HSV-1-Based Vectors for Gene Therapy of Neurological Diseases and Brain Tumors: Part II. Vector Systems and Applications[1]

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Abstract
Many properties of HSV-1 are especially suitable for using this virus as a vector to treat diseases affecting the central nervous system (CNS), such as Parkinson's disease or malignant gliomas. These advantageous properties include natural neurotropism, high transduction efficiency, large transgene capacity, and the ability of entering a latent state in neurons. Selective oncolysis in combination with modulation of the immune response mediated by replication-conditional HSV-1 vectors appears to be a highly promising approach in the battle against malignant glioma. Helper virus-free HSV/AAV hybrid amplicon vectors have great promise in mediating long-term gene expression in the PNS and CNS for the treatment of various neurodegenerative disorders or chronic pain. Current research focuses on the design of HSV-1-derived vectors which are targeted to certain cell types and support transcriptionally regulatable transgene expression. Here, we review the recent developments on HSV-1-based vector systems and their applications in experimental and clinical gene therapy protocols.

Keywords: glioma, gene therapy, recombinant HSV-1, amplicon.

Recombinant HSV-1 Vectors

Recombinant herpes viruses were first constructed for the purpose of vaccination against HSV-1 and HSV-2 and to study the functions of individual virus genes. Only later were they considered suitable gene transfer vehicles, especially for use in the central nervous system (CNS). A protocol for site-specific modification of the virus genome was first reported by Post and Roizman [1] and is reviewed in detail elsewhere [2,3]. In principle, two types of recombinant HSV-1 vectors can be distinguished depending on the target tissue and purpose of gene delivery: i) for therapeutic gene transfer/replacement in neurons, mutation/deletion of some essential genes can serve to prevent the expression of toxic virus proteins and initiation of the lytic viral replication cycle (replication-defective HSV-1 mutants); ii) for therapeutic treatment of tumors (virus therapy), where toxicity is the main purpose, one or more genes are deleted which are essential for virus replication in nondividing cells of the surrounding tissue, but can be complemented by proliferating tumor cells (replication-conditional HSV-1 mutants). Both types of vectors can incorporate single or multiple therapeutic genes accounting for at least 30 kb of the 152-kb genome. In principle, recombinant HSV-1 mutants are generated by homologous recombination. The frequency of recombination is proportional to the length of the homologous flanking sequences and usually less than 5%.

Gene Transfer into Neurons

The wild-type (*wt*) HSV-1 genome can persist in neurons during latency in a nuclear life-long episomal location without disturbance of host-cell metabolism and without eliciting an immune response. This suggests that if viral neuropathogenesis and reactivation could be avoided by manipulation of the viral genome, it should be possible to express transgenes from a silent virus genome in a nerve cell-specific manner. However, several obstacles have to be overcome: i) replication-defective mutants of HSV-1 can cause cytopathic effects in primary cultures of neural cells and inflammatory responses in neural tissue *in vivo*; ii) most viral and nonviral promoters are silenced after injection into the brain; and iii) the activity of the LAT (latency associated transcript) promoter is weak in CNS neurons [4]. 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.

Two main approaches have been taken to reduce toxicity: i) the engineering of mutants in which virus gene expression is blocked in an early stage; and ii) the use of mutants which lack the neurovirulence gene $\gamma 34.5$. As the initiation of the viral replicative machinery depends on the concerted action of at least five viral proteins (VP) or infected cell proteins (ICP), including VP16, ICP 0, 4, 22, and 27, single or double deletion mutants involving various combinations (ICP 0 alone, ICP4 alone, ICP4+22, ICP4+27, ICP4+47) were initially created. Although expression of viral early and late

genes is dramatically reduced in these mutant backgrounds, they still cause some toxicity and alteration of cell morphology and chromatin structure [5–9], most likely due to expression of remaining immediate-early (*IE*) genes. Supporting this hypothesis, mutations affecting the activation function of VP16 did reduce the toxicity of an ICP4⁻ mutant significantly [10], probably due to the overall reduction in *IE* gene expression in the absence of VP16 transactivation. Substantially reduced vector cytotoxicity was also reported for replication-defective HSV-1 mutants that contain deletions in multiple genes [4,11–14]. These multiple deletions included the *IE* genes encoding ICP4, ICP22, ICP27, and ICP47 [11,12,14] and prevented early and late viral gene expression, but major histocompatibility complex (MHC) class I antigen expression remained intact in the absence of ICP47. Viral mutants deleted simultaneously for the *IE* genes encoding ICP4, ICP22, and ICP27 showed substantially reduced cytotoxicity when compared to viruses deleted for ICP4 alone or ICP4 in combination with either ICP22, ICP27 or ICP47 [14]. Infection of cultured neurons with these triple IE deletion mutants substantially improved cell survival and supported transgene expression for over 21 days [11,12]. However, infection of Vero and human embryonic lung cells leads to inhibition of host cell DNA synthesis and pronounced and distinct alterations of nuclear morphology, most likely due to the accumulation of ICP0 [14,15]. The elimination of ICP0 together with VP16 and ICP4 [16,17], or together with ICP4 and ICP27 [15], or together with all other *IE* genes [18], greatly reduced cytotoxicity, but also reduced the level of transgene expression. Therefore, transgenes inserted into the genome of these mutants may be expressed at reduced levels, depending on the promoter used to regulate them. Nevertheless, these types of vectors have been successfully used to express bcl-2 and to reduce 6-hydroxydopamine-induced apoptosis of neurons in the substantia nigra in experimental animal models [19].

The *γ34.5* deletion mutants, where both copies of the *γ34.5* gene are deleted, were first generated for the assessment of its phenotype [20], tested in animal models [21], and later adapted for virus-mediated therapy of brain tumors [22,23]. In proliferating cells, these mutants can replicate as efficiently as *wt* virus, whereas in most neurons, they are replication-defective, but still able to establish latency [24]. Delivery of a constitutively active form of a heat shock factor using this type of vector protected neurons from thermal and ischemic stress [25] as well as from apoptosis [26]. However, a mutant, where the *lacZ* reporter gene driven by the LAT promoter was inserted into a nonessential gene, *UL43*, of recombinant HSV-1 **1716** (ICP34.5 double-deletion) failed to mediate transgene expression for more than 2 weeks after injection into the peripheral nervous system (PNS) and CNS [24]. The same vector did not transduce enteric neurons efficiently and, in addition, exhibited some toxicity [27].

The choice of the promoter driving the gene of interest is important to achieve long-term (several weeks to months) gene expression in neurons. In principle, three different types of promoters can be used: i) the HSV-1 LAT promoter;

ii) other viral promoters; or iii) cellular promoters. The LAT promoter is of special interest, as it mediates specific gene expression and is active in latency in certain neurons [28,29]. Recombinants of HSV-1 that contained *lacZ* reporter genes placed under transcriptional control of the LAT promoter at both LAT loci, mediated synthesis of *β*-galactosidase in sensory neurons for 2 months [30], and in neurons located in facial and hypoglossal nerve nuclei and in the upper cervical spinal cord for up to 10 months [31]. Control of *β*-glucuronidase expression from the TATA box-containing latency promoter, LAP1, led to production of the enzyme for up to 4 months after inoculation of the vector into the trigeminal ganglia or brainstem of MPS VII mice [32]. Although the alternate LAT promoter, LAP2, is primarily responsible for LAT expression during lytic infection, LAP2 has also been shown to be transcriptionally active during latency [33]. *LacZ* mRNA could be detected in hippocampal neurons for up to 4 weeks after inoculation of recombinant HSV-1 vectors that express this reporter gene from LAP2 [7]. LAP2 was also capable of supporting long-term (4 weeks) expression of the gene for nerve growth factor, *β*-NGF, in latently infected neurons *in vivo*, providing protection of dorsal root ganglion neurons from peroxide toxicity [34]. HSV-1 recombinants that express the transgene from constitutive viral promoters, such as the retrovirus LTR or cytomegalovirus IE promoter, or even from neuron-specific promoters, support in general only short-term (several days) expression in the CNS [5–7,34–37]. Heterologous promoters introduced into the virus genome are prone to come under the influence of the transcriptional machinery of the virus, which can result in unspecific expression, at least in the CNS [7]. Moreover, the tightly condensed nucleosomal configuration of the HSV-1 genome in latency may restrict access of transcriptional activators. Two studies, however, demonstrated that the MoLMV LTR promoter can support *lacZ* gene expression in primary sensory neurons for up to 18 months [38,39].

Virus/Gene Therapy of Gliomas

HSV-1-based therapy of brain tumors relies on the selective killing of tumor cells by the virus replication *per se* and/or the virus-mediated expression of anti-tumor acting genes. Anti-tumor acting genes can encode: i) prodrug-activating enzymes (e.g., thymidine kinase [40], *Escherichia coli* cytosine deaminase [41,42], *E. coli* guanine phosphoribosyl transferase [43,44], cytochrome P450 [45–47], deoxycytidine kinase [48]); ii) cell-cycle regulating proteins (e.g., p53) [49]; iii) factors which inhibit angiogenesis; or iv) immunomodulating cytokines (reviewed in Ref. [50]).

For virus therapy of brain tumors, different single- or multiple-mutated replication-conditional HSV-1 vectors have been created, which have reduced neuronal toxicity and replicate *selectively* in dividing, and not in quiescent, cells. The deletions/mutations include the *tk* gene (e.g., HSV-1 **RH105** [51]; *dl*sptk [52,53]; **KOS-SB** [54,55]; **G92A** [56,57]), the ribonucleotide reductase (RR) gene (e.g., HSV-1 **hrR3** [58–62]), the *γ34.5* gene (e.g., HSV-1 **R3616** [20,22,23]; **1716** [63–65]) or the *γ34.5* gene

together with either the RR gene or the gene encoding uracyl N-glycosylase (HSV-1 **G207** [66]; **MGH-1** [67]; **3616UB** [68]).

First generation HSV-1 mutants, such as the dlsptk vector, exhibited a substantial therapeutic effect in terms of killing glioma cells in culture and in vivo, but their application was limited for two reasons: first, the recombinant virus lacks a functional tk gene and, therefore, potential dlsptk-induced encephalitis cannot be controlled by ganciclovir (GCV); second, in some studies, intracranial inoculation caused fatal encephalitis at high vector doses [22,52,54]. Elimination of the neurovirulence gene, γ34.5, substantially reduced the risk of developing focal encephalitis, and still retained the therapeutic effect in primary glioma, medulloblastoma, and metastatic brain tumor models [22,23,69–72]. The LD50 in mice increased by a factor of $\sim 10^6$ compared to wt HSV-1 [20,64]. Attempts to recover virus from brains of infected animals were either unsuccessful or yielded very small amounts of infectious particles [20,21]. Histopathological studies of immunocompetent mice which were injected into the left cerebral hemisphere with 1×10^6 PFU of 1716, a γ34.5 double-deletion mutant derived from HSV-1 strain syn17, exhibited a low grade meningoencephalitis with a limited inflammatory response at early times after inoculation [73]; virus gene expression was confined to the site of inoculation. By 28 days after inoculation, the CNS appeared histopathologically normal and virus-encoded antigens and virus-induced immune responses were no longer detectable. These findings demonstrate that infection of the CNS by γ34.5 null mutants results in a finite, self-limiting response and highlights the potential usefulness of these vectors for clinical protocols [73,74]. In a similar study [75], the recombinant HSV-1 R3616, a γ34.5 double-deletion mutant derived from HSV-1 strain F, has been shown to infect neurons, astrocytes, oligodendrocytes, and ependymal cells and, hence, did not discriminate at the level of infection among CNS cell types. The transduced cells have been cleared entirely from the nervous system by day 7 after infection [75]. This study supported the view that the neuroattenuation of γ34.5 deletion mutants is based on a gross reduction in the ability of the virus to replicate and spread cell-to-cell and not on a restricted host range in the CNS. However, other data suggest that this type of vector needs further attenuation before it can be used in clinical applications for the following reasons: i) the vector can replicate in and destroy the ependyma, leading to a persistent loss of the ependymal lining in mice [76]; ii) after inoculation into the CNS of rats or mice, the vector is able to spread to distant sites where it induces inflammatory reactions [77,78]; iii) the vector causes cytopathic effects in the immunocompromised host [79]. Moreover, HSV-1 strain-related differences of toxicity and the possibility of a second site suppressor mutation occurring in HSV-1 mutants, where a single gene is deleted or mutated, possibly affecting neurovirulence or other activities of the vector should be kept in mind when preparing an HSV-1 mutant for clinical use. Of note, the capacity of γ34.5 double-deletion mutants to kill human cells derived from glioblastoma multiforme, anaplastic astrocytoma, anaplastic glioma, gliosarcoma, and normal human astrocytes in culture varies [69]. Moreover, cultured astrocytes were two to three orders of magnitude less susceptible to killing than were malignant glia [69], indicating a cell-type dependent therapeutic effect. Insertion of different immunomodulating interleukin genes into the γ34.5 locus induced significant alterations of the host immune response and demonstrated its important role as part of the therapeutic effect of these vectors [80]. An enhanced anti-tumor effect of recombinant HSV-1 R3616 was also achieved by ionizing radiation, which caused a two- to five-fold enhanced replication in irradiated tumor cells, resulting in a significantly greater reduction in volume, or total regression, of tumors than either radiation or infection alone [81].

Other first-generation vectors, such as recombinant HSV-1 hrR3, bear a lacZ-insertional mutation within the large subunit of the gene encoding RR [61], thereby restricting replication of the vector to proliferating cells, e.g., tumor cells, which express complementary RR activity (Figure 1). This vector has been used for the selective destruction of tumor cells in different experimental tumor models in the brain and other organs [58,60,62,82–84]. For example, in in vivo studies, animals harboring human U-87MG gliomas were randomly divided and treated intraneoplastically with either 5×10^6 PFU of recombinant HSV-1 hrR3 or medium alone. The virus-treated animals showed significant inhibition of tumor growth [62]. The vector retains an intact HSV-1 tk gene, which can be used as "marker gene" to monitor vector replication and spread using non-invasive in vivo imaging methods [85] as well as to sensitize transduced cells to GCV. Administration of GCV to tumor-bearing animals treated with hrR3 reduced the vector-mediated inflammatory response in a disseminated brain tumor model [83] and served to increase the therapeutic capacity of the vector in animals bearing intracranial and intrathecal rat 9L gliosarcomas [60,83]. A similar observation was made with a variant of hrR3, **rRp450**, carrying a cytochrome P450 (CYP2B1) insertional mutation at the RR gene locus [86]. This mutant replicated selectively in dividing rat and human tumor cells which express high levels of mammalian RR, and addition of cyclophosphamide potentiated oncolytic effects against subcutaneous (s.c.) tumor xenografts established in athymic mice without compromising replication of the vector. In a similar experimental tumor model, injection of this vector with application of both cyclophosphamide and GCV resulted in enhanced tumor regression after pro-drug activation [87]. In view of a potential clinical application of these vectors, it should be noted that pre-existing HSV-1 immunity decreases, but does not abolish HSV-1 vector-mediated gene transfer to experimental brain tumors [88]. Moreover, in two different latency models in rats, intracerebral injection of hrR3 did not reactivate latent wt HSV-1 in either the corneal or the cerebral model. This indicates that intracranial injection of partially defective recombinants of HSV-1
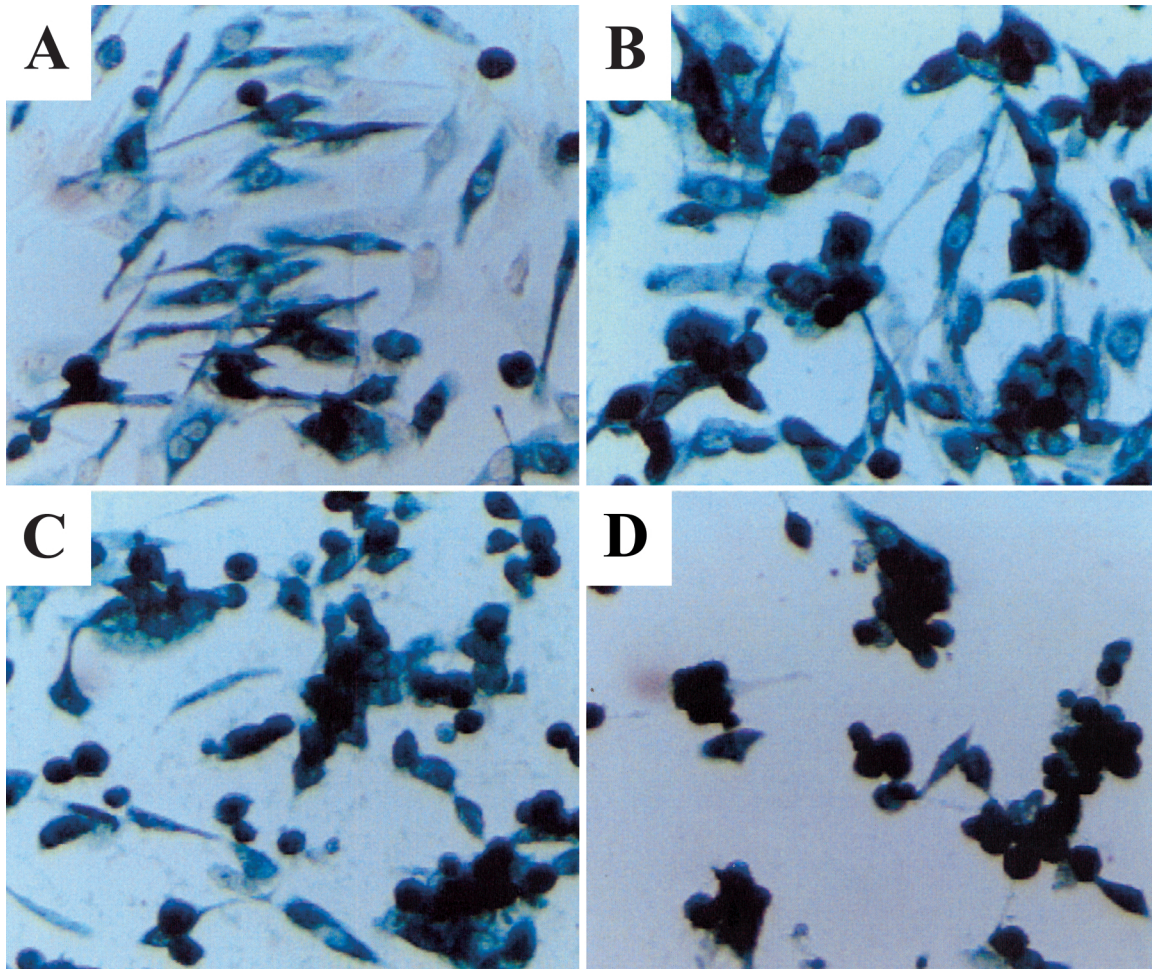
Figure 1. *Time‑course of cytopathic effect and lacZ gene expression mediated by replication‑conditional HSV‑1 mutant **hrR3** [61] after infection of cultured rat 9L gliosarcoma cells. MOI = 1.5 PFU/cell. (A) 19 hours postinfection (p.i.); (B) 28 hours p.i.; (C) 42 hours p.i.; (D) 50 hours p.i. Replication and spread of the recombinant leads to progressive cytopathic effects and, eventually, to death of all cells in the culture (D).*

may bear little or no risk of reactivating latent *wt* virus present in sensory ganglia or brain [89], although it must be kept in mind that rats are, in general, less infectable with HSV‑1 than humans.

Second generation HSV‑1 vectors, such as triple mutants carrying deletions at both $\gamma 34.5$ gene loci and a transgene insertion that inactivates the ribonucleotide reductase gene (G207, MGH‑1, and rRp450), were found to be especially suitable for treating human malignant brain tumors for several reasons [66,67,90]: i) high replication‑competence in glioblastoma cells (and other dividing cells); ii) neuroattenuation; iii) temperature sensitivity; iv) GCV hypersensitivity [91]; and v) the presence of a detectable histochemical marker (*lacZ*) or therapeutic gene coding for a pro‑drug activating enzyme. In nude mice harboring s.c. or intracerebral U‑87MG gliomas, intraneoplastic inoculation with G207 caused decreased tumor growth and/or prolonged survival [66]. Similar therapeutic effects were observed in human F5 malignant meningioma [90], human malignant breast cancer [92], mouse CT26 colorectal carcinoma, and M3 melanoma [93], but not in rat 9L gliosarcoma cell lines [67], demonstrating again a cell‑dependent therapeutic

effect. Most importantly, such therapeutic vectors produce vi) a strong and highly tumor antigen‑specific cytotoxic T‑lymphocyte (CTL) response which seems to play a major role in the therapeutic effect of this vector in immune competent recipients [93]. The mutants are avirulent on intracerebral infection of both mice and highly HSV‑sensitive nonhuman primates. They are therefore considered interesting candidate vectors for the clinical evaluation in the treatment of glioblastomas [94]. The presence of two mutations reduces the possibility of recombinational events *in situ*, thereby reducing the risk of generating virulent progeny during virus therapy [68].

HSV‑1‑Based Amplicon Vectors

Historical Background

The serial passage of HSV‑1 at high multiplicities of infection results in the production of both *wt* and defective HSV‑1 viruses [95]. The genomes of these naturally occurring defective viruses consist of multiple reiterations of specific HSV‑1 sequences (repeat units), including a DNA

cleavage/packaging signal (pac) and an origin of DNA replication (ori), arranged as head-to-tail concatemers comprising ~152 kb of DNA, the size of the wt HSV-1 genome [95–97]. Based on these observations, Spaete and Frenkel [99] developed bacterial plasmids that contained single repeat units with ori and pac and demonstrated that, in mammalian cells in the presence of HSV-1 helper functions, the plasmids were replicated and packaged into virus particles. The genomes of these particles were composed of amplified head-to-tail-linked concatemers of the seed plasmid, which was therefore termed "amplicon" (Figure 2A) [98,99].

The HSV-1 amplicon has several properties that make it a promising candidate gene transfer vehicle: i) The two HSV-1 sequence elements, ori and pac, that are sufficient to support replication and packaging into virions are smaller than 1 kb and do not encode any virus proteins. Consequently, the amplicon has the potential to accommodate large fragments of foreign DNA (theoretically up to 152 kb), including multiple and large transgenes or large cell type-specific promoters; ii) Depending on the size of the seed amplicon, several copies of the transgene can be packaged into a single vector particle, thereby increasing the transgene dose per infected cell; iii) HSV-1 can infect most mammalian cell types, including the nondividing cells of the nervous system; iv) The HSV-1 virion is quite nontoxic.

As a major disadvantage, however, replication and packaging of amplicons into HSV-1 particles depends on helper functions which have conventionally been provided by replication-conditional helper viruses (Figure 3A). Initially, a temperature-sensitive (ts) mutant, HSV-1 tsK [100], was employed as a helper virus to package amplicons into HSV-1 particles [101]. HSV-1 tsK carries a missense mutation in the essential IE3 gene which encodes a ts form of ICP4, allowing virus replication to proceed at 31°C, but not at 37°C. To prepare vector stocks, cells cultured at 31°C were transfected with the seed amplicon and then infected with the tsK helper virus, yielding vector stocks that contained both packaged amplicon vector and helper virus. Although replication of this helper virus is inhibited in target cells that are cultured at the restrictive temperature (37°C), HSV-1 tsK can still express many genes that are toxic and not compatible with cell survival. Moreover, reversion of ts ICP4 to wt phenotype occurs at a relatively high rate. Consequently, further developments were aimed at: i) reducing the frequency of reversion to wt HSV-1; ii) reducing the toxicity of the helper virus; and iii) increasing the proportion of vector particles relative to helper virus.

To reduce the frequency of reversion to wt phenotype, HSV-1 tsK was replaced by a mutant of HSV-1 that contained a deletion in the essential IE3 gene (HSV-1 D30EBA [102], with packaging performed in IE3-complementing, M64 cells [103,104]). The reversion frequency was further reduced by replacing M64 cells with RR1 cells [8], which contain a smaller fragment of the HSV-1 genome carrying the IE3 gene than do M64 cells, thereby reducing the chance of homologous recombination and potential generation of replication competent virus. Mutants of HSV-1 that carry deletions in IE genes are useful helper viruses because virus replication in nonpermissive cells is blocked at a very early stage; however, of the five HSV-1 IE genes, only IE3 (ICP4) and IE2 (ICP27) are essential for HSV-1 replication. Lim et al. developed a packaging system with an IE2 deletion mutant helper virus (HSV-1 5dl1.2; [105]) using IE2-expressing 2-2 cells [106], which produced vector stocks with relatively high amplicon titers (10^6 to 10^7 transducing units, t.u., per milliliter), an acceptable vector to helper virus ratio (up to 1), and low levels ($<10^{-7}$) of revertants with wt HSV-1 phenotype [107]. As a modification of the deletion-mutant packaging system, the so-called "piggyback" amplicon encodes a gene that complements a deletion-mutant helper virus, allowing packaging to be performed in any cell line that supports HSV-1 replication [108–110]. Moreover, replication and packaging of amplicon
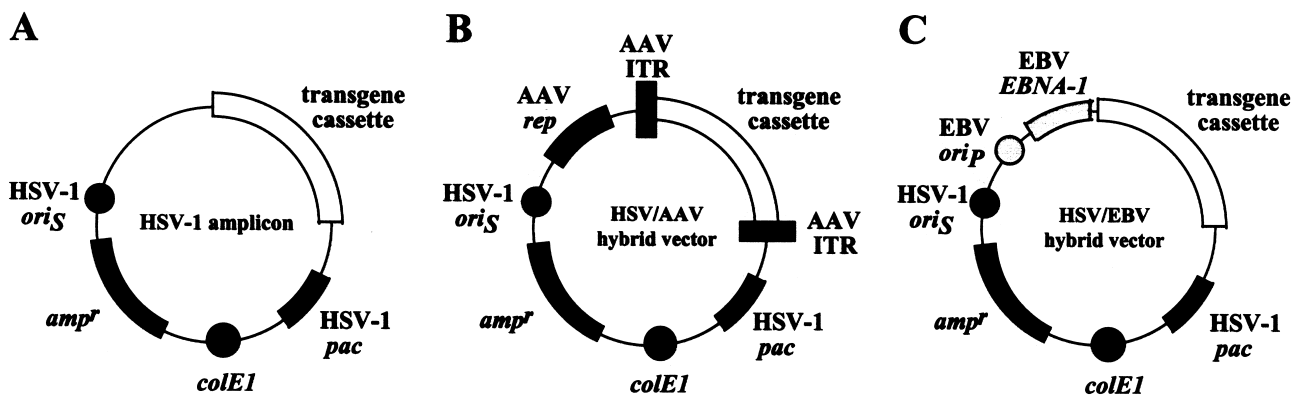

Figure 2. *Amplicon structures. (A) The standard HSV-1 amplicon is composed of three types of genetic elements: i) sequences from bacteria, including an origin of DNA replication (colE1) and an antibiotic resistance gene (amp^r), which allow propagation of plasmid DNA in E. coli; ii) sequences from HSV-1, in particular an origin of DNA replication (ori$_S$) and a DNA cleavage/packaging signal (pac), which support replication of amplicon DNA and subsequent packaging into HSV-1 particles in mammalian cells in the presence of helper functions; and iii) a transgene cassette with one or more gene(s) of interest. (B) In addition to the standard amplicon elements, HSV/AAV hybrid amplicons contain the adeno-associated virus (AAV) rep gene and a transgene cassette that is flanked by AAV inverted terminal repeats (ITR). (C) HSV/EBV hybrid amplicons contain the EBV EBNA1 gene and the EBV latent origin of DNA replication oriP in addition to the standard amplicon elements.*
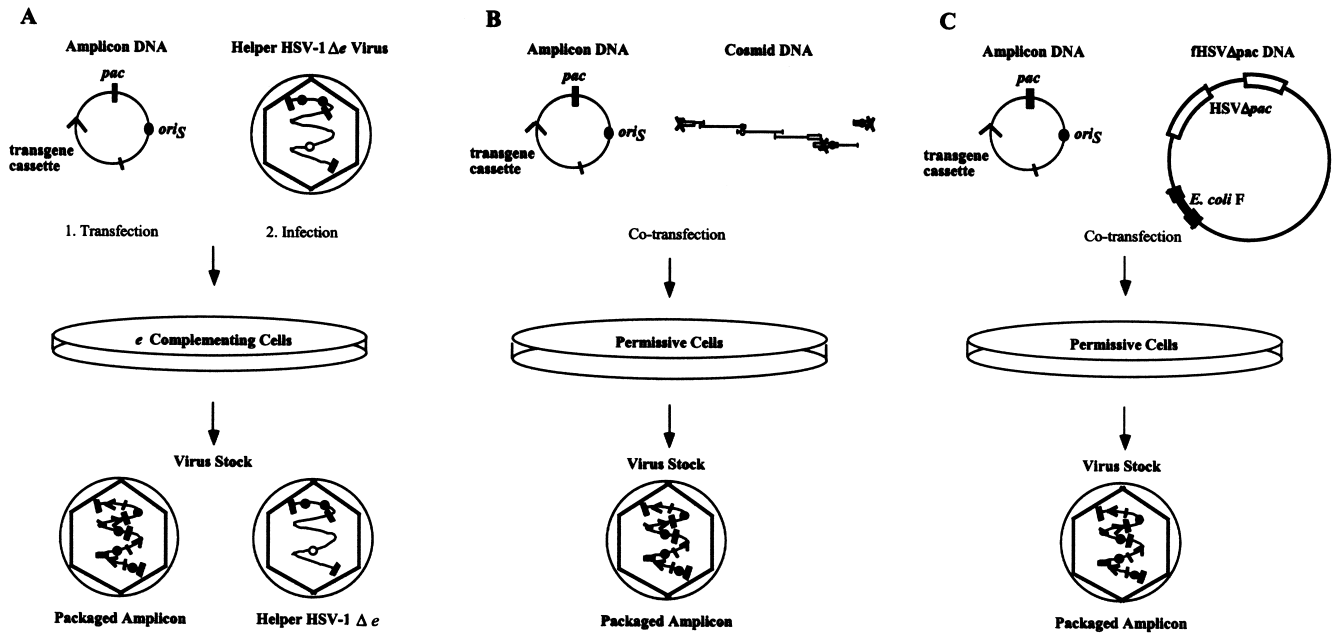
Figure 3. *Packaging systems for HSV-1 amplicon vectors. (A) Helper virus-dependent packaging system using a replication-defective mutant of HSV-1 containing a deletion in an essential gene (△e). Cells which complement the deleted virus gene (e), under transcriptional control of a VP16 responsive promoter, are transfected with amplicon DNA and then superinfected with the deletion-mutant helper virus. The resulting vector stocks contain both packaged amplicon vector and helper virus. (B) Cosmid-based, helper virus-free packaging system. Cells that are permissive for HSV-1 replication are cotransfected with amplicon DNA and DNA from cosmid set C6△a48△a. While helper HSV-1 genomes reconstituted from the cosmid clones are not packageable in the absence of pac signals, they still can provide all the trans-acting functions required for the replication and packaging of the co-transfected amplicon DNA. (C) F-plasmid-based helper virus-free packaging system. Permissive cells are co-transfected with amplicon DNA and fHSV△pac plasmid DNA. The fHSV△pac helper DNA is not packageable in the absence of pac signals, but it can still provide all the trans-acting functions required for the replication and packaging of co-transfected amplicon DNA.*

and helper virus mutually depend on each other, thereby eliminating helper viruses propagating independently and increasing the ratio of amplicon to helper virus.

Although the development of improved helper virus-dependent packaging systems has continuously increased the safety of the amplicon system, many problems associated with the helper virus still remained. These include: i) acute cytopathic effects and pronounced immune responses induced by gene expression from the helper virus; ii) reversion of the helper virus to *wt* HSV-1 phenotype; iii) potential interactions with endogenous viruses, such as reactivation and recombination; and iv) unreliability of transgene expression. Many of these problems have been eliminated by the recent development of helper virus-free packaging systems.

Helper virus-free packaging systems use replication-competent, packaging-defective genomes of HSV-1 to provide the functions necessary for replication and packaging of co-transfected amplicon DNA. The packaging of the HSV-1 helper DNA itself is prevented by deletion of *pac* signals, which are essential for entry of viral DNA into capsids, cleavage of the HSV-1 DNA, and closing of the capsid before virion production. In the initial helper virus-free packaging system, the helper functions were provided by a set of five overlapping cosmids that cover the HSV-1 genome [111], with *pac* signals deleted (cosmid set C6△a48△a; Figure 3B) [112]. On transfection into cells, the cosmids can form a complete, replication-competent HSV-1 genome, through homologous recombination be-

tween the individual clones, and provide all helper functions necessary for the replication and packaging of co-transfected amplicon DNA. However, the packaging of the reconstituted HSV-1 genome is blocked by virtue of absence of the *pac* signals. The resulting vector stocks have amplicon titers of up to 10^6 to 10^7 t.u./ml of cell culture medium, contain levels of helper virus less than 1 of 10^7 amplicon vectors, and can efficiently transduce many different cell types, including postmitotic neurons (Figure 4), while causing minimal to no cytopathic effects at MOIs≤50 [112–116]. To simplify this packaging system, the HSV-1 genome, deleted for the *pac* signals, has recently been cloned as a single-copy, F-plasmid-based bacterial artificial chromosome in *E. coli* (fHSV△pac), which reduces the numbers of clones representing the HSV-1 genome from five (cosmid set C6△a48△a) to one (fHSV△pac; Figure 3C) [117–119].

Amplicons and Hybrid Vectors

The recent development of improved packaging systems has greatly reduced the toxicity, but had little effect on extending the stability of amplicon-mediated transgene expression. A more fundamental improvement of long-term gene expression has been achieved by new vector designs, which included the use of cell type-specific promoters and the combination of components of the HSV-1 amplicon with genetic elements from other virus vectors.

Viral immediate-early promoters, such as the HSV-1 *IE4/5* (induced by VP16) or CMV *IE1* promoters that are typically
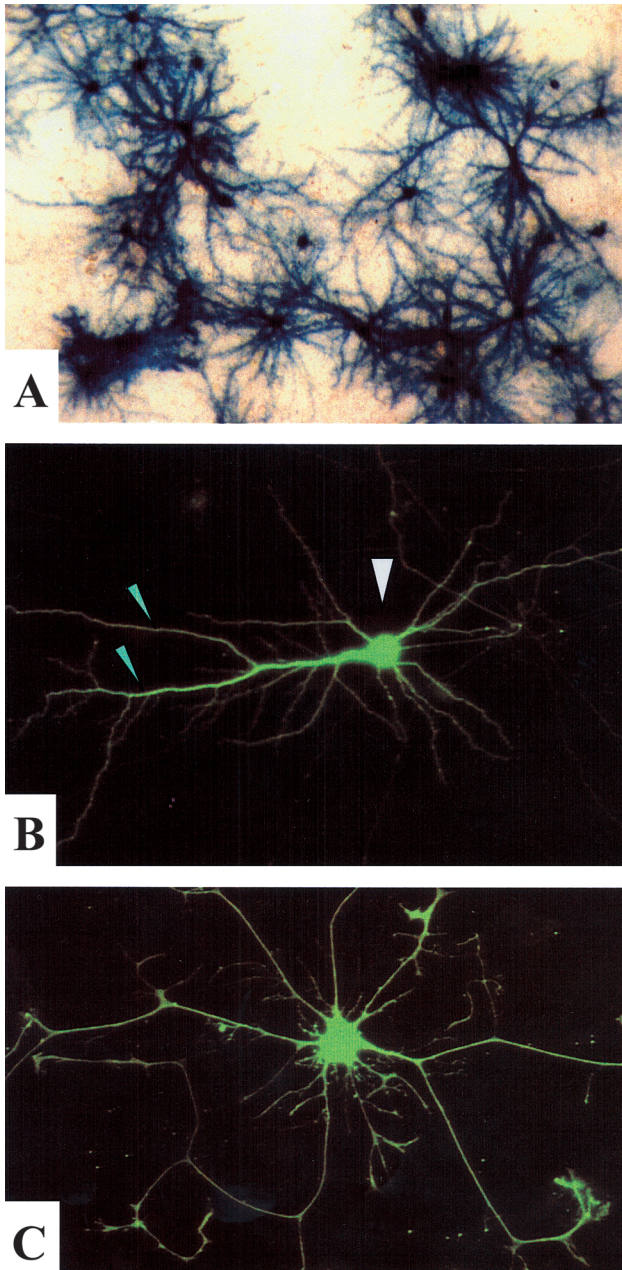
Figure 4. Helper virus-free amplicon mediated laZ (A) or gfp (B,C) expression in cultured rat cortical cells. Axons and dendrites (▼) and cell bodies (▽) are clearly delineated by expression of the reporter genes. The use of gfp as marker gene facilitates the assessment of the kinetics of transgene expression in living cells over time.

used in amplicon vectors, support strong, but transient expression of the amplicon-delivered transgene in most target cells [112,114,120,121]. Neuron-specific promoters with large 5' regulatory sequences, such as the 2.7-kb preproenkephalin (PPE) promoter and regulatory sequence, and the 9-kb tyrosine hydroxylase (TH) promoter and regulatory sequence, have been demonstrated to mediate long-term, cell type-specific gene expression from amplicon vectors in nondividing cells [122,123]. As one of the greatest attractions, the HSV-1 amplicon has a very large transgene capacity (theoretically up to ~152 kb) and can easily

accommodate large cell type-specific promoters, including their large 5' regulatory sequences and intron elements. In practice, however, amplicons with inserts of >20 kb have not yet been described. Conventional amplicon vectors use plasmid backbones which allow medium to high copy-number plasmid propagation and are, therefore, not suitable to explore the full transgene capacity of HSV-1. The development of a single-copy, F-plasmid-based amplicon (famplicon) may increase the genetic stability and allow the packaging of very large inserts (unpublished material).

The large transgene capacity of the amplicon provides also the ability to introduce genetic elements from other viruses to achieve specific properties, including genetic stability and long-term gene expression. Optimally, such hybrid vectors combine the advantageous components of their parent virus vector systems without including the disadvantages. AAV vectors are nontoxic, have a broad host range, and can either persist in an episomal form or integrate site-specifically or randomly into the host cell genome, and support stable, long-term gene expression [124–129]. However, the transgene capacity of AAV vectors is less than 5 kb, which reduces their usefulness for many applications in gene therapy. HSV-1 amplicon vectors are also nontoxic in the absence of helper virus, also have a broad host range and, in addition, have a very large transgene capacity. As the major drawback, however, transgene expression from amplicon vectors is in general transient in dividing cells as vector DNA is lost during mitosis. The combination of the advantageous elements from these two virus vector systems resulted in the HSV/AAV hybrid vector [116]. In addition to the standard amplicon elements, HSV/AAV hybrid amplicons contain those elements from AAV that allow for stable maintenance of the transgene, in particular the *rep* gene and the ITR, which flank the transgene cassette (Figure 2B). Because hybrid amplicons are packaged into HSV-1 virions, the advantages of the amplicon system, including the availability of a helper virus-free packaging system and the large transgene capacity, are conserved. After delivery into the host cell genome, the hybrid vector has the potential to act like an AAV vector with *rep*-mediated amplification of the transgene cassette and genomic integration. The functionalities of the HSV-1 and AAV elements in the context of the hybrid vector have been demonstrated: the vector can i) be packaged into HSV-1 particles, ii) efficiently transduce many different cell types, and iii) support both amplification of the ITR-flanked transgene cassette and long-term gene expression in dividing and nondividing cells [114,116,130]. Moreover, 20% to 30% of 293 cells initially transduced with a hybrid vector, bearing a transgene cassette consisting of the genes for green fluorescent protein and neomycin resistance, formed stable, green fluorescent, neomycin resistant colonies. In comparison, only ~4% of the cells transduced with a standard amplicon formed such colonies under these conditions (D.R.J., unpublished material).

Epstein-Barr virus (EBV)-based vectors, are bacterial plasmids that contain the EBV EBNA 1 gene and the EBV latent origin of DNA replication (*oriP*) which support episomal

replication of the vector and segregation to daughter cells, and up to 180 kb of foreign DNA [131–135]. As a major disadvantage of this vector system, the host range of EBV is limited to human epithelial, muscle, and some hematopoietic cells, as well as B and T lymphocytes. By inserting the EBNA 1 gene and *oriP* into an HSV-1 amplicon vector, Wang and Vos have developed a hybrid vector that replicates episomally and supports long-term transgene expression in a broad range of host cells (Figure 2*C*) [136,137]. Taking advantage of the broad host range and retention properties of HSV/EBV hybrid amplicons, a new triple hybrid vector (HER) has been constructed, which contains additional retrovirus genes *gag-pol* and *env* (GPE), to efficiently convert human and dog primary glioma cells to retrovirus vector producer cells after single step transduction [138]. This system serves an intriguing approach to circumvent disadvantages of retroviral vectors, such as low transduction efficiency, vector instability and producer cell line immunogenicity, in gene therapy of brain tumors.

HSV-1 Amplicon Vector-Mediated Gene Transfer into Cells of the Nervous System

The large transgene capacity, which allows the use of cell type-specific promoters and/or the expression of multiple transgenes, as well as the natural tropism for the CNS, make the HSV-1 amplicon an ideal vector for CNS-directed gene transfer (Figure 4). Amplicon vectors have been employed to study neuronal physiology, such as the effect of expression of GAP43 or the low affinity nerve growth factor (NGF) receptor on morphology and growth of neurons [139,140]. In hippocampal slice cultures, amplicons have been used to mediate both kainate receptor-induced toxicity [141] and glucose transporter-induced protection of neurons [142]. Amplicon-mediated expression of brain-derived neurotrophic factor induced the growth of neuritic processes in cultures prepared from spiral ganglia of the murine ear [143], and promoted neuronal survival in dissociated avian cochlear cultures [144]. Amplicons have also been used to deliver various therapeutic genes in models of CNS disease *in vivo*. For example, expression of glucose transporter protected neurons in an induced seizure model and stroke [142,145,146], expression of *bcl-2* rescued neurons after focal ischemia [147,148], and production of tyrosine hydroxylase-induced behavioral changes in parkinsonian rats [149].

An interesting approach by Brooks *et al.* employed amplicon vectors to generate mouse somatic mosaics [150]. Transgenic mice were developed that carried a NGF gene containing an inactivating insertional element, flanked by *loxP* sites, between promoter and transcript. The delivery of amplicon-encoded cre recombinase activated the expression of NGF in these animals at specific sites. The ability to induce conditional transgene expression in a spatial and temporal fashion will have broad applications for the generation of transgenic animals, especially with genes that encode toxic products or for which germline deletion is lethal.

Another property of HSV-1 relevant for CNS-directed gene transfer, is the ability of the virus particles to travel retrogradely along axons. The localization and spread of HSV-1 within the CNS has been investigated by using recombinants of HSV-1 or HSV-1 amplicon vectors that express the *E. coli lacZ* reporter gene [5,36,151,152]. After single injections into certain brain areas, transduced neurons and glia were detected at the injection site, but also at distant brain areas in neurons that make afferent connections with the cells at the primary site. For example, following striatal injection, amplicon vector-transduced cells have been demonstrated in both substantia nigra pars compacta and locus coeruleus [122,130].

Traditionally, the stability of transgene expression, as well as the toxicity and immunogenicity of the helper virus were the limiting factors for amplicon-mediated gene transfer to cells of the CNS *in vivo* [112,152–156]. However, the recent advancements discussed above have largely addressed these problems. Vector-associated toxicity and immune responses have been greatly reduced by eliminating the helper virus, and the stability of gene expression has been increased fundamentally by using hybrid vectors and/or neuron-specific promoters.

HSV-1 Amplicon Vectors for Gene Therapy of Gliomas

We evaluated the efficiency of HSV-1 amplicon and HSV/AAV hybrid amplicon vectors expressing the *tk* gene to transduce and sensitize rat and human glioma cells to GCV in culture and *in vivo* [157]. The vectors were able to efficiently sensitize cultured cells to GCV in a vector dose-, GCV dose-, cell density- and time-dependent manner. However, transduction of s.c. rat 9L gliosarcoma tumor cell grafts in nude mice with 1×10^6 t.u. of vector particles did not result in consistent tumor regression, although the transduction rate was high around injection sites (1% to 10%) as assessed by immunohistochemistry (Figure 5). The efficiency of these vectors *in vivo* may be increased by developing methods that improve the distribution of vector particles throughout the tumor [158,159]. Similarly, replication-defective HSV-1 vectors that co-express *tk* and *TNF-α* also had little therapeutic effect in an intracerebral U-87
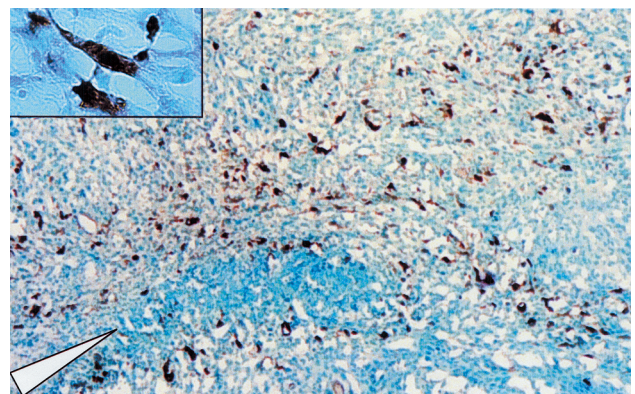

Figure 5. *Immunohistochemical analysis of a rat 9L gliosarcoma grown s.c. in a nude mouse 2 days after direct injection of 1×10^6 t.u. of HSV/AAV hybrid amplicon vectors that express tk. The immunohistochemical staining for thymidine kinase (brown cells) reveals a transduction rate of approximately 1% to 10% around the injection site (▽).*

MG tumor model, also apparently due to low transduction efficiency (<1%) [160]. By contrast, expression of *tk* from an amplicon vector that was packaged by using a $\gamma34.5$ deletion mutant of HSV-1 (R3616) as the helper virus resulted in significant growth inhibition of s.c. tumors in a syngeneic, immune-competent mouse tumor model (Gl261) [161]. In this case, the therapeutic effect was thought to be mediated by the combination of *tk*-mediated GCV sensitivity and the cytotoxicity of the helper virus, which is able to replicate in and spread within the tumor. However, no therapeutic effect was observed in human glioma cells (U87MG) grown in athymic mice, suggesting that the major factor responsible for tumor killing in the immune-competent animals may have been the immune response against the helper virus, possibly mediating enhanced immune recognition of tumor antigens. An intriguing approach would therefore be the boosting of tumor-specific immune responses by both cytotoxic, replication-conditional HSV-1 vectors and amplicon vectors encoding IL-12 [93,162].

Targeted and Regulated HSV-1 Vectors

Tissue- or cell-specific and regulatable targeting of vectors is critical to the overall success of gene therapy approaches. For HSV-1-derived vectors, this may be achieved by: i) modification of glycoprotein-mediated cell binding and entry; ii) gene complementation; iii) use of tissue-specific and/or regulatable promoters; and iv) improving the efficiency of physical vector transfer (reviewed in Ref. [163]).

The initial phase of virus attachment mediated by charge interaction between glycoproteins gB/gC and heparan sulfate moieties of the target cell membrane might be alterable by deletion or modification of essential glycoproteins. However, construction of targeted HSV-1 vectors is complicated by the highly complex process of infection. In addition, most of the glycoproteins have multiple functions and, therefore, some domains must be preserved while limiting the number that can be replaced by a new functional element. Laquerre *et al.* have constructed a recombinant HSV-1 vector for targeted binding to a non−HSV-1 cell surface receptor for erythropoietin by deletion of gC and the heparan sulfate binding domain of gB, and inclusion of chimeric proteins composed of mutated forms of gC and the erythropoietin hormone [164]. Moreover, an HSV-1 amplicon vector has been constructed expressing HSV-1 gC modified with His-tag replacing the heparan binding domain to achieve targeting vector toxicity to tumor cells [165]. These experiments demonstrate that targeted HSV-1 binding to a non−HSV-1 cell surface receptor is possible, in principle [164]. A similar approach has been very successful in targeting adenoviral vectors [166] either by direct genetic alterations of the HI loop of the adenoviral fiber knob [167,168] or by use of bifunctional antibody conjugates to adenoviral fiber and either epidermal growth factor receptor [169], folate receptor [170], or fibroblast growth factor receptor [171]. This approach can greatly increase the specificity and efficiency of gene transduction into appropriate cell types.

Replication-conditional HSV-1 vectors carrying mutations in genes that are neccessary for nucleic acid metabolism in nondividing cells, such as *tk* or RR, can be used to target proliferating tumor cells with complementary TK or RR activity [61]. A similar strategy has been employed to design an adenoviral vector for the selective killing of p53-deficient human tumor cells [172]. Apparently, the replication specificity of this vector does not reside entirely in the p53 status [173].

Transcriptional targeting can be achieved by the use of tissue-specific promoters. For example, incorporation of the glial fibrillary acidic protein (GFAP) promoter into HSV-1 recombinant 1716 yielded transgene (*lacZ*) expression predominantly in astrocytes both in culture and *in vivo* [174,175]. To target lytic virulence to gliomas, the recombinant HSV-1 **Myb34.5** was engineered by deleting the RR gene and the two endogenous copies of the $\gamma34.5$ gene and by re-introducing one copy of $\gamma34.5$ under control of the E2F-responsive, cellular B-myb promoter [176]. Whereas neurovirulence of recombinant HSV-1 Myb34.5 (PFU/$LD_{50}=2.7\times10^{7}$) was similar to that of recombinant HSV-1 R3616 (PFU/$LD_{50}>1\times10^{7}$) after direct intracerebral inoculation into BALB/c mice, Myb34.5's oncolytic efficacy against a variety of human glioma cells in culture and *in vivo* was enhanced compared to that of R3616, and was similar to that of wild-type F strain and of HSV-1 mutants that possess a wild type $\gamma34.5$ gene [176]. These results indicate that transcriptional regulation of $\gamma34.5$ by cell cycle-regulated promoters may be used to target HSV-1 virulence toward tumors, while maintaining the desirable neuroattenuated phenotype of a $\gamma34.5$ mutant. Selective destruction of hepatocellular carcinoma cells by a replication-conditional HSV-1 vector was achieved by inserting an albumin enhancer/promoter-ICP4 transgene into the *tk* locus of HSV-1 mutant *d*120, which contains deletions in both copies of the ICP4 gene [56]. The hepatocyte-specific expression of the essential *IE3* gene (ICP4) supported tissue-specific viral replication and cytolysis in dividing hepatoma cells, but not in the surrounding nondividing hepatocytes. In comparison, strategies for tumor-selective expression of therapeutic genes by adenoviral vectors make use of: i) an E2F-responsive promoter [177,178], presuming that tumor cells with mutations affecting the p16/cyclinD1/pRB/E2F pathway would have elevated levels of E2F; ii) a hypoxia-responsive promoter; or iii) a radiation inducible promoter. The different promoters direct selective transgene expression to cells with elevated E2F levels [179], hypoxemic cells [180], or to cells which have been radiosensitized [181]. All approaches result in increased control of tumor growth with limited damage to normal tissue [179,181].

Both constitutive (Gal4/VP16) and regulatable (RU486) transcriptional enhancers have been inserted into replication-conditional HSV-1 vectors. The Gal4/VP16 fusion protein consists of a DNA binding domain of the yeast transcriptional activator (Gal4) and the activation domain of the HSV-1 transactivator VP16 [182], which strongly activates promoters targeted by a Gal4-binding site [183]. Reporter gene expression was induced up to 35-fold in different models with the activator and the response

promoter present either in the same or in different replication-conditional HSV-1 recombinant viruses [184,185]. Modification of this transactivation system produced a drug-inducible system, which uses a truncated form of the hormone binding domain (HBD) of the progesterone receptor fused to the Gal4/VP16 transactivator to form a HBD-Gal4/VP16 fusion protein. Binding of the specific activator RU486 to the modified HBD [186,187] alters the conformation of the fusion protein, which is necessary to allow binding to the Gal4 promoter elements with subsequent activation of transcription. Reporter gene expression was induced 30- to 150-fold by RU486, when the activator and the responsive promoter were placed on the same replication-conditional vector [188]. HSV-1 amplicon vectors containing tetracycline-regulatable promoter elements [189–192] have also been constructed [193,194]. These vectors express reporter genes from a combined minimal CMV promoter and a heptameric tetracycline operator. This promoter element is activated by a tetracycline-responsive hybrid protein which is also encoded by the vector. Maximal repression of reporter gene expression by tetracycline in hippocampal cultures was about 50-fold and withdrawal of tetracycline de-repressed gene expression, reaching maximal levels within 10 to 12 hours [193]. In adult rat hippocampus, reporter gene expression was repressed by tetracycline 9- to 60-fold [193]. In another report [194], after injection of amplicon vector stocks into the mouse hippocampus and administration of tetracycline, transgene expression could be repressed by a factor of 10 only. Moreover, the repression factor was reduced with increasing numbers of helper viruses present in the vector stock [194], and tetracycline-regulatable constructs in other amplicon vectors have proven leaky in the "off" state due to transcriptional override by HSV-1 elements and proteins [195].

The route of vector administration is crucial for the efficient killing of tumor cells in gene therapy protocols for malignant brain tumors. The delivery of viral vectors to the brain for treatment of intracerebral tumors is most commonly accomplished by stereotaxic inoculation directly into the tumor mass. However, diffusion of the vector is limited, which reduces the efficacy of viral therapy of large or disseminated tumors [157]. This obstacle can be overcome by intracarotid delivery of vector and concurrent, selective blood-tumor barrier disruption by bradykinin or RMP-27, as described previously [196–200]. The feasibility of this approach in the clinical application, however, needs to be demonstrated in the future. Other studies suggest that access of virus to tumors can be mediated through the cerebrospinal fluid [83,84] or through migratory neural progenitor cells [201,202].

Conclusions

HSV-1 structure and function has been studied extensively by others, which has facilitated the design and engineering of HSV-1-derived vectors as gene transfer vehicles for various treatment strategies in experimental gene therapy models. The natural neurotropism of HSV-1, as well as its large transgene capacity and high transduction efficiency, make HSV-1-derived vectors suitable for applications in the nervous system. Combined selective oncolysis and modulation of the immune response mediated by replication-conditional, multiple-mutated HSV-1 vectors appears to be a highly promising approach in the battle against malignant glioma. Helper virus-free HSV/AAV hybrid amplicon vectors have great promise in mediating long-term gene expression in the PNS and CNS for the treatment of neurodegenerative disorders, injury or chronic pain. The design of HSV-1-derived vectors, which are targeted to a certain cell population and support transcriptionally regulatable transgene expression, are the focus of present and future research. The importance of developing different modes of mechanical vector delivery, which enable sufficient vector distribution, cannot be underestimated. Gene therapy is a multi-variate approach with each variable being equally important for the overall success in a clinical situation. Therefore, the design of efficient gene therapy protocols relies on the concerted research and interaction of basic and clinical scientists.

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