demonstrated by PCR and sequence analysis of *ITR*-AAVS1 junctions in transduced human 293 cells. The junctions were similar to those that had been identified in cells infected with wt AAV [133, 134, 138, 139, 216-218]. Similar results were obtained by Wang and colleagues who have used also 293 cells and extended the study to other cell lines, including glioma cells (gli36) and primary myoblasts [178]. These investigators used HSV/AAV hybrid vectors that contained *rep68* and *rep78*, or no *rep*, and an *ITR*-flanked transgene cassette that consisted of an EGFP reporter gene and a neomycin resistance gene. In order to overcome the low-titer packaging problem inherent to the *rep* gene, they worked on position/orientation effects and found that a decent amplicon vector titer is achieved when the *rep* genes are placed downstream of the *ITR* cassette in the forward orientation. Rep mediated a significantly improved efficiency of stable tranduction in all human cells tested, including 293 cells, glioma cells and primary myoblasts. Although neomycin selection was employed for cell cloning, a high proportion of the stably transduced cells had the transgene sequences correctly integrated at the AAVS1 site. In summary, inserting the AAV *ITRs* and *rep* genes into an HSV-1 amplicon considerably improved the frequency of stable transgene expression in various proliferating human cell types. Integration events of 4-5 kb *ITR*-flanked transgene cassettes occurred at a rate of approximately 10-30 % of the HSV/AAV hybrid vector infected cells, and about 50% of those events occurred specifically at the AAVS1 locus [91, 178]. The potential for AAVS1-specific integration and expression of an entire gene under control of its endogenous promoter using the HSV/AAV vector has also been evaluated. Large functional inserts (approximately 100 kb) could be integrated at the AAVS1 site but with a reduced efficiency [219, 220].

While the expression of *rep78/68* has been demonstrated to be essential for the ability of HSV/AAV hybrid vectors to mediate site-specific integration, Rep proteins have a strong inhibitory effect on the HSV-1 replication machinery [91, 132, 178, 221]. As a consequence, the titers of HSV/AAV hybrid vectors are up to 2000-fold lower than those of standard amplicon vectors [91]. This could be due to (i) the toxicity of Rep, resulting in compromised cell metabolism [222], (ii) the ability of Rep to inhibit HSV-1 replication [131, 132], or (iii) the excision of *ITR*-flanked sequences from the amplicon DNA during packaging.

Potential improvements of the HSV/AAV amplicon vectors may rely on the appropriate use of the p5 promoter sequence. Indeed, the p5 promoter driving the expression of *rep78/68* in the afore described HSV/AAV hybrid vectors [91, 178] may promote vector-backbone integration [144, 223] owing to its location outside of the therapeutic transgene cassette. In addition, it may also interfere with site-specific integration of the p5-free *ITR*-flanked transgenes. Transferring the p5 promoter sequence from the *rep* expression cassette to the transgene cassette may not only solve the problem of inadvertent integration of vector backbone sequences but also increase the efficiency of site-specific integration of the *ITR* cassette [144, 223].

Liu *et al.* developed a strategy to overcome the negative effect of AAV Rep on hybrid vector replication and packaging [224]. These investigators designed an HSV/AAV hybrid vector in such a way that little or no *rep* was expressed during packaging. However, *rep* was expressed in transduced cells if Cre-recombinase was provided; following site-specific integration, *rep* was suppressed again. These vectors mediated stable expression in 22% of transduced Cre-expressing 293 cells. Of those cells, approximately 70% transduction efficiency was achieved by Rep-mediated site-specific integration.

The finding that concatameric plasmid replication products from the AAV p5 replication origin can be packaged into HSV-1 virions if HSV-1 *pac* is included on the plasmid [108] could lead to the construction of a novel generation of HSV/AAV hybrid amplicon vectors which replicate from a heterologous origin of DNA replication. Such a vector system would have several advantages: first, as described by Philpott and coworkers, the AAV p5 element can efficiently mediate site-specific vector integration into AAVS1 on human chromosome 19 and support long term

transgene expression [144, 225]. Second, the AAV p5 replication origin is not inhibited by *rep* expression, but instead depends on the presence of AAV Rep protein in the replication/packaging process [108, 116, 117].

The HSV-1 virion contains three proteinaceous compartments for delivery - envelope, tegument, and capsid – which could all be used to deliver functional foreign proteins by fusion with virion components [226]. For example, AAV Rep could be fused with VP16, an abundant HSV-1 tegument protein that enters the cell nucleus along with the virus genome. This would allow eliminating the *rep* gene from the HSV/AAV hybrid vector genome, as Rep protein could enter the cell nucleus as a fusion with VP16 and there may mediate efficient site-specific integration of the transgene sequences *via* p5 or *ITRs*.

The full potential of HSV/AAV hybrid vectors still needs to be evaluated for site-specific integration *in vivo*, for example in transgenic mice that carry the human-specific AAVS1 genomic element [227]. As murine [228] and simian [229] AAVS1 orthologs have been found, AAV2 likely can mediate site-specific integration in other species as well.

Future perspective and clinical use of HSV/AAV hybrid vectors are closely linked to standard HSV-1 amplicon vectors as both vector systems depend on the same packaging procedure. Helper virus-free packaging systems require transient transfection of vector DNA and packaging-defective HSV-1 helper DNA, which limits scale up potential. The use of amplicon vectors for clinical trials depends, therefore, on the design of novel packaging procedures that allow the production of large amounts of vector stocks with high titers. Strategies to overcome the adverse effects of the AAV rep gene on the titers of HSV/AAV hybrid vectors have been discussed above. The presence of the genetic elements from AAV on HSV-1 amplicon vectors should not add additional safety concerns to the amplicon system, as AAV is not known to be pathogenic in humans and AAV vectors are already being used in clinical trials.

OTHER HYBRID AMPLICON VECTORS

HSV/EBV Hybrid Vectors

Epstein-Barr virus, a human Gammaherpesvirus, has also been used as a hybrid partner with HSV-1 amplicons, due to its potential to persist as an extrachromosomal element in B-lymphocytes [230]. The EBV nuclear antigen (EBNA-1) and the origin of DNA replication (oriP) are the sole elements necessary for the long-term episomal retention and are therefore incorporated into the HSV-1 amplicon backbone to support replication and mitotic segregation of the amplicon concatenate in the host cell nucleus [231, 232] (Fig. **2D**). HSV/EBV hybrid amplicon vectors have been demonstrated to efficiently transduce various human cells in culture and to support retention of vector sequences in dividing human cells [205]. Stable expression from large transgenes has also been demonstrated [233, 234]. Maintenance of transgene DNA in an episomal state as opposed to genomic integration reduces adverse effects in the host cell. However, long-term expression by these vectors depends on selective pressure and expression of EBNA-1 [95, 235]. In order to circumvent the potential immunogenic and oncogenic properties of EBNA-1 [236], the use of a human episomal retention element (scaffold/matrix attachment region (S/MAR) from the human β-interferon gene to generate a novel HSV-1 amplicon-based episomal vector has shown great potential even in the absence of selection pressure [93].

HSV/RV Hybrid Vectors

Elements from retroviruses (RV) have been combined with HSV-1 amplicons in order to achieve prolonged transduction of transgenes. Retroviruses, such as Moloney murine leukaemia virus (MoMLV), integrate randomly into the genome of dividing cells, and produce viral progeny without killing the host cell [237]. Due to the low efficiency of gene transfer, MoMLV –based vectors have been mostly used for *ex vivo* gene therapy protocols [238, 239]. Although this strategy has shown some therapeutic success in experimental brain tumors [240, 241] it is not effective when used in human trials [242-245]. HSV/RV hybrid amplicon vectors containing genetic elements from MoMLV have been developed in order to transduce genes required for the *de novo* synthesis of small defective retrovirus vectors. These hybrid vectors contain the long terminal repeat sequences (LTRs) flanking a transgene cassette, and the *gag*, *pol*, and *env* genes in a separate cassette (Fig. **2E**). The LTRs and *psi* sequence comprise the signals necessary for packaging of virion RNA, reverse transcription, and integration into host cell genome. HSV/MoMLV hybrid vectors have indeed been demonstrated to support the packaging of genomic retrovirus RNA expressed from the amplicon vector into MoMLV particles and accomplish integration and transgenic expression in infected naïve cells [246]. One point of caution, however, is the danger of endogenous retroviruses complementing retroviral elements in hybrid vectors. The possibility that endogenous integrases can act on LTRs in hybrid amplicon vectors has indeed been demonstrated [247]. In order to enhance the transduction efficiency of a therapeutic gene *in vivo* and increase its expression stability, hybrid vectors containing elements from more than 2 viruses have been developed as well. These tribid vectors are based on HSV-1 amplicon vectors and contain elements from MoMLV and either EBV or AAV [205, 248].

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