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Methods for Gene Transfer to the Central Nervous System

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Abstract

Gene transfer is an increasingly utilized approach for research and clinical applications involving the central nervous system (CNS). Vectors for gene transfer can be as simple as an unmodified plasmid, but more commonly involve complex modifications to viruses to make them suitable gene delivery vehicles. This chapter will explain how tools for CNS gene transfer have been derived from naturally occurring viruses. The current capabilities of plasmid, retroviral, adeno-associated virus, adenovirus, and herpes simplex virus vectors for CNS gene delivery will be described. These include both focal and global CNS gene transfer strategies, with short- or long-term gene expression. As is described in this chapter, an important aspect of any vector is the *cis*-acting regulatory elements incorporated into the vector genome that control when, where, and how the transgene is expressed.

1. INTRODUCTION

The purpose of this chapter is to provide a detailed background on vectorology as it relates to central nervous system (CNS) gene transfer. Ever since Hershey and Chase discovered that DNA was the inherited genetic material in 1952 (Hershey & Chase, 1952), and that viruses carried DNA, the notion to utilize viruses to treat genetic conditions became a natural next step. Since that time the use of gene transfer vectors as tools for research and therapeutic purposes has been extensively explored. While this chapter will touch on the use of vectors for therapeutic purposes, it will mostly describe their capabilities as general CNS gene transfer reagents.

To begin with, a few relevant terms will be defined. A *vector* is a vehicle used to move genetic information into a target cell. While *gene transfer* refers to the movement of genetic material, *transduction* more specifically refers to the successful delivery and expression of a foreign nucleic acid within a target cell. *Transduction* typically refers to virus-mediated gene transfer, while *transfection* refers to nonviral gene transfer. While *gene transfer* can refer to any movement of genetic material, it should not be termed *gene therapy* until the gene transfer results in a positive therapeutic outcome.

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The scope of this chapter will cover the most common vectors for CNS gene transfer, including plasmid-containing nanoparticles, and recombinant vectors derived from retroviruses, adeno-associated virus (AAV), adenovirus, and herpes simplex virus (HSV). Retroviral and AAV vectors have become the prominent CNS delivery tools, and these will be covered in the most detail. Each of these vectors has advantages and disadvantages for specific gene transfer needs. For each viral vector, background will be provided on the virus from which the vectors were derived and on the process that was undertaken to engineer a wild-type (wt) virus into a gene transfer vector. Further modifications, such as transcapsidation or pseudo-enveloping, are described that alter the targeting of the vectors based on the needed gene transfer application. The nanoparticle design, viral capsid, or viral envelop dictates what cells are targeted. Within that population of cells, which receive the transgene, expression and vector genome persistence is dictated by the design of the expression cassette. This is an extremely important aspect of the over vector design and key *cis*-acting regulatory elements and expression systems will be discussed.

There are other vectors that have been used for CNS gene transfer, notably including a large range of strategies to develop antitumor therapies. Many of these approaches utilize replicating lytic viruses to specifically infect and eliminate cancer cells. These strategies and vectors are not covered in this chapter except for some examples provided in the section on HSV vectors, but they are covered well in recent review articles (Assi et al., 2012; Tobias, Ahmed, Moon, & Lesniak, 2013). Similarly, the topics covered will be limited to the delivery of transgene expression constructs. For the purpose of this chapter, this excludes the delivery of small oligonucleotides which by some definitions could be considered gene therapy.

2. VECTORS FOR CNS GENE TRANSFER

2.1 Plasmid DNA/Nanoparticles

Perhaps the simplest, cheapest, and least toxic approach to gene therapy is to utilize naked plasmid DNA to express a foreign transgene. The normally transient nature of gene expression from plasmids is usually seen as a detriment, but it could be a benefit in certain applications where only short-term gene expression is needed or risks of long-term transgene expression are uncertain (such as delivery of growth factors). In contrast to some viral vectors, the use of plasmid DNA minimizes safety complications such as high frequency of insertional mutagenesis and immunogenicity to the viral carrier. Moreover, the plasmid could be injected repeatedly as needed, similar to pharmacologic intervention. Unfortunately, the uptake of naked DNA into cells, subsequent trafficking to the nucleus, and long-term retention of transcriptionally active plasmid is an extremely inefficient process. Moreover, repeated direct injection of vector into the brain parenchyma is not practical. Approaches to deliver plasmids to the brain are mostly focused on packaging the plasmids within nanoparticles that can be administered peripherally. The use of various transfection reagents complexed to the DNA, as well as *cis*-elements in the plasmid design to promote stability, is the main area of optimization using nonviral DNA vectors.

The field of nanoparticle design is rapidly evolving, and although it is often developed for protein or small molecule delivery to the brain it can often be adapted to carry plasmid

cargo. A common nanoparticle design is to use a polyethylene glycol polymer as a basic scaffold of the nanoparticle, to provide stability and basic structure. Specific receptor ligands can be incorporated into the nanoparticle to target them to specific tissues or cells, often termed “Trojan horse liposomes” (reviewed by Boado and Pardridge (2011), Boado (2007), Tosi et al. (2013)). As an example, Zhang and colleagues created polyethylene glycolylated immunoliposome (PIL) that incorporated receptor ligands for blood–brain barrier (BBB) transporters. After intravenous infusion of reporter plasmids contained in these PILs, widespread and efficient neuronal expression of the reporter genes was achieved in mouse and rat brains (Zhang, Boado, & Pardridge, 2003). This approach was applied in a therapeutic preclinical setting for mucopolysaccharidosis (MPS) type VII, wherein a plasmid-containing nanoparticle expressing the GUSB gene was administered to MPS VII mice intravenously and led to thereapeutic levels of GUSB enzyme production in the brain and peripheral organs (Zhang, Wang, Boado, & Pardridge, 2008).

Expression is typically transient, requiring repeated administration of the plasmid-containing nanoparticles. This is due to either silencing of the plasmid DNA and/or failure to replicate with dividing cells. Incorporation of specific *cis*-acting chromatin modulators into the plasmid can increase the efficiency of plasmid retention and lead to long-term gene expression through the inhibition of heterochromatin formation. The ubiquitous chromatin-opening element and insulator elements such as the chicken beta-globin hypersensitive site 4 (HS4) were identified as optimal elements to enhance gene expression strength and length (Hagedorn, Antoniou, & Lipps, 2013). Another common modification to prolong expression and improve the safety of plasmid vectors is to remove CpG dinucleotides from the plasmid sequence, which are known to promote both heterochromatin formation as well as inflammatory responses (Takahashi, Nishikawa, & Takakura, 2012; Tolmachov, 2009).

In conclusion, plasmid-based vectors have a potential advantage over viral-based vectors due to their reduced immunogenicity and safety. In certain applications, the transient nature of expression is an advantage. Evolving improvements in nanoparticle design and incorporation of *cis*-DNA elements may increase their equivalence to virus-based approaches for gene transfer, allowing efficient gene transfer and long-term transgene expression after a single dose. A technical challenge is the scalability of increasingly complex nanoparticle designs for large animal and human use.

2.2 Adeno-Associated Virus

2.2.1 Background and Advantages/Disadvantages—AAV has emerged as one of the safest and most commonly used vectors for the delivery of therapeutic genes (reviewed in Lentz, Gray, and Samulski (2012)). AAV belongs to the Parvoviridae family in the *Dependovirus* genus, which depend on the coinfection of a helper virus (adenovirus or HSV) for replication in host cells (Atchison, Casto, & Hammon, 1965). In the absence of a helper virus, AAV may stably integrate, albeit at relatively low frequency, into the host gene cell and remain quiescent. Consequently, wt AAV and recombinant AAV (rAAV) used for gene therapy do not have any known associated pathologies and cause a very mild immune response. The human population has widespread exposure to a variety of AAV serotypes,

however. Preexisiting immunity and the presence of anticapsid neutralizing antibodies to AAV is a serious challenge to overcome in human clinical trials utilizing rAAV.

AAV is a small, non-enveloped virion that is only ~20 nm in diameter and has an icosahedral protein capsid encompassing ~4.7 Kb of linear single-stranded DNA (Cassinotti, Weitz, & Tratschin, 1988; Rose, Maizel, Inman, & Shatkin, 1971; Xie et al., 2002). The benefit of a small genome is that scientists can easily manipulate AAV to package a transgene of interest into rAAV for the purpose of gene delivery. A disadvantage of a small vector genome, however, is that the size of the transgene that can be packaged is limited and large genes are not suitable for use in a standard AAV vectors. Multiple serotypes of AAV exist with distinct tissue selectivity and transduction efficiency due to differences in the capsid protein composition. Extensive research of naturally occurring serotypes and manipulation of these serotypes in the laboratory has provided the knowledge for engineering capsids of rAAV vectors that have a broad selection of specifically targeted tissues with minimal transduction of off-target cells, tissues, and organs. Importantly, the duration of AAV-delivered transgene expression is essentially permanent in nondividing cells following just a single dose. AAV-delivered transgenes express for more than 6 months in the mouse brain (Klein et al., 1999) and can persist in other tissues for at least 6 years in primates (Rivera et al., 2005) and at least 8 years in dogs (Niemeyer et al., 2009; Stieger et al., 2009). Importantly, a recent gene therapy trial has shown that the therapeutic effects of AAV-delivered transgenes can persist for at least 10 years in the human brain (Leone et al., 2012). AAV vector plasmids have been further improved to allow for the large-scale production of highly pure vector necessary for the treatment of humans. Overall, rAAV vectors have emerged as a viable delivery method for human gene therapy as they can be designed to meet the precise treatment needs of a given disease by delivering a gene to specific cell types within the affected tissues with a minimal immune response.

A final concern, similar to other viral vectors, is the possibility of immune clearance of the vector and vector-infected cells (Vandenberghe & Wilson, 2007). An estimated 25–30% of the human population carries neutralizing antibodies against AAV2, the most common serotype for rAAV-mediated clinical trials (Halbert et al., 2006; Hildinger et al., 2001; Xiao et al., 1999). The use of other AAV serotype capsids that are less prevalent in the human population, as well as modification of immunogenic epitopes on the capsid surface, may also circumvent this problem.

2.2.2 Basic Biology and How AAV Is Used as a Vector—The single-stranded DNA AAV genome consists of three open reading frames that are flanked on either side by 145 base-pair inverted terminal repeats (ITRs) (Lusby, Fife, & Berns, 1980; Sonntag, Schmidt, & Kleinschmidt, 2010; Srivastava, Lusby, & Berns, 1983). The ITRs are predicted to form a stem loop structure. ITRs are the only *cis*-acting elements that are necessary for genome replication, integration, and packing into the capsid (Lusby et al., 1980; Nash, Chen, & Muzyczka, 2008; Straus, Sebring, & Rose, 1976). The wt viral genome encodes four replication proteins (Rep 78, 68, 52, 40) that are critical for many aspects of the viral life cycle, three viral structural proteins (VP1, 2, 3) that form the virion capsid and the assembly activating protein (AAP), which is thought to localize the AAV capsid proteins to the nucleolus and function in capsid assembly (reviewed in R. H. Smith (2008)). The capsid is

the protein shell of a virus that protects the genetic material of a virus while interacting with the host environment. As such, the capsid determines the tissue specificity or tropism of a given virus by regulating the immediate cellular response to the virus, mediating pathways for internalization into the cell, and functions in the uncoating process within the nucleus. The capsid is icosahedral and has 20 equilateral triangular faces, with each face consisting of VP1, VP2, and VP3 proteins that are estimated to combine in a ratio of 1:1:10 in wt AAV (Kronenberg, Kleinschmidt, & Bottcher, 2001). VP1 and VP2 are identical to VP3 except that they have an additional N-terminus. Specific regions of the capsid proteins interact with receptors and coreceptors on the host cellular surface to mediate the viral infection process and serotypes can differ with respect to the receptors that they bind to. AAV infects a host cell through receptor-mediated endocytosis via clathrin-coated pits (Bartlett, Wilcher, & Samulski, 2000). Following phagocytosis, the virus must escape from the early endosome and be transported to the nucleus where uncoating occurs to release the viral genome that is then transformed to double-stranded (ds) DNA (Ferrari, Samulski, Shenk, & Samulski, 1996). In the absence of a helper virus, wt AAV DNA can be retained in linear and circular episomal forms (Duan et al., 1998; Schnepp, Jensen, Chen, Johnson, & Clark, 2005) or it can be stably integrated into the host cell genome on human chromosome 19 (Cheung, Hoggan, Hauswirth, & Berns, 1980; Kotin et al., 1990; Samulski et al., 1991). Coinfection with a helper virus or cellular stress triggers a lytic cycle where AAV transcription and DNA replication are reactivated to produce AAV viral particles.

Over the last 30 years, wt AAV plasmids have been drastically transformed to create nonpathogenic, pure rAAV vectors that can be used for human gene therapy (Figure 3.1). rAAV vectors are derived from a wt AAV plasmid construct that retains only the ITRs that flank a transgene cassette that consists of a promoter with a gene of interest. AAV2 was the first extensively used AAV serotype and as a result, the majority of rAAV vectors today contain the ITRs from AAV2. The AAV genome is limited to a packaging capacity of ~4.7 kb, and the genome cannot exceed this size (Dong, Nakai, & Xiao, 2010; Dong, Fan, & Frizzell, 1996; Hirsch, Agbandje-McKenna, & Samulski, 2010; Lai, Yue, & Duan, 2010; Wu, Yang, & Colosi, 2010), so the AAV *rep* and *cap* coding sequence are replaced with the transgene cassette in rAAV vectors. The *rep* and *cap* genes are then expressed on a separate plasmid, called an AAV packaging plasmid or AAV helper plasmid. The separation of these genes from the vector plasmid DNA is critical to prevent the formation of wt AAV. rAAV also loses the specificity of integration into human chromosome 19 and appears to integrate randomly at an infrequent rate while most genomes are maintained as episomes. To generate rAAV, the vector and packaging constructs must be cotransfected into cells that have been infected with a helper virus, such as adenovirus. Adenovirus functions as a helper virus by supplying the E1a, E1b, E2a, E4orf6 and viral-associated RNA genes for rAAV production (Xiao, Li, & Samulski, 1998).While coinfection of adenovirus and rAAV vectors into producer cells is an effective means of generating rAAV, it also results in the production/ contamination of adenovirus particles. To circumvent this issue, an adenovirus helper plasmid, called pXX6, was developed that contains only the essential adenovirus helper genes (Xiao et al., 1998). Using a triple transfection method, the vector DNA plasmid with the transgene cassette flanked by AAV ITRs, the AAV packaging plasmid containing the *rep* and *cap* genes of a specific AAV serotype, and the adenovirus helper plasmid are

cotransfected into cells, such as HEK 293, for rAAV production (Xiao et al., 1998). Together these optimized plasmids and methods enable the large scale production of pure rAAV with low immunogenicity that can be used for gene transfer, including human gene therapy.

2.2.3 Transcapsidation to Change Tropism—Over 100 AAV serotypes and variants have been described (Gao, Vandenberghe, & Wilson, 2005; Wu, Asokan, & Samulski, 2006), each of which differs in amino acid sequence, particularly in the hypervariable regions or looped out domains that are found on the capsid surface (Gao et al., 2003). The most studied serotype is AAV2, which binds the primary receptor heparan sulfate proteoglycan and the coreceptors αvβ5 integrins (Qing et al., 1999; Summerford & Samulski, 1998; Summerford, Bartlett, & Samulski, 1999). AAV3 also binds to heparan sulfate, although with an elution profile that is quite distinct from AAV2, suggesting that these serotypes interact differently (Rabinowitz et al., 2002). In contrast, AAV4 and AAV5 do not interact with heparan sulfate but instead interact with sialic acid moieties, although through different linkages (Kaludov, Brown, Walters, Zabner, & Chiorini, 2001; Walters et al., 2001). See Table 3.1 for an expanded list of the receptor specificity for each serotype. Different cell types have different cell surface receptors, so each AAV serotype transduces multiple cell types with distinct specificity between serotypes and with varying efficiency. This was well exemplified in a study using AAV serotypes 1–9 packaging a luciferase reporter gene that was injected into the tail vein of mice (Zincarelli, Soltys, Rengo, & Rabinowitz, 2008). It is also thought that serotype may determine the particular mechanism of viral trafficking from the cell surface to the nucleus and in the nuclear viral uncoating process, which may in turn regulate the efficiency of transduction (Keiser, Yan, Zhang, Lei-Butters, & Engelhardt, 2011).

The driving force behind the manipulation of the AAV capsid is to map and understand the contribution of the capsid amino acids, as well as to exploit this information to alter the tropism and/or transduction efficiency. The simplest way to alter the capsid is through transcapsidation, which is the packaging of a genome containing ITRs from one serotype in the capsid to a different serotype. This technique has been adopted for rAAV vectors so one recombinant genome construct can be easily packaged in multiple capsids, so that gene transfer can be targeted to different tissues. Historically, AAV2 was the most widely used serotype and early vectors that used the ITRs of serotype 2 could only be packaged in the corresponding capsid serotype to obtain sufficient yields. Rabinowitz et al. (2002) developed a cross-packaging system that allowed a vector with serotype-2 ITRs to be transcapsidated with the capsids of other AAV serotypes. In the AAV packaging plasmid, the AAV2 rep sequence downstream of the p19 promoter was replaced with the rep sequence corresponding to the desired capsid serotype. These packaging plasmids were named pXR1-5 to denote replication and packaging of a serotype-2 ITR genome into serotypes 1–5. A benefit of transcapsidation is that serotype tropism can be directly compared in vivo when they package equivalent genomes and the only variable is the AAV capsid (Rabinowitz et al., 2002; Zincarelli et al., 2008). This technique has now been expanded to mosaic capsids that are a packaged mixture of unmodified capsid proteins from different serotypes. To generate mosaic vectors, a mixture of AAV packaging constructs are used that encode

capsid proteins from different serotypes or wt and mutant capsid proteins of the same serotype or even from two different capsid subunit of the same serotype (reviewed in Choi, McCarty, and Samulski (2005)). In doing so, features of each source can be handpicked that synergistically enhance transgene expression in addition to altering tropism.

2.2.4 Capsid Engineering: Rational Design, Shuffing, Peptide Insertion—One of the most critical aspects of viral-mediated gene therapy is the specific targeting of tissues that require the gene of interest with minimal transduction of off-target cells, tissues, or organs. Transduction of off-target tissues can have deleterious effects including insufficient delivery of therapeutic genes to target tissue and large therapeutic doses to compensate for this. Tissue tropism is controlled via the viral capsid and extensive work has been done to identify naturally occurring AAV serotypes and to expand their tissue tropism through transcapsidation. Utilizing knowledge gained from naturally occurring serotypes and mosaic capsids, researchers are now able to engineer rAAV capsids to enhance tissue selectivity and specificity as well as to evade host neutralizing antibodies. Second-generation AAV vectors are generated through methods including (1) rational design based on known AAV structure and biology, (2) use of directed evolution through mutagenesis and DNA shuffling, and (3) peptide insertion of ligands into the AAV capsid (reviewed in Gray, Woodard, and Samulski, (2010)). Together, these newly engineered AAV vectors offer a broad range of selection to meet different experimental and therapeutic needs.

The rational design method of vector engineering uses the current body of knowledge of capsid protein structure–function to insert or exchange small epitopes into the capsid shell as a means of retargeting AAV tropism. As the functions of each component of the capsid shell are delineated, this information can be used to select or remove specific components of the viral tropism, until only the desired properties are combined into a single capsid coating. In addition to altering cell specificity, rational design can be used to enhance the efficiency of gene transduction and improve the safety profile of a given vector. Rational design has been used to develop chimeric AAV capsids, which have critical amino acids and/or domains from one serotype incorporated into the capsid of another. The choice and locations of these amino acid modifications are determined by analysis of known capsid function combined with structural information. For example, using the AAV2 capsid, Bowles et al. (2012) used sequence alignment and site-directed mutagenesis to swap five AAV1 amino acids into the AAV2 capsid that were different from the AAV2 sequence and were located in a structurally variable region on the capsid surface to create the AAV2.5 vector. The AAV2.5 vector had improved muscle targeting properties of AAV1, as well as reduced cross-reactivity with antibodies against both AAV1 and AAV2, and was successfully used in Phase 1 clinical trials for Duchenne muscular dystrophy to deliver the minidystrophin gene into the muscles of patients with no adverse effects (Bowles et al., 2012). In another example of capsid mutagenesis, Pulicherla et al. (2011) introduced point mutations into AAV9 to knock down its liver tropism, potentially creating a safer version of AAV9 to deliver intravenously to the CNS. Rational design of AAV capsids can also be accomplished by mutating tyrosine residues on the capsid shell. Phosphorylation of tyrosine residues on the capsid of AAV2 has been shown to negatively impact the intracellular trafficking of virus following uptake and transgene expression in vivo (Zhong et al., 2008). Mutation of surface exposed capsid

protein tyrosine residues to phenylalanine results in increased transduction efficiency due to reduced intracellular trafficking to the proteasome and improved intracellular trafficking to the nucleus (Zhong et al., 2008). A similar increase in transduction efficiencies was also found when capsid tyrosine residues on AAV2, AAV8, and AAV9 were mutated to prevent phosphorylation (Petrs-Silva et al., 2009).

Directed, or molecular, evolution is an unbiased method by which investigators randomly mutate and/or shuffle the cap protein coding sequence to develop novel AAV vectors with altered tropism. This approach expands upon the DNA shuffling technique originally developed by Stemmer, which uses random fragmentation of a gene to create pools of selected mutant genes that are then reassembled into full-length sequences using a polymerase chain reaction (PCR)-like process (Stemmer, 1994). In directed evolution, the *cap* genes from multiple AAV serotypes are fragmented, reannealed using a primerless PCR reaction, and then in a second PCR reaction are assembled into full-length chimeric *cap* genes. Chimeric viral libraries produced in this manner can then be administered systemically, the target tissue isolated, and viral capsid sequences within this tissue amplified through PCR. Using these recovered library capsids, a new library can be produced to undergo subsequent rounds of genetic diversification and selection. This approach enables the investigator to select a capsid with the desired vector phenotype from a pool of diverse mutants and does not require prior knowledge of AAV's structure–function biology. Recently, Gray, Blake, et al. (2010) have used DNA shuffling and directed evolution to create novel AAV vectors with the ability to target therapy transgene to sites of seizure damage in the brain of an epileptic rat while being detargeted from the liver and other off-target tissues. Specifically, the capsid DNA from AAV serotypes 1–6, 8, and 9 was fragmented, shuffled, and recombined to create a library of chimeric AAVs that were then injected intravenously into rats following the induction of a seizure. Three days later, seizure-prone brain sites were harvested and viral DNA was isolated from these tissues. Through four cycles of selection, two novel AAV vectors were identified that were able to cross the seizure-compromised BBB and efficiently transduced brain cells. A potential caveat of the directed evolution approach, however, is that vectors designed in cell lines and small animal models may not have fully recapitulated properties in large animals and humans.

Peptide insertion is a method in which known ligands are directly inserted into the AAV *cap* gene as a means of expanding the cell or tissue tropism of the wt vector. Recently, this approach has been used to target AAV2 vectors to the CNS. For example, in one study, Xu, Ma, Bass, & Terwilliger, 2005 inserted peptides into the AAV2 capsid that were derived from an N-methyl-D-aspartate receptor agonist and a dynein binding motif to increase the axonal retrograde transport of AAV2 to the CNS by 10- to 100-fold. In a second study, Chen, Chang, and Davidson (2009) used phage-display biopanning in two different lysosomal storage diseased mouse models and in wt mice to identify peptides that bound the blood vasculature under diseased and normal conditions. By incorporating these peptides into the AAV2 capsid, AAV2 tropism could be expanded to include selective targeting of virus to the CNS vasculature of a disease model but not wt mice, or to wt mice but not to pathogenic mice (Chen et al., 2009). A major challenge with peptide insertion, however, is

the disruption of the stability and function of both the ligand and the transducing vector. Additionally, a given peptide insertion allows the targeting of a single receptor, so that targeting of a new receptor requires additional genetic modification of the capsid proteins.

2.2.5 Self-Complementary AAV—Researchers have created an altered version of AAV termed scAAV that packages two complementary copies of the genome that are linked in *cis* through a mutated ITR (McCarty, Monahan, & Samulski, 2001). One of the factors affecting the transduction efficiency of rAAV vectors is the conversion of the single-stranded DNA vector genome into dsDNA to achieve gene expression. By avoiding synthesis of a second strand, scAAV can form a stable ds intermediate more quickly, leading to better vector genome retention and faster transgene expression. scAAV vectors are produced by deleting the terminal resolution site from one rAAV ITR, so that replication cannot be initiated from the mutated ITR (McCarty et al., 2003). These constructs result in single-stranded, inverted repeat genomes with a wt ITR at each end and a mutated ITR in the middle. After uncoating, it is thought that intramolecular base pairing begins at the mutant ITR and then proceeds through the vector genome to fold the genome into a ds or self-complementary form. Benefits of using scAAV vectors over traditional single-stranded AAV vectors include the quicker onset of transgene expression and a 10- to 100-fold increase in transduction efficiency (Gray, Matagne, et al., 2011; McCarty et al., 2003, 2001). As a caveat though, scAAV can only encode half of the already limited capacity of AAV. The AAV vector, including the ITRs, is a maximum of ~4.7 kb in length. If carrying single-stranded DNA, AAV vectors can deliver ~4.5 kb of unique transgene sequence; however, scAAV vectors are only able to carry ~2.2kb because the unique transgene sequence is in duplex form. Thus, as part of AAV vector development, much emphasis has been placed on the design of minimal promoters, 3′ and 5′ untranslated regions, and polyadenylation (polyA) signals to increase the remaining amount of available coding sequence for the transgene (reviewed in (Gray (2013)).

2.2.6 Utilizing Specific Serotype Capsids and Routes of Administration to Transduce CNS Targets—Recent advancements in vector design and the use of alternative routes of viral administration place rAAV at the forefront of vectors for gene delivery to the CNS (reviewed in (Gray (2013)). AAV vectors have been extensively used to deliver genes to neurons in both basic and clinical applications due to their ability to infect nondividing cells, high transduction efficiency, long-lasting expression from a single dose, and relatively low host immune response (Kaplitt et al., 1994; McCown, Xiao, Li, Breese, & Samulski, 1996). The most commonly used AAV serotypes in the CNS include AAV1, AAV2, AAV4, AAV5, AAV6, AAV8, and AAV9. The early characterized AAV2 vector has been most extensively used by researchers and in clinical studies, but the spread and transduction in the brain is rather limited compared to more recently characterized serotypes. While AAV2 preferentially transduces neurons after direct injection into the brain parenchyma, AAV1 and AAV5 have been shown to be more efficient at targeting neurons and transduce some glia as well, in multiple regions of rat and nonhuman primate brain regions (Mandel & Burger, 2004). When injected intracerebrally, AAV7, AAV8, and AAV9 primarily transduce neurons and AAV9 vector shows the greatest spread from the site of injection (Cassia N. Cearley & Wolfe, 2006). Viral spread is dependent upon both

extracellular and intracellular transport, the latter of which can occur in either the anterograde or retrograde direction along axons (Kaspar, Llado, Sherkat, Rothstein, & Gage, 2003; Kaspar et al., 2002). Axonal transport varies amongst the AAV serotypes and can be exploited to enhance therapeutic efficacy by infecting both the cell types targeted by a vector as well as the projection field of those cells. For example, when injected into the ventral tegmental area, both AAV1 and AAV9 have been shown to disseminate along axonal projections in both directions (Cearley & Wolfe, 2007). AAV1, AAV5, and AAV9 show the greatest spread and highest transgene expression. When administered intrathecally, AAV6, AAV8, and AAV9 infect cells in the spinal cord and dorsal root ganglia, with the affected cell population dependent on the serotype (Snyder et al., 2011; Storek et al., 2008; Towne, Pertin, Beggah, Aebischer, & Decosterd, 2009). AAV4 preferentially targets ependymal cells through intracerebral ventricular injection, and this strategy was successfully employed to treat mice with MPS VII (Liu, Martins, Wemmie, Chiorini, & Davidson, 2005). Given that AAV genomes do not persist in dividing cell populations, the long-term efficacy of this approach is questionable and remains to be tested, since the ependyma has a turnover rate of approximately 130 days (Chauhan & Lewis, 1979). One of the challenges of targeting the brain for gene delivery is identifying vectors that are able to cross the BBB so that, ideally, a gene therapy can be administered peripherally. Work by Duque et al. (2009) and Foust et al. (2009) demonstrated that AAV9 crosses the BBB in mice and cats when injected intravenously in both neonatal and adult animals; AAV8 was also found to cross the BBB in mice, although to a lesser extent than AAV9 (Gray, Matagne, et al., 2011). Importantly, both neurons and astrocytes were transduced by intravenously injected AAV9 vectors, demonstrating that it is possible to deliver gene therapy to a large portion of the brain and spinal cord without having to inject directly into the CNS. The ability of AAV9 vectors to cross the BBB following intravenous injection in mice, rats, cats, and nonhuman primates has been reported by multiple groups (Bevan et al., 2011; Gray, Matagne, et al., 2011; Zhang et al., 2011), and found to be at least 10 times more efficient when performed with scAAV vectors rather than single-stranded AAV vectors (Gray, Matagne, et al., 2011; Wang et al., 2010). In mice, intravenous delivery of AAV9 has been successful in treating disorders of the CNS, including spinal muscular atrophy (SMA) (Foust et al., 2010) and MPS IIIB (Fu, Dirosario, Killedar, Zaraspe, & McCarty, 2011) in mice. Conversely, the use of AAV9 vectors to treat specific CNS disorders may be precluded by altered receptor expression on the cell surface of target tissue as part of the disease phenotype. For example, in a mouse model of lysosomal storage disease, peripherally administered AAV9 failed to transduce CNS tissue due to increased brain levels of sialic acid, which covered the terminal galactose residues used by AAV9 (Chen, Claflin, Geoghegan, & Davidson, 2012). There are several additional challenges in moving gene therapies from small animals to humans, including the presence of anti-AAV9 neutralizing antibodies in the human population, the large amounts of vector that must be produced for therapy when scaled to larger animals and humans, and the off-target distribution of virus to peripheral tissues.

One strategy for translating AAV9 gene therapy from small animals to humans is to utilize alternate viral delivery routes. Though more invasive than an intravenous injection, one approach that researchers are currently exploring is the use of intra-CSF delivery via

intrathecal injection into the lumbar cistern or cisterna magna (Bevan et al., 2011; Federici et al., 2012; Gray, Nagabhushan Kalburgi, McCown, & Jude Samulski, 2013; Samaranch et al., 2012; Wang et al., 2014). Intra-CSF delivery could better target cells in the spinal cord, reduce expression in off-target peripheral organs, and lower viral doses compared to those used for intravenous delivery. In contrast to direct intracranial injection to the brain parenchyma, use of the CSF for viral delivery would still allow volume dose scaling across different species. In pigs, AAV9 delivery via intrathecal injection transduced the majority of motor neurons across the entire spinal cord with minimal targeting of virus to peripheral organs (Federici et al., 2012). In a study that compared the use of intravascular versus intracisternal injection of AAV9 in nonhuman primates, greater transduction of the CNS was achieved using intra-CSF vector delivery at a lower dose than the intravascular injections (Samaranch et al., 2012). Interestingly, circulating anti-AAV9 neutralizing antibodies were detected following intra-CSF injection (Samaranch et al., 2012). In a second study by Gray et al. (2013), intra-CSF delivery via intrathecal or intracisternal injections were compared in nonhuman primates. Intra-CSF delivery using a single injection by either method resulted in widespread transduction of AAV9 throughout the entire CNS, particularly in the spinal cord. Biodistribution to peripheral organs was detected, although at much lower levels than seen with intravascular delivery. Low levels of circulating anti-AAV9 neutralizing antibodies did not appear to have inhibitory effects on targeted gene transfer in the CNS by intra-CSF administration, although higher levels did show some inhibition (Gray et al, 2013; Samaranch et al., 2012). Overall, multiple groups have now shown that intra-CSF delivery of AAV9 vectors results in widespread expression of transgenes in large animals and support the use of intra-CSF AAV9 vector delivery for gene therapy in humans (Haurigot et al., 2013).

2.3 Retrovirus/Lentivirus

2.3.1 Introduction—HIV-1 based (lentiviral) vectors are among the most intensely studied vectors utilized for virus-mediated gene transfer. These studies established the foundation of exploiting lentiviral vectors as vehicles for efficient gene delivery into broad range of tissues and organs. The capacity of efficient integration into the host genome, ability to infect nondividing cells and shuttle large genetic payloads, and maintenance of stable, long-term trans-gene expression are attributes that have brought lentiviral vectors to the forefront of gene therapy.

A little more than 30 years after the first retroviral gene transfer experiment demonstrated transfer of a HSV, thymidine kinase (*tk*) gene into the genome of a mouse cells (Shimotohno & Temin, 1981; Wei, Gibson, Spear, & Scolnick, 1981), a retroviral gene transfer field has reached a stage of great diversity and progress. This development is attributed to better understanding of biology of the Retroviridae family members. As a hallmark, all members of this family are capable of converting single-stranded RNA (*ss*RNA) of the retrovirus into dscDNA (*ds*DNA), which can be then stably integrated into the host genome and replicated along with it (Baltimore & Huang, 1970; Mizutani, Boettiger, & Temin, 1970; Temin & Mizutani, 1970). As highly evolved parasites retroviruses act in concert with cellular host factors to ensure delivery of their genetic payload into the nucleus, where they exploit host machineries to fulfill replication and long-term expression.

The Retroviridae family comprises seven major genera: alpha-retroviruses (prototype ALSV), beta-retrovirus (prototype MMTV), delta-retrovirus (prototype HTLV-I, HTLV-II), gamma-retrovirus (prototype MLV), epsilon-retrovirus (prototype WDSV), lenti-retroviruses (prototype HIV-1), and spuma viruses (prototype SFV) (Figure 3.2) (classified in (Coffin (1992)). Many of these retroviruses have been studied and developed for retroviral gene transfer. Although all the aforementioned viruses have potential interest for retroviral gene transfer, so far the focus has almost exclusively been on the two genera: simple gamma-retroviruses (mammalian C-type viruses), exemplified by murine leukemia virus (MLV) (reviewed in Baum, Schambach, Bohne, and Galla (2006)), and complex lentiviruses, exemplified by HIV-1. However, inability of transducing nondividing cells has restricted employment of the gamma-retroviruses primarily for gene transfer into hematopoietic cells (Lewis & Emerman, 1994; Roe, Reynolds, Yu, & Brown, 1993; Suzuki & Craigie, 2007).

Lentiviral vectors, in contrast, have evolved the ability to transduce non-dividing and slowly dividing cells (Bukrinsky et al., 1993; Lewis, Hensel, & Emerman, 1992), an attribute that significantly broadened the use of the lentiviral vectors for the gene delivery into numerous tissues and organs, including the CNS.

2.3.2 HIV-1: Structure and Life Cycle—HIV-1, as mentioned above is a member of the Lentivirinae genus also including HIV-2, simian immunodeficiency virus (SIV) and nonprimate lentiviruses, such as visna virus, equine infectious anemia virus (EIAV), caprine arthritis-encephalitis virus (CAEV), and the feline and bovine immunodeficiency viruses (FIV and BIV) (classified in (Coffin (1992)) (Figure 3.2). Although, all of the aforementioned lentiviruses were intensively studied and engineered into vectors, the first lentiviral vector that developed, HIV-1 based, is still the most promising among them (Naldini, Blomer, Gage, Trono, & Verma, 1996; Naldini, Blomer, Gallay, et al., 1996). This is attributed to a better understanding of the basic biology and the life cycle of the HIV-1 (for more comprehensive review see Coffin, Hughes, and Varmus (1997)).

A capsid of the HIV-1 is an enveloped protein shell that is 80–100 nm in diameter and contains the viral genome. The HIV-1 genome is encoded by an approximately 9 kb positive sense single-stranded RNA molecule, which is packaged within lipid-enveloped viral particles. The HIV-1 *env* gene encodes the envelope glycoprotein of the virus. The native envelope of HIV is a glycoprotein, gp120, that is essential for viral entry into cells as it plays a vital role in attachment to specific cell surface receptors via a specific interaction with the CD4 receptor and coreceptors, which are located on macrophages, dendritic cells, and particularly on helper T-cells. Binding to CD4 induces the start of a cascade of conformational changes in gp120 and its internal part, gp41, that lead to the fusion of the viral and host cell membranes. Interaction between gp120–gp41 and the host receptor and coreceptors is under intensive investigation, since it is hoped that better understanding of this step will be essential for the design of a vaccine against HIV-1 (Julien et al.; Lyumkis et al.; Merk & Subramaniam).

Following attachment and entry into host cells, viral reverse transcription takes place in the host's cytoplasm. The process of reverse transcription generates a ds linear DNA that serves

as a precursor for integration (Figure 3.3). This DNA is collinear with its RNA template, but it contains terminal duplications on both ends: U3 region at the 5′-LTR (long terminal repeat) harbors the promoter sequence, while U5 region at the 3′-LTR carries the poly-A signal of HIV. Viral LTRs are fully restored prior to integration. Other indispensable elements within the HIV genome include the primer binding site (PBS) and polypurine tract (PPT). A PBS is a region proximal to the 5′-LTR where a primer binds to initiate minus-strand synthesis. The primer of HIV is delivered by a $tRNA_3Lys$, although the virus can utilize other tRNAs (Hansen, Schulze, Mellert, & Moelling, 1988; Panganiban & Fiore, 1988).The plus-strand DNA primer is provided by a 15-nucleotide PPT, a purine-rich sequence generated from viral RNA by the RNase H activity of reverse transcriptase (reviewed in Rausch and Le Grice, (2004)). The PPT is highly conserved in most retroviruses and has been shown to be selectively used as the site of plus-strand initiation. Soon after completion of the DNA synthesis, viral integrase protein (*Int*) recognizes and cleaves within the *att* sites located on the both ends of the viral DNA, eliminating the terminal two bases from each 3′ end. The resulting recessed 3′-OH group defines the pro-virus attachment sites utilized by the viral cDNA for integrating into the host chromosomes (Colicelli & Goff, 1985, 1988a, 1988b; Bushman & Craigie, 1990; Bushman, Fujiwara, & Craigie, 1990; Craigie, Fujiwara, & Bushman, 1990; Leavitt, Rose, & Varmus, 1992). Following integration, the DNA of the virus replicates along with the host genome and passes on to the cell's progeny; thus, all descendants of the infected cell will also bear proviruses in their genomes (Buchow, Tschachler, Gallo, & Reitz, 1989; Farnet & Haseltine, 1991; Shin, Taddeo, Haseltine, & Farnet, 1994). Following replication, viral mRNA is transcribed by the host RNA polymerase II.

In addition to core proteins encoded by three genes of the HIV-1: *gag* (encodes viral matrix, capsid, and nucleocapsid proteins), *pol* (encodes a protease, reverse transcriptase, and integrase), and *env* (encodes a surface envelope protein), complex retroviruses, such as HIV-1, harbor six additional genes: two regulatory (*rev* and *tat*), and four accessory genes (*nef, vif, vpr*, and *vpu*), involved in the viral entry, replication, and particle release (reviewed in Coffin et al. (1997)). The accessory genes are dispensable for the vector's production, and can be deleted, thus creating space for the insertion of transgenic sequences (Blomer et al., 1997; Dull et al., 1998; Kafri, Blomer, Peterson, Gage, & Verma, 1997; Kim, Mitrophanous, Kingsman, & Kingsman, 1998; Zufferey, Nagy, Mandel, Naldini, & Trono, 1997). In contrast, the regulatory protein *Rev* is essential for exporting full-length and partially spliced RNAs harbored a Rev response element from the nucleus to the cytoplasm (reviewed in Cockrell and Kafri (2007)). When transcription initiates, the host RNA-splicing machinery in the nucleus quickly splices the RNA so that only the regulatory proteins *Rev* and *Tat* and the accessory protein *Nef* are generated. In the presence of *Rev* protein, RNA is exported from the nucleus before it is spliced, thus affirming transcription of the viral proteins. This mechanism allows a positive feedback loop to allow HIV to overwhelm the host's defenses, and provides time-dependent regulation of replication (Dayton, Powell, & Dayton, 1989; Emerman, Vazeux, & Peden, 1989; Hadzopoulou-Cladaras et al., 1989). After HIV-1 reassembles in the cytoplasm, it escapes the cell by budding out from the cellular membrane (reviewed in (Gomez & Hope, 2005; Sundquist & Krausslich).

2.3.3 Lentiviral Vectors: Transgenic, Packaging, and Envelope Cassettes—As mentioned above, the first lentiviral vectors were evolved from the HIV-1 virus. In contrast to gamma-retroviral vectors, HIV-1 based vectors retain the ability of transducing, nondividing, and slowly dividing cells. Yet, they share the ability of gamma-retroviral vectors to integrate into the host chromosomes, without triggering a significant inflammatory response (Consiglio et al., 2001; Kordower et al., 2000).

Due to the relative complexity of the HIV-based vector system, production of viral stocks at high titers was a challenge initially. Furthermore, instability of the *Env* protein further contributed to the problem (Akkina et al., 1996). Nevertheless, it has been found that HIV-based vectors are capable of incorporating heterologous proteins that can replace the native envelope. In fact, early studies have shown that coinfection of the HIV-1 with other viruses may result in phenotypically mixed particles acquiring a broader host range. (Canivet, Hoffman, Hardy, Sernatinger, & Levy, 1990; Chesebro, Wehrly, & Maury, 1990). Following these publications, Page, Landau, and Littman (1990) demonstrated that the wild type HIV-1 was rendered replication defective by replacing the gp120 protein with a guanine-phosphoribosyl transferase (*gpt*) gene driven by the simian virus 40 (SV40) early promoter. These early observations extended in the experiments demonstrated that the envelope glycoprotein of the vesicular stomatitis virus (VSV-G) is capable of being efficiently incorporated into Moloney murine leukemia virus (MoMLV)-based retroviral vectors encoding the gene for neomycin phosphotransferase (Neo) (Emi, Friedmann, & Yee, 1991) and HIV-1 particles (Akkina et al., 1996; Reiser et al., 1996). Furthermore, VSV-G envelope was found to be significantly more stable allowing vector concentration by ultracentrifugation (Burns, Friedmann, Driever, Burrascano, & Yee, 1993). In addition, pseudotyping with VSV-G dramatically broadened vector tropism, as it has been initially suggested that VSV-G utilizes phosphatidyl serine-contained receptors on target cells (Schlegel, Tralka, Willingham, & Pastan, 1983). However, more recent data has demonstrated that phosphatidylserine is not the cell surface receptor for VSV-G, although it may play role in a postbinding step of virus entry (Coil & Miller, 2004). It also has been shown that VSV-G guides the vector to endocytic pathway, reducing thus the requirements for viral accessory proteins (Aiken, 1997). Nevertheless, works by Croyle et al. (2004); DePolo et al. (2000); Higashikawa and Chang (2001) have demonstrated that transduction of the mammalian cells with lentiviral vector can be hampered by complement- and antibody-mediated immune responses directed against the VSV-G envelope. As an alternative, lentiviral vectors can be successfully pseudotyped with other envelops including simple retroviral vector's envelope proteins, for example, the glycoprotein of the lymphocytic choriomeningitis virus (LCMV); and the hemagglutinin of the avian influenza virus (reviewed in Cronin, Zhang, and Reiser (2005)) and discussed below.

To reduce risk of replication-competent viruses (RCVs), packaging and envelope cassettes of the vectors are expressed separately from two different plasmids delivering respective proteins *in trans* (Naldini, Blomer, Gage, et al., 1996) (Figure 3.4). Because the above plasmids share almost no homology, it is very unlikely that they are capable of reconstituting a wt virus. Furthermore, if replication competent virus is inadvertently generated by recombinations between the plasmids, it will lack all of the accessory proteins

and the pathogenic properties of the HIV. To avoid transfer of the HIV-gene coding sequences into target cells, the packaging and envelope cassettes of the virus are deleted from viral *cis*-elements, including a packaging signal and LTRs, yet it retains the *Rev* binding site, and a parental splice donor site.

To maximize expression of viral mRNA, heterologous promoters, such as cytomegalovirus (CMV) and the Rous sarcoma virus (RSV) promoters, were successfully incorporated into the packaging cassette in place of the parental LTRs. In addition, insulin or bovine growth hormone poly-adenylation signals replaced the relatively weak endogenous poly-A of the virus, potentiating mRNA stability (Dull et al., 1998; Kafri et al., 1997; Kim et al., 1998; Naldini, Blomer, Gage, et al., 1996; Zufferey et al., 1997). Deletion of the accessory proteins, *Vpu*, *Vpr*, *Vif*, and *Nef* (Dull et al., 1998; Kafri et al., 1997; Kim et al., 1998; Naldini, Blomer, Gage, et al., 1996; Zufferey et al., 1997), (Figure 3.4) resulted in second-generation packaging cassette harboring only the *tat* and the *rev* genes (Zufferey et al., 1997). Further separation of the *gag/pol* and *rev* sequences into two different cassettes, combining with *tat* deletion resulted in third-generation packaging system (Dull et al., 1998). All of these improvements further decreased a likelihood of generating RCVs. Importantly, they neither reduced vector yield nor hampered the ability of lentiviral vectors to transduce nondividing cells, such as terminally differentiated neurons (Dull et al., 1998; Kafri et al., 1997; Kim et al., 1998; Naldini, Blomer, Gage, et al., 1996; Zufferey et al., 1997). In all, the expression (transgenic) cassette had undergone significant changes resulted in improvement of biosafety, yield, and expression of the vectors (Figure 3.5).

By replacing the parental HIV-promoter located in the 5′-LTR with CMV or RSV promoters, the vector acquires independence from the *Tat* protein, and, therefore, enables the vector to be packaged with a third-generation packaging cassette (Kim et al., 1998; Miyoshi, Blomer,Takahashi, Gage, & Verma, 1998; Zufferey et al., 1998). Subsequently, deletions within the 3′-region of the 3-LTR that included the enhancer/promoter sequence′ and the TATA box, helped to create a self-inactivating (SIN) lentiviral vectors (Iwakuma, Cui, & Chang, 1999; Miyoshi et al., 1998; Zufferey et al., 1998). Since the region relocates to the 5′-LTR, during the reverse transcription, SIN-vectors are completely devoid of HIV-parental enhancer/ promoter sequences; thus, it lacks the ability of generating a full-length RNA that could be packaged into the virions. Significantly, the deletion did not affect vector production, and vector's yield remains comparable with those of non-SIN vectors. Development of a SIN platform further reduced the likelihood of generating RCVs. Furthermore, it minimized a likelihood of mobilizing the vector's mRNA by the replication-wt virus. In addition, absence of enhancer/promoter sequences reduced the risk of inadvertent activation of silent host-cell promoters by the provirus.

Finally, incorporation of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (Zufferey, Donello, Trono, & Hope, 1999) and the central polypurine tract (*cPPT*) (Zennou et al., 2000) into the vector cassette significantly enhanced expression of transgenes delivered by lentiviral vectors and efficiency of transduction, respectively (Figure 3.5). Interestingly, WPRE has also been shown to have a strong positive effect on the vector's yield and expression when integrated into adeno-associated and gamma-retroviral vectors (Loeb, Cordier, Harris, Weitzman, & Hope, 1999; Zufferey et al., 1999).

2.3.4 Production of the Retroviral Vectors and Lentiviral Vectors; Stable Cell Lines—Conventional method for generating retro- and lentiviral vectors based on a calcium-phosphate and polyethylenimine (PEI) protocol (Figure 3.6) (reviewed in details in Cockrell and Kafri, (2007)). Briefly, highly permissible, human embryonic kidney (HEK) 293T cells are usually utilized for the transfection. These cells express a polyomavirus-derived large-T antigen, which is exploited to enhance a vector's yield through binding to the origin of replication (Ori) sequence of SV-40 virus harbored in the expression cassette. While efficient, the transient transfection protocol has several disadvantages, including a risk of DNA recombinations, variability in the quality of vector's stocks, and difficulties in scaling up production process. For this reason, a number of stable packaging cell lines have been recently developed (Cockrell, Ma, Fu, McCown, & Kafri, 2006; Throm et al., 2009) (Figure 3.7).

Development of stable packaging cell lines are impeded by cytotoxic effects associated with constitutive expression of the *VSV-G* (Ory, Neugeboren, & Mulligan, 1996), protease (Konvalinka et al., 1995), and *Vpr* (Bartz, Rogel, & Emerman, 1996). The successful development of inducible packaging systems was an important improvement to address the problem (Cockrell et al., 2006; Kafri, van Praag, Gage, & Verma, 2000; Reiser, Lai, Zhang, & Brady, 2000; Xu, Ma, McCown, Verma, & Kafri, 2001).

Commonly employed inducible systems are based on a tetracycline (*tet*) regulator (Gossen, Bonin, Freundlieb, & Bujard, 1994) (Figure 3.7). To develop the system, producer cells are introduced by transfection with a constitutively expressed tetracycline transactivator (*tTA*) that is maintained in the "off state" in the presence of tetracycline analog, doxycycline (Dox). Initially, SIN vector platform could not be used, because of its inability to generate full-length mRNA, implying a need for a non-SIN, *Tat*-dependent system (Farson et al., 2001; Kafri, van Praag, Ouyang, Gage, & Verma, 1999; Kaul, Yu, Ron, & Dougherty, 1998). Development of *Tat*-independent, CMV promoter-driven vectors (Klages, Zufferey, & Trono, 2000) has been an important step toward establishing a conditional SIN system, included heptameric repeats of a tetracycline response elements (TRE), and CMV-minimal promoter in the LTRs of the vector (Xu et al., 2001). This enables the vector's production in the packaging cells constitutively expressing the *tTA* transactivator, while concomitantly maintaining the SIN phenotype in the *tTA*-negative target cells (Haack et al., 2004; Xu et al., 2001). Similarly, packaging and envelope cassettes can be equipped with the inducible promoters allowing *tTA*-dependent regulation (Haack et al., 2004).

2.3.5 Risk of Insertional Mutagenesis; Non-integrating Lentiviral Vectors—Employment of gamma-retroviruses for correcting human diseases is hampered by a relatively high risk of insertional mutagenesis associated with these vector systems. Thus, initially successful treatments of ADA-SCID, SCID-X1, and X-linked CGD with retroviral vectors were unfortunately troubled by leukemias developed by several patients. It has been demonstrated that the leukemia's patients harbored provirus DNA in the vicinity of proto oncogenes deregulating their expression (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina, von Kalle, Schmidt, Le Deist, et al., 2003; Hacein-Bey-Abina, Von Kalle, Schmidt, McCormack, et al., 2003). Similar to gamma-retroviruses, lentiviral vectors are capable of integrating into the host genome, thus potentially retaining the ability to induce onco- and

tumorigenicity. Furthermore, lentiviral vectors are not completely detached from the potential for insertional mutagenesis. In fact, EIAV vectors have been shown to be associated with the formation of tumors in the livers of mice following in utero and neonatal vector administration (Themis et al., 2005). A causal relationship between EIAV vectors and tumorigenesis has yet to be established; nevertheless, it is important to note that in the same study the use of HIV-based vectors were not associated with formation of any detectable tumors (Themis et al., 2005). Despite the evidence from this study, the lack of any precedent for HIV-based vectors to be associated with tumorigenecity and oncogenicity, and presumption that the risk of insertional mutagenesis in nondividing cells is not as immense as in dividing cells, lentiviral vectors that would obviate insertional mutagenesis are most desirable (Bayer et al., 2008; Kantor et al., 2011).

A strategy to modify the lentiviral vector-packaging cassette is being pursued to avert insertional mutagenesis based on developing a nonintegrating vector platform. This approach premises on findings that the HIV-1 and other retroviruses generate extrachromosomal (episomal) genomes over the course of infection. Furthermore, episomal DNA is observed to constitute the vast majority of viral genomes (Chun et al., 1997; Kantor, Ma, Webster-Cyriaque, Monahan, & Kafri, 2009; Pang et al., 1990; Teo et al., 1997), and found to be exceptionally stable in nondividing cells (Bayer et al., 2008; Butler, Johnson, & Bushman, 2002; Kantor et al., 2011; Pierson et al., 2002). Extrachromosomal DNA appears in the four major forms: linear episomes, which are the precursor for integration; 2-LTR (double-LTR) and 1-LTR (single-LTR), are circular forms generated by host-mediated repair mechanisms; and auto-integration episomes (Figure 3.8) (Bayer et al., 2008; Kantor et al., 2011).

Nonintegrating vectors can be generated by introducing nonpleiotropic mutations within the open reading frame (ORF) of *Int* protein of the packaging cassette. These mutations have been shown to specifically target the integration process (Engelman, Englund, Orenstein, Martin, & Craigie, 1995; Nakajima, Lu, & Engelman, 2001). Our data demonstrated that an *Int*-deficient genome is capable of being efficiently transcribed, although the levels of protein expression are significantly lower than that of integrase wt vectors (Bayer et al., 2008; Kantor et al., 2009). Nevertheless, even the reduced expression levels of nonintegrating vectors have been shown to be sufficient for correcting genetic disorders in experimental animals (Philippe et al., 2006; Yanez-Munoz et al., 2006). In regards to the mechanism of gene repression of nonintegrating vectors, the reduced level of episomal expression is attributed to formation of repressive chromatin structure around the episomal DNA (Kantor et al., 2009). Furthermore, this chromatin has been found to be enriched in posttranslational histone modifications typically associated with transcriptionally silenced genes (Kantor et al., 2009). Remarkably, the reduced expression of the episomal genome associated with vector transduction or viral infection can be improved by treatment of transduced or infected cells by histone deacetylase inhibitors (*HDACi*). Importantly, these *HDACi* are endogenously generated in the gastrointestinal tract in the forms of short-chain fatty acids by normal microbial flora (Kantor et al., 2009). Depletion of the HDACs could be achieved also by mutating *cis*-acting elements within the U3′- region of the vector's LTRs, enriched in HDACs-interacting negative transcriptional regulators. In fact, by

mutating these elements a significant improvement in gene expression of nonintegrating vectors can be achieved both in vitro and in vivo (Bayer et al., 2008), (Kantor et al., 2011), and (Suwanmanee et al., 2014). Remarkably, these mutations have not changed the relative abundances of the episomal forms, suggesting that the increase in expression is not attributed to a distinct episomal form appearance (Bayer et al., 2008). Furthermore, the effect of the deletion has been shown to be tissue-specific in the rat's brain, which is in line with earlier observations demonstrating cell-type-dependent gene expression of the nonintegrating vectors (Bayer et al., 2008; Philippe et al., 2006; Vargas, Gusella, Najfeld, Klotman, & Cara, 2004). These studies altogether suggest that nonintegrating lentiviral vectors may provide an effective means of delivery of therapeutic transgenes to nondividing and slowly dividing cells.

2.3.6 Lentiviral Vector for Use in the CNS—Lentiviral vectors have been used extensively as gene transfer tools for the CNS throughout the past two decades since they transduce most cell types in the brain, resulting in robust and long-lasting transgene expression. In fact, in the very first publication reported the use of the lentiviral vectors for gene transfer in vivo, Naldini and coworkers demonstrated efficient transduction into the neurons of the brain (Naldini, Blomer, Gage, et al., 1996). Following this report, hundreds of publications have demonstrated successful gene transfer utilizing both integrase-competent and integrase-deficient platforms in the CNS (de Almeida, Zala, Aebischer, & Deglon, 2001; Azzouz et al., 2002; Baekelandt et al., 2002; Bayer et al., 2008; Consiglio et al., 2001; Kantor et al., 2011; Perrin et al., 2007; Sergijenko et al., 2013; Wong et al., 2004).

As mentioned above, lentiviral vectors are considered attractive tools for gene transfer into the CNS, due their ability to transduce nondividing and slowly dividing cells. Lentiviral vectors have been demonstrated to be safer in comparison to gamma-retroviruses. Moreover, significant improvements of the packaging and expression cassettes of the vector, described above immensely contributed to reduce the likelihood of generating RCVs (Dull et al., 1998; Zufferey et al., 1997, 1998). Importantly, these modifications have not resulted in reduction of the vector yield nor did they hampered the ability of the vectors to transduce nondividing cells (Dull et al., 1998; Kafri et al., 1997; Naldini, Blomer, Gage, et al., 1996; Naldini, Blomer, Gallay, et al., 1996; Zufferey et al., 1997). Pseudotyping the vector with different envelopes tremendously expanded the range of transduction (Table 3.2). Multiple studies demonstrated that lentiviral vectors are capable of transducing most cell types within the CNS in vivo, including terminally differentiated neurons, dendritic cells, glial cells, astrocytes, and oligodendrocytes (Cheng et al.; Bayer et al., 2008; Blomer et al., 1997; Consiglio et al., 2004; Jakobsson, Ericson, Jansson, Bjork, & Lundberg, 2003; Kafri et al., 1997). Interestingly, it has been demonstrated that although other types of lentiviral vectors, such as SIV, EIAV, and FIV are capable to deliver transgenes into the CNS, the most robust and efficient delivery has been achieved employing the HIV-1-based vectors. This is likely because of species-specific restrictions hampering the transduction of nonhuman lentiviral vectors (Wiznerowicz & Trono, 2005).

2.3.7 Tissue-Specific Promoters Used in Lentiviral Gene Transfer into the CNS —Transduction efficiency and posttransduction gene expression of the lentiviral vectors

depend on a promoter type that employed (Table 3.3). At the early days, ubiquitous promoters such as CMV and phosphoglycerate kinase (PGK) were utilized to drive the transgene expression (Jakobsson et al., 2003; Naldini, Blomer, Gage, et al., 1996). These promoters are predominantly active in the neuronal cells, but this could be partially attributed to a greater abundance of the neurons, and the lower rate of transduction in the glial cells. Moreover, transgenic expression driven from the CMV promoter is weakened over the time, likely because of the DNA methylation (Grassi et al., 2003; Mehta, Majumdar, Alam, Gulati, & Brahmachari, 2009). Development of tissue-specific promoters provides an important tool to control lentiviral vector's gene expression in multiple cell lineages of the CNS (Dittgen et al., 2004; Jakobsson et al., 2003; Lai & Brady, 2002). Moreover, both neuron- and glial-specific promoters have been demonstrated to confer cell-type-specific transgene expression in the desired cell type (Dittgen et al., 2004; Jakobsson et al., 2003; Lai & Brady, 2002) (Table 3.3). These promoters appear to be highly specific, at least in the experiments when reporter GFP transgene was employed. Notwithstanding these findings, expression driven by the glial-specific promoter, glial fibrillary acidic protein (GFAP) was detected not exclusively in the glial cells when the glial cell line-derived neurotropic factor was carried (Jakobsson et al., 2003). It is possible that the gene of interest can affect the specificity of transduction and expression. Alternatively, integration of silenced transgene into active chromatin environment may stimulate the promoter activation via interaction with the surrounding enhancer sequences. To increase specificity of transduction, a combinatory approach should be considered, in which cell-type-specific promoters combined with cell-specific envelopes ensuring thus more specific targeting of the desired cell population.

2.3.8 Envelopes for Gene Delivery into the CNS—As was mentioned before, VSV-G is the most common envelope utilized the vector transduction (Table 3.2). The widespread use of this protein for pseudotyping lentiviral vectors transduction has made it, in effect, the standard against which the effectiveness of other viral envelopes is compared. Other glycoproteins are also capable of governing vector delivery into the CNS. Among them LCMV envelope mentioned above, Mokola virus (MV), Moloney murine leukemia virus (MoMLV), Ross River virus (RRV) and Rabies virus (RV) have been demonstrated to be effective in pseudotyping vectors (reviewed in Cronin et al. (2005)). RV and MV belong to the same genus, Lyssavirus, and are closely related. Glycoproteins derived from these viruses were the first to be incorporated into the HIV-1-based vectors demonstrating robust transduction into the brain (Conzelmann, Cox, Schneider, & Thiel, 1990; Mochizuki, Schwartz, Tanaka, Brady, & Reiser, 1998). Furthermore, Watson and coworkers demonstrated that the lentiviral vector pseudotyped with the glycoprotein of MV injected into the rats' striatum efficiently expressed the *β-gal* reporter. In addition, they found that the pattern of transduction governed by the envelope of MV was similar to that of VSV-G-pseudotyped vectors, with efficient delivery of both vectors into neuronal cells (Watson, Kobinger, Passini, Wilson, & Wolfe, 2002) (Table 3.2). In a related study, Desmaris and colleagues tested and compared the ability of the glycoproteins of MV and VSV to govern retrograde transport following reporter transfer with lentiviral vectors (Desmaris et al., 2001). In this study lentiviral vectors pseudotyped with either VSV-G, or MV-GP were injected into the nasal cavity, or the limb muscles of rats. Both vectors efficiently transduced

neurons of the olfactory bulb following nasal delivery; however no *β-gal* expression was detected in the motor neurons in the spine for either vector (Desmaris et al., 2001). The study concluded that lentiviral vectors pseudotyped with VSV-G and MV-GP envelopes are similar in the efficiency of retrograde axonal transport, although both are incapable of infecting via neuromuscular junctions. In contrast, lentiviral vector pseudotyped with the glycoprotein of RV injected into the rat's striatum was efficiently delivered into thalamus and substantia nigra suggesting both retrograde and anterograde transport (Sacramento, Badrane, Bourhy, & Tordo, 1992) (Table 3.2).

The original report of VSV-pseudotyped lentiviral vectors showed transduction of both neurons and glial cells in the hippocampus and striatum of adult mice (Naldini, Blomer, Gage, et al., 1996), implying the ubiquitous nature of transduction utilizing this envelope. In the related study, Cannon and colleagues demonstrated that a lentiviral vector pseudotyped with the glycoprotein of MV delivered by the intranigral infusion in the rat's brain provided a similar pattern of expression to that observed after infusion of the glycoprotein of VSV (Cannon, Sew, Montero, Burton, & Greenamyre, 2011). They suggested that because it is straightforward to generate high-titer lentiviral vector stocks pseudotyped with the G-protein of VSV, the lentiviral vectors pseudotyped with the glycoprotein of MV may confer no advantage for gene transfer to the rat substantia nigra (Table 3.2).

A different pattern of transduction was observed when the lentiviral vectors pseudotyped with LCMV and MoMLV envelopes were employed for gene delivery into the mouse brain (Watson et al., 2002). In this study reporter gene expression was detected in the white matter after striatal infusion, showing only limited neuronal transduction after infusion near the hippocampus. In comparison, lentiviral vectors pseudotyped with the glycoprotein of MoMLV efficiently transduced striatal cells of undetermined nature and granule neurons of the hippocampus after hippocampal infusion (Watson et al., 2002). In contrast, Cannon and coworkers have found that both vectors pseudotyped with LCMV and MoMLV envelopes demonstrated selective transduction of astrocytes in the rat substantia nigra (Cannon et al., 2011). Similarly to the envelopes of VSV and MV, the glycoprotein derived from the RRV-governed efficient lentiviral transduction and GFP-expression in both neurons and glial cells of the brain (Jakobsson, Rosenqvist, Marild, D, & Lundberg, 2006). Using two cell-type-specific promoters, neuron-specific enolase, and human GFAP protein, Jakobsson and colleagues demonstrated cell-specific transgene expression in aforementioned cell types (Jakobsson et al., 2006). In the same publication, the glycoprotein protein of the RRV was found capable of transducing human neural progenitor cells in vitro, suggesting that receptors for the envelope of the RRV are present on human neural and glial cells (Jakobsson et al., 2006) (Table 3.2).

2.3.9 Peripheral Administration of Lentivirus to Target the CNS—Systemic route of delivery of retro- and lentiviral vectors would be the most desired option for introducing a transgenic DNA into the CNS. In fact, the necessity of utilizing intracranial route reduces the feasibility of employing vector protocols for clinical applications. Since, the BBB serves as a significant impediment to the vector's transport, developing a system that will penetrate the CNS after peripheral administration is greatly important. In this regard, vectors that are capable of reaching the brain by retrograde transport are in great demand. As discussed

earlier in this chapter, pseudotyping the vectors with glycoproteins that support a retrograde transport, such RV, beneficial for exploring the peripheral route of delivery (Sacramento et al., 1992) (Table 3.2). These vectors already has been successfully implemented for treating animal models of amyotrophic lateral sclerosis and SMA, in which lentiviral vectors expressing either vascular endothelial growth factor or the human survival motor neuron gene were retrogradually transported to motor neurons after intramuscular injection (Azzouz et al., 2004; Wong et al., 2004).

One strategy to target the CNS after peripheral delivery of the gene transfer vector is to exploit the secreted form of the lentivirus regulatory protein, *Tat*, to invade the CNS (reviewed in (Rapoport & Lorberboum-Galski, 2009; Wang et al., 2009). The *Tat* protein can destabilize a monolayer of blood–brain covering endothelial cells, thus allowing permeation of high molecular weight cargoes (Cooper et al., 2012). Furthermore, it has been shown that β-gal protein fused to *Tat* protein was efficiently delivered into the brain by penetrating the BBB (Schwarze, Ho, Vocero-Akbani, & Dowdy, 1999). Subsequently, various groups have used the *Tat*-based delivery approach to administrate a variety of therapeutically relevant cargoes to the brain (reviewed in Rapoport & Lorberboum-Galski (2009); Wang et al., 2009)). Specifically, fusion of the *Tat* to the proteins which bind the postsynaptic density protein, PSD-95, was found to be effective in protecting neurons from excitotoxicity (Aarts et al., 2002). Furthermore, studies in rodents in vivo revealed that not only intact *Tat* protein can cross the BBB, but also *Tat* derivatives contained the protein transduction domain are capable of penetrating the BBB and accumulate in brain tissue (reviewed in (Rapoport & Lorberboum-Galski, 2009)).

2.3.10 Conclusions on the Use of Lentivirus for CNS Gene Transfer—The ability of lentiviral vectors to transduce nondividing or slowly dividing cells and deliver large genetic payloads throughout maintaining stable and long-term transgene expression are attributes that brought lentiviral vectors to the forefront of the gene delivery field. Clinical trials utilizing lentiviral vectors clearly underlined their efficacy for therapies of hereditary diseases including adrenoleukodystrophy, β-thalassemia, Wiskott–Aldrich syndrome, and metachromatic leukodystrophy (discussed in details in the next chapter). In comparison to gamma-retroviral vectors, which were commonly employed in the first clinical trials, lentiviral vectors own favorable features including a potentially safer integration profile. Nevertheless, stable integration of the retroviral and lentiviral vectors, risk of appearance of RCVs, insertional mutagenesis that may deregulate expression profiles of neighboring genes, aberrant splicing, and germ-line transmission are points of major concern from the gene therapy perspective. Therefore, along with appreciation of the substantial benefits of using lentiviral vectors for gene transfer into the CNS, it is important to consider other vector platforms while considering therapeutic outcomes.

2.4 Adenovirus

Adenovirus had been the traditional workhorse of gene therapy, but it has mostly fallen out of favor for CNS gene transfer in lieu of AAV and retroviral vectors. Adenovirus is a common and pathogenic human dsDNA virus, and the recombinant gene therapy vector can package up to 35 kb of foreign DNA, depending on the version of the vector (Campos &

Barry, 2007; Volpers & Kochanek, 2004). Adenovirus vectors have the advantages of a large packaging size, high functional titers, the ability to transduce dividing and nondividing cells, and fast transgene expression (1–2 days postdelivery). Following transduction, the vector DNA does not normally integrate in the host genome and is instead maintained as an episome with minimal risk of insertional mutagenesis. Adenovirus vectors have several disadvantages that have limited their application in CNS gene transfer, including the often transient nature of transgene expression and their higher preponderance to provoke an inflammatory response (Lentz, Gray, & Samulski, 2012).

Adenovirus vector genomes have improved significantly since their original development, such that current vectors have much lower immunogenicity and longer transgene expression (Campos & Barry, 2007; Chen et al., 1997; Schiedner et al., 1998). First-generation recombinant adenovirus vectors have the E1 genes removed, but they still contain and express at low levels the E2, E3, E4, and late genes, which are highly immunogenic (Campos & Barry, 2007). Low-level expression of these viral antigens leads to elimination of transduced cells by the host immune system, thereby limiting the duration of transgene expression in vivo (McConnell & Imperiale, 2004). Second-generation vectors lack the E2, E3, and E4 genes, and third-generation “gutless” vectors do not express any viral proteins (Amalfitano, 1999; Chen et al., 1997). Even with these improvements, third-generation adenovirus vectors still generate significant immune responses against the viral capsids, which creates a patient-specific limit to the amount of vector that can be administered (Muruve, 2004). Switching to canine adenovirus serotype 2 (CAdV2), rather than the commonly used human adenovirus serotype 5, has the advantage of reduced immune responses and prolonged transgene expression (Keriel, Rene, Galer, Zabner, & Kremer, 2006; Perreau et al., 2007). Using CAdV2 vectors, MPS VII mice showed stable expression, reduced pathology, and improved phenotype for at least 16 weeks after a single intracranial vector injection (Ariza et al., 2014).

2.5 Herpes Simplex Virus

2.5.1 Introduction—HSV is a member of *Herpesviridae* and belongs to the subfamily *Alphaherpesvirinae*. Depending on the cellular site of latency, herpesviruses are classified as HSV-1 or as HSV-2. HSV-1 is the primary cause of orolabial cold sores caused by the establishment of latency in the trigeminal nerve; whereas genital herpes is caused by the latency of HSV-2 in the sacral ganglia. HSV-1 is acquired during childhood or adolescence by primary infection of the oral mucosa. About 40% of adults are seropositive for HSV-1 in developed countries (Smith & Robinson, 2002). Latency occurs when progeny viruses produced at the site of infection enter sensory nerve terminals and translocate their nucleocapsid to the soma of neurons by microtubule dependent retrograde axonal transport (Tomishima, Smith, & Enquist, 2001).

2.5.2 Structure—The HSV-1 virion is made up of an icosahedral capsid covered by a lipid bilayer viral envelope (Figure 3.9). The envelope is embedded with glycoproteins essential for viral entry into hosts mediated by attachment of these glycoproteins to cellular receptors. Between the envelope and the nucleocapsid lies the tegument which contains structural proteins and regulatory enzymes encoded by the viral genome. The main role of the

tegument is thought to be the ability to supply proteins essential in DNA replication and evasion of the immune system (Baron, 1996). The ds viral genome is 152 kb in length and encodes about 90 proteins for replication and cellular attachment (Donald W Kufe et al., 2003). The genome contains unique long (U_L) and unique short (U_S) segments and is flanked on either end by inverted repeat sequences (Osten, Grinevich, & Cetin, 2007).

2.5.3 Life Cycle of HSV—Cellular attachment is mediated by the interaction of the glycoproteins gB and gC of the viral envelope with heparan sulfate of the host-cell membrane. Cellular entry is then mediated by the envelope protein gD by binding to the viral entry receptor, herpesvirus entry mediator. The fusion of the cell membrane and the viral envelope is mediated by gB and the gH–gL complex, resulting in the release of the nucleocapsid into the cytoplasm of the host cell. In neurons, the nucleocapsid is transported to the cell body by retrograde axonal transport mediated by the interaction of the host microtubule network with the tegument proteins of HSV-1 (Diefenbach, Miranda-Saksena, Douglas, & Cunningham, 2008).

Upon cellular entry, HSV-1 can establish either of these two states: lytic or latent. The lytic pathway ultimately results in the death of the host cell whereas latent HSV establishes as an extrachromosomal episome within host nuclei. However, latent HSV is capable of reactivation where the expression of the ICP0 gene is thought to play a key role (Lachmann, 2004).

Replication and transcription of viral DNA is a multistep process requiring the production and coordination of multiple proteins. The lytic pathway is mediated by the sequential expression of three classes of viral gene products: these proteins are classified as α-proteins or immediate-early (IE) proteins, which encode major transcriptional regulatory proteins, β-proteins or early (E) proteins, which encode viral DNA polymerase while altering the intracellular composition to favor viral replication and γ -proteins or late (L) proteins, which primarily encode structural proteins. Among these, the IE genes are most essential for the lytic pathway as these genes are necessary for the synthesis of the β- and γ -proteins (Bernard Roizman, 2001). There are 37 essential genes in the HSV genome out of which 36 are located in the U_L region. This allows for the deletion of a large amount of viral DNA containing the nonessential genes in the U_S region (Ho et al., 1995; Roizman, 1996, 1999), which can then accommodate large amounts of transgene DNA in vectors.

2.5.4 Vectors Derived from HSV—Vector properties required to correct genetic deficiencies are vastly different from the properties of a vector used to target cancer cells. The versatility of HSV-1 vectors cannot only provide long-term gene expression with lack of cytotoxicity but can also provide high levels of expression of toxic products malignant cells. The adaptations that make HSV vectors suitable for neurological and cancer therapy applications are discussed below:

HSV-1 adaptations for neurological applications:

1. The virus can be disseminated transynaptically from neuron to neuron.
2. Movement of the virus is possible in retrograde and anterograde directions.

3. Glycoproteins on the envelope recognize receptors such as nectin that are particularly useful for viral entry into neurons.
4. Upon entry into the nucleus, the virus is able to persist as an extrachromosomal episome and all lytic genes are suppressed establishing latency in neurons, which are immune privileged.

The properties of HSV-1 that make it an attractive candidate for cancer gene therapy are

1. HSV vectors efficiently infect a number of human tumor cell lines in vitro.
2. Several HSV vectors take advantage of underlying defects in molecular pathways of tumor cells.
3. HSV-1 vectors express the *tk* gene, which encodes thymidine kinase, which results in apoptosis of host cells (Kuriyama et al., 1995; Vile & Hart, 1993)
4. Modification of envelope proteins can increase infectivity of tumor cells, which bear the corresponding receptors (Grandi, Spear, Breakefield, & Wang, 2004; Grandi, Wang, et al., 2004)

These various properties can be exploited by engineering various classes of vectors. The three categories of HSV-1-derived vectors are as follows, which are discussed in detail in the subsections below.

1. Replication-defective vectors
2. Replication-competent vectors
3. Amplicon vectors

2.5.4.1 Replication-Defective HSV Vectors: As the name suggests, replication-defective vectors have deletions of the essential genes from the viral genome, which prevents the proliferation of the HSV-1 particles in vitro. These essential genes are IE genes and are required for the E and L cascade responsible and functions such as viral entry, replication, splicing, polyA, and mRNA stability. The first generation of these vectors were mutants, which lacked the expression of the IE gene encoding ICP4 (DeLuca & Schaffer, 1985), which is essential in viral replication and induction of early and late gene expression (DeLuca & Schaffer, 1988). These vectors showed reduced pathogenicity but were found to be cytotoxic in neurons. Several combinations of deletions in five essential genes namely ICP0, ICP4, ICP22, ICP27, and ICP47 produced a variety of replication defective vectors that are highly defective mutants (Krisky et al., 1998; Samaniego, Neiderhiser, & DeLuca, 1998). Deletion of all five IE genes reduced cytotoxicity and allowed the viral genes to persist in the cells for prolonged periods of time (Kaplitt & Makimura, 1997). Deletion of these IE genes allowed for the accommodation of transgene cassettes as well as regulatory elements (Krisky et al., 1998).

Due to their reduced toxicity, replication-defective vectors have a wide range of therapeutic applications in neurological diseases like lysosomal storage disorders (Martino et al., 2005), Alzheimer's (Hong, Goins, Goss, Burton, & Glorioso, 2006) and chronic pain (discussed in detail below) (Goss et al., 2001). Martino et al. have shown successful clearance of the GM2

ganglioside, the lysosomal storage material that accumulates due to defects in the gene encoding the enzyme hexaminidase A α-subunit, in a murine model of Tay–Sachs disease (Martino et al., 2005). These vectors have also been used in the treatment of motor neuron disease due to its ability to deliver multiple neurotrophic factors to specific cell populations (Marconi et al., 2005; Perez, Hunt, Coffin, & Palmer, 2004).

Attenuated HSV vectors have been used in cancer therapy primarily as anticancer transgene delivery vehicles including suicide genes such as thymidine kinase, immune-stimulatory molecules such as cytokines and anti-angiogenic factors to tumors such as melanomas (Krisky et al., 1998) and glioblastomas (Niranjan et al., 2000).

2.5.4.2 Replication-Competent Vectors: Deletion of certain nonessential genes responsible for HSV-1 immune evasion and virulence gives rise to replication-competent vectors, which proliferate in vitro but are compromised in vivo (Todo, 2008). These vectors are modified such that they have attenuated ability to replicate in healthy cells but do so rapidly in dividing cells present in tumors (Hu & Coffin, 2003) and can be used to deliver therapeutic agents to tumors. Thymidine kinase, ribonucleotide reductase, virion host shut-off, and ICP34.5 protein are nonessential gene products that are responsible for the virulence and pathogenicity of HSV-1 (Samady et al., 2003). Deletion of these genes allows these vectors to take advantage of physiological changes in tumors that allow for the lytic infection of the vector specifically in these tumor cells (Hu & Coffin, 2003; Todo, 2008).

Replication-competent vectors have been used in the treatment of peripheral nervous system disorders. These vectors are allowed to carry out their natural life cycle and establish latency in the cell bodies after retrograde transport from the periphery. Transduction of the dorsal root ganglia by proenkephalin A mediated by these vectors has been used in various pain studies (Goss et al., 2001). These vectors have also shown efficient transduction of primate retinal pigmented epithelial cells and retinal ganglion cells with limited inflammatory and cytotoxic responses, which make them amenable to clinical applications in models of optic nerve degeneration (Liu et al., 1999).

Among the various types of HSV vectors, replication-competent vectors have been one of the most commonly used vectors in cancer therapies at preclinical and clinical trials (Chiocca, 2002; Mullen & Tanabe, 2002).These viruses selectively replicate and lyse cancer cells by using the cancer cell machinery while sparing the nondividing healthy cells. The first generation of oncolytic viruses contained three distinct mutations:

1. Deletion of the thymidine kinase gene (Martuza, Malick, Markert, Ruffner, & Coen, 1991), which slowed tumor growth and prolonged survival.
2. Insertion of the *lacZ* gene in the E gene creating ICP6 (Jeyaretna, Rabkin, & Martuza, 2007), which showed enhanced killing of tumors and improved survival.
3. Deletion of the nonvirulence factor ICP34.5 (Dambach,Trecki, Martin, & Markovitz, 2006) essential for HSV pathogenicity, showed antiglioma activity.

Among the first-generation replication-competent vectors, only the ICP34.5 mutant is currently in clinical trials (Liu et al., 2003) as the other two cause fatal encephalitis in

animals (Martuza et al., 1991). The second-generation replication-competent vector G207 that contained multiple mutations (deletion of the ICP34.5 and insertion of the *lacZ* gene in ICP6 gene (Mineta, Rabkin, Yazaki, Hunter, & Martuza, 1995) has a higher safety profile and low neurotoxicity which allowed it to move into Phase Ib/II trials for malignant tumors (Markert et al., 2000). Chemotherapy and radiation are required in combination with the oncolytic viruses to increase the success rates in cancer treatment (Post, Fulci, Chiocca, & Van Meir, 2004).

2.5.4.3 Amplicon Vectors: Amplicon vectors are prepared by transfecting cells with the amplicon plasmid and superinfecting the cells with helper HSV-1. This leads to the production of vector particles that are similar to wt HSV-1 structure and immunogenicity but contain a bacterial plasmid DNA instead of the viral genome (Frenkel, Singer, & Kwong, 1994). This concatemeric plasmid is capable of packaging more than one transgene cassette, a origin of replication, and a cleavage/package signal (Epstein, 2005). The advantages of amplicon vectors are numerous: these have a large packaging capacity of about 150 kb and reduced toxicity due to the lack of viral genes. The repetitive nature of the amplicon plasmid ensures multiple copies of transgene are delivered to each cell transduced. However, one disadvantage of these vectors was the inability to produce helper-free vector stocks. Helper-contaminated vectors induced significant inflammatory responses as well as cytotoxicity. To overcome this issue, helper-free vector stocks were produced by supplying the HSV-1 genome in *trans* through bacterial artificial chromosomes (Saeki et al., 1998) or by employing the Cre/loxP-based site-specific recombination to remove helper virus (Logvinoff & Epstein, 2001).

Helper-free vectors show reduced cytotoxicity in neurons as well as long-term expression of transgenes. The use of these vectors has been multi-faceted in the field of neuroscience. Transgenes expressing neurotransmitters and/or receptors have been useful in behavioral studies involving learning and memory (Adrover et al., 2003; Brooks, Cory-Slechta, Bowers, Murg, & Federoff, 2000). These vectors have also been used as therapeutic agents when packaged with transgenes such as brain-derived neurotrophic factor (Geschwind et al., 1996), nerve growth factor (NGF) (Pakzaban, Geller, & Isacson, 1994), and antiapoptotic genes (Antonawich, Federoff, & Davis, 1999) to confer neuroprotection in experimental settings. These vectors have been essential in experiments studying the treatment of Parkinson’s disease in culture by delivery of tyrosine hydroxylase (Geller, During, Oh, Freese, & O’Malley, 1995; Geller, Yu, Wang, & Fraefel, 1997).

Due to the nonreplicating nature of amplicon vectors, their application in cancer therapy is limited to genes which stunt tumor growth by inducing hypoxia and attenuate angiogenesis (Shah & Breakefield, 2006). The major application of amplicon vectors in cancer gene therapy has been the delivery of the synergistic prodrug-activating enzymes, thymidine kinase and cytosine deaminase (Jacobs et al., 2003, 2007), and the delivery of tumor necrosis factor-related apoptosis-inducing ligand for in vivo imaging and treatment of tumors (Shah, Tang, Breakefield, & Weissleder, 2003; Shah, Tung, Breakefield, & Weissleder, 2005).

2.5.5 High-Impact Application of HSV Vectors in the Treatment of Chronic Pain—Latency, neurospecificity, and neuroinvasiveness are several adaptations of HSV, which make it useful in the treatment of neurological conditions. Retrograde transport allows these vectors to be transported from the periphery to the cell bodies where it remains latent by means of the latency-active promoter system. These mechanisms can be used favorably to obtain long-term transgene expression in neurons of the central and peripheral nervous systems (Chattopadhyay et al., 2005; Goins et al., 1999) and have been used for the development of novel therapies for chronic pain.

Chronic pain is pain that lasts for prolonged periods of time in the absence of injury or stimulus. Chronic pain can result from a number of causes including arthritis, fibromyalgia, peripheral neuropathies, etc., and can last longer than 6 months.The relief obtained from analgesics and opiods is limited due to tolerance and the adverse systemic effects produced. Gene therapy can be used to produce localized transcription of therapeutic genes which have analgesic effects or inhibit pain signaling without producing systemic effects.

HSV vectors have been used for delivery of therapeutic agents to sensory neurons that inhibit pain signaling pathways. Inhibitory tone is increased in phasically active neurons, which are interneurons that transmit signals between the dorsal horn and the brain stem, by replication-defective HSV vector-mediated transfer of endogenous opiod peptide encephalin (ENK) in the dorsal root ganglia. Rodents that received this treatment showed reduced pain response to noxious stimuli or underlying inflammation (Braz et al., 2001; Goss et al., 2001, 2002). In a dose escalation gene therapy clinical trial using an HSV vector expressing ENK, 10 patients reported focal pain relief with no adverse effects (Fink et al., 2011). HSV-mediated gene therapy using GABA and endomorphin has also been successful in treating neuropathic pain, spinal cord injury, and inflammatory pain in animal models (Hao et al., 2005; Hao, Wolfe, Glorioso, Mata, & Fink, 2009; Liu et al., 2004). GABA is the dominant inhibitory neurotransmitter in the spinal cord and exerts tonic inhibitory control over nociceptive transmission and endomorphin is a natural ligand of the mu opioid receptor, which is the major site of action for narcotic drugs such as morphine. HSV vectors have also been used to deliver anti-inflammatory cytokines such as IL-4 and IL-10, which decrease neuroimmune stimulation and provide analgesia in several models of chronic pain due to chronic inflammation (Cunha, Poole, Lorenzetti, Veiga, & Ferreira, 1999; Poole, Cunha, Selkirk, Lorenzetti, & Ferreira, 1995). For a detailed review of the applications of HSV vectors in chronic pain, please refer to (Goins, Cohen, & Glorioso, 2012; Goss, Krisky, Wechuck, & Wolfe, 2014; Srinivasan, Fink, & Glorioso, 2008).

3. GENOME DESIGNS FOR OPTIMAL EXPRESSIONS

In recent years, gene therapy has begun to make the advance from proof of concept to proof of practice. An often underappreciated aspect of vector design is the means and control of transgene expression. Until recently, the majority of constructs have used only ubiquitous viral promoters to drive expression from simple gene expression cassettes lacking introns, matrix attachment regions (MARs), WPRE, and other components of the expression cassette. In fact, these elements have a key role in determining the levels and permanence of gene expression in vivo, regardless of the vector system used. In conjunction with

modification of vector tropism and strategies to limit their immunogenicity, vector genome optimization should create superior vectors for highly specific research and clinical applications involving gene transfer.

A myriad of neurological disorders including Parkinson's disease, Huntington's disease, epilepsy, Alzheimer's disease, and many others may be treatable using gene therapy. Each disorder has differing requirements in terms of cell type and level/range of therapeutic protein necessary to fall within the therapeutic window for that particular disease. For example, gene therapy has been identified as a potential therapeutic for Rett Syndrome. Rett syndrome is a neurodevelopmental disorder that is characterized by normal early growth and development followed by a slowing of development, loss of use of the hands, distinctive hand movements, slowed brain and head growth, problems with walking, seizures, and intellectual disability. Delivery of the *MeCP2* gene to neurons and glial cells has given positive results to alleviate disease symptoms on a sustained basis (Gadalla et al., 2013; Garg et al., 2013). Although this gene holds therapeutic promise for Rett patients, overexpression of *MeCP2* is known to have detrimental effects and in those studies there was toxicity observed in areas such as the liver where the transgene was overexpressed (Gadalla et al., 2013). In this instance, a safe and effective gene therapy approach for Rett syndrome is likely to require tightly controlled transgene expression that is limited to specific cell populations (Gray, 2013).

By creating these custom expression cassettes, expression or down regulation of a particular gene can be controlled in many different dimensions. These dimensions include the strength, time lapse, and modulation of the specific transgene inside the vector. This section aims to highlight some of the principle considerations of gene expression in vitro and in vivo, and the means by which it may most effectively be achieved through modifications of the expression cassette. A list of promoters is provided in Table 3.4 and a summary of other regulatory elements is provided in Table 3.5.

3.1 Viral Promoters

The majority of pioneering gene therapy work has utilized viral promoters. A promoter is a sequence of DNA where transcription is initiated. Usually found near the beginning of a gene, the promoter has a binding site for the enzyme used to make a messenger RNA (mRNA) molecule. Their compact size and ability to induce often higher levels of transcriptions than eukaryotic promoters make them an attractive component in vector cassettes. These include the SV40, cytomegalovirus immediate-early promoter, Rous sarcoma virus long terminal repeat, Moloney murine leukemia virus (MoMLV) LTR, and other retroviral LTR promoters. The use of viral promoters has thus been widespread and successful in vitro and for certain applications in vivo.

However, the use of viral promoters has become increasingly limited due to eukaryotic cells' ability to detect and silence viral transgene expression. Viral promoters have been commonly shown to be transcribed by many eukaryotic constructs in vivo but in many instances been shown to be specifically down regulated by tumor necrosis factor and interferons. It is certainly clear that powerful nonspecific promoters such as CMV are prone

to silencing in the CNS (Gray, Foti, et al., 2011), and specific eukaryotic promoters or *cis*-acting regulatory elements may be used to avoid this effect.

3.2 CNS Promoters

Although endogenous eukaryotic promoters trail viral promoters in terms of expression intensity, ongoing modifications are continuously closing this gap. Eukaryotic genes are highly regulated and it is to be expected that success in their use will depend upon the use of an appropriate promoter and the incorporation of additional elements to maximize its expression, in contrast to the frequently more compact, self-contained viral promoters. The catalog of tissue- and disease-selective eukaryotic promoters employed to drive target-specific transgene expression has expanded considerably over the past few years and reflects attempts to improve the strength and longevity of transgene expression in vivo.

Several diseases similarly require expression restricted to the CNS. Smith-Arica et al. utilized the astrocyte-specific GFAP and neuronal- specific endolase (NSE) promoters to generate inducible neurospecific vectors, and demonstrated selective expression in vivo (Smith-Arica et al., 2000). Xu et al. found several brain-specific promoters superior to CMV in a panel of neuron-specific AAV vectors in vitro and in vivo, including the NSE, GFAP, preproenkephalin (PPE), and nonspecific elongation factor EF1α-promoters (Xu et al., 2001). In primary cell cultures in vitro the EF1-α and NSE promoters were at least 10 times the strength of CMV, whilst the GFAP and PPE promoters induced a comparable level of reporter gene expression. Following in vivo stereotactic injection of these AAV vectors, the NSE promoter was found to induce almost 100-fold greater levels of expression than the CMV promoter, while the EF1α, GFAP, and PPE promoters gave up to several-fold greater levels of expression.

3.3 Modifications to Enhance Expression

3.3.1 WPRE—Addition of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) to the NSE and PPE constructs improved expression levels by up to a further 10-fold in vitro and in vivo. WPRE is a DNA sequence that, when transcribed, creates a tertiary structure enhancing transgene expression. Insertion of the WPRE in the 3′ untranslated region of coding sequences carried by either oncoretroviral or lentiviral vectors substantially increased their levels of expression in a transgene, promoter, and vector-independent manner. Using adenovirus vectors, Xu et al. found that the WPRE in the sense orientation cloned between the luciferase gene and the polyA sequence stimulated 2- to 7-fold more luciferase expression in vitro and 2- to 50-fold more in the liver, kidney, and lung of mouse than occurred without the use of the WPRE (Xu, Mizuguchi, Mayumi, & Hayakawa, 2003). The most efficient Ad vector in this study, which contained an improved CMV promoter and the WPRE, showed more than 700-fold luciferase expression in mouse liver than did the Ad vector containing the conventional CMV promoter but no WPRE. These results indicate that inclusion of the WPRE, combined with the optimization of transcriptional regulatory elements in Ad vectors, can permit a given therapeutic goal to be achieved with substantially fewer viral particles. This and other studies utilizing WPRE provided novel information that incorporated this element in CNS gene therapy projects.

3.3.2 MARs and Chromatin Insulators—Gene transfer in eukaryotic cells and organisms suffers from epigenetic effects that result in low or unstable transgene expression and high clonal variability. Use of epigenetic regulators such as MARs is a promising approach to alleviate such unwanted effects. As discussed in Section 2.1, utilization of MARs in nonviral vectors shows an increase in the expression of recombinant proteins from cultured cells as well as mediating high and sustained expression in mice. Though most of these studies used nonviral vectors, the proof of concept shows promise in the use of this element in viral vectors studies.

To overcome chromatin-dependent repressive transgenic states, chromatin regulatory elements can be used to “insulate” transgene expression. Insulators, or chromatin boundaries, separate a gene locus into independent and separately regulated modules. These are able to protect a transgene against chromatin position effects at their genomic integration sites, or the effects of neighboring enhancer/promoter elements, and they are able to maintain transgene expression for long periods of time. Therefore, these elements may be very useful tools in gene therapy applications for ensuring high-level and stable expression of transgenes. This is also of particular importance for integrating vectors, where a strong vector promoter could influence the expression neighboring proto-oncogenes with deleterious consequences. The chicken beta-globin locus control region contains an insulator, HS4, which has been used in gene transfer studies (Li, Peterson, Fang, & Stamatoyannopoulos, 2002). The 1.2 kb HS4 was inserted into the MoMLV LTR without any adverse effects on vector production, and insulated integrated viral DNA from insertional position effects (Rivella, Callegari, May, Tan, & Sadelain, 2000). Steinwaerder et al. inserted an expression cassette driven by a metal (Zn2+) inducible promoter into an adenoviral vector, and observed that the background transgene expression increased significantly as a result of *cis*-acting viral sequences (Steinwaerder & Lieber, 2000). Flanking the expression cassette by the HS4 sequence increased the induction ratio by 15-fold in vivo. In an AAV vector, flanking the transgene construct with the 250 bp HS4 insulator stabilized expression levels to constant levels regardless of position effects (Inoue et al., 1999).

3.3.3 MicroRNA—One major obstacle in gene therapy trial is peripheral transduction in other tissue types. To circumvent this, Xie et al. (2010) used tissue-specific, endogenous microRNAs (miRNAs) to repress rAAV expression outside the CNS, by engineering perfectly complementary miRNA-binding sites into the rAAV9 genome (). miRNAs are a class of naturally occurring, small noncoding RNA molecules, about 21–25 nucleotides in length. miRNAs are partially complementary to one or more mRNA molecules, and their main function is to downregulate gene expression in a variety of manners, including translational repression, mRNA cleavage, and dead-enylation. This approach allowed simultaneous multitissue regulation and CNS-directed stable transgene expression without perturbing the endogenous miRNA pathway. In this instance, AAV9 was able to be administered systemically, targeting both the liver and the brain. By incorporating liver-specific miRNA-binding sites into the vector genome, expression in the brain was allowed to occur whereas expression in the liver was silenced. Similarly, target sequences of the mir-142-3p incorporated into lentiviral vectors suppressed expression in hematopoietic

lineages, whereas expression was maintained in nonhematopoietic cells (Brown, Venneri, Zingale, Sergi Sergi, & Naldini, 2006). This expression profile enabled stable gene transfer into immunocompetent mice, thus overcoming a major hurdle to successful gene therapy (Brown et al., 2006).

3.3.4 PolyA Site—The polyA tail is a long chain of adenine nucleotides that is added to a mRNA molecule during RNA processing. The polyA tail makes the RNA molecule more stable and prevents its degradation and allows the mature mRNA molecule to be exported from the nucleus and translated into a protein by ribosomes in the cytoplasm. A comparison of different polyA signals, including the mouse α-globin polyA, the bovine growth hormone polyA, and a synthetic polyA showed that by utilizing different polyA sequences expression could be modulated over a 7-fold range (Wu et al., 2008).

3.4 Regulatable Expression Systems

3.4.1 Ligand-Inducible Systems—The majority of regulative systems engineered to date are ligand-inducible promoters, which have the distinct advantage of permitting pharmacological control of transgene expression following vector administration in vivo (reviewed in Zoltick & Wilson (2001)).

The most prominent regulative system has been the tetracycline (tet) on/off system (Figure 3.10). In this system, the transactivator (TA) is constitutively expressed, and the transgene is under control of the tet response element (tet operator). The TA consists of the tet repressor fused to a transcription factor activation domain. The same or two separate vectors may encode the TA and transgene in this system, but the latter “dual” vector system has had more success in maintaining regulatory stringency. The original wt repressor requires constant administration of the drug to keep the transactivator (tTA) displaced from the response element and the switch “off ” (tet-OFF system). The most useful tet switches for many gene therapy applications are those that use the mutant reverse tet repressor (rtTA), which binds in the presence of tetracycline or a suitable analogue. This switch is constitutively off and permits the “on” signal to be transmitted upon administration of tetracycline (the tet-ON system), necessitating its administration only when transgene expression is required rather than vice versa. A very large number of studies have made use of this system, which permits regulation of transgene expression by simple oral administration of Dox.

Blesch et al. used a tetracycline-regulatable gene expression system was generated to determine whether controllable release of NGF and green fluorescent protein (GFP) from primary rat fibroblasts could modulate biological responses (neurite outgrowth) in vitro (Blesch, Uy, Diergardt, & Tuszynski, 2000). Using a tetracycline-repressible construct, it was found that NGF mRNA, NGF protein, and NGF-induced neurite outgrowth could be tightly regulated within a 24-h period, and in a dose-dependent fashion, by exposure to the tetracycline analog Dox. Similarly, levels of green fluorescence could be regulated in GFP-transfected cells. These findings in a neurobiological system lay the framework for future studies using regulated neurotrophin delivery in in vivo models of neurodegenerative diseases and CNS injury.

Steroid response switches using nonhuman analogues have also proved successful because of their suitable pharmacokinetic properties as small fast acting and rapidly metabolized lipophilic molecules (Hoppe, Marban, & Johns, 2000). This system utilizes a modular recombinant receptor consisting of the mutated ligand-binding domain fused to appropriate transcription factor DNA-binding and activation domains. The receptor is constitutively expressed and activates transcription from the target gene when ligand is present and binds to it. One study that evaluated the tet-on and antiprogestin switches in a dual adenoviral vector system in vitro demonstrated up to 1800- and 600-fold induction levels with the two switches, respectively (Molin, Shoshan, Ohman-Forslund, Linder, & Akusjarvi, 1998). Spiga et al. developed a novel glucocorticoid-inducible adenovirus vector that overproduces MMP1 only in the presence of dexamethasone, a synthetic corticosteroid drug (Spiga & Borras, 2010). The availability of this vector sets up the foundation for the development of gene therapy drugs for the potential treatment of ocular hypertension in steroid-responsive patients.

Another ligand-inducible system takes advantage of the dimerizing function of the antibiotic rapamycin, which links the two proteins FK506-binding protein (FKBP) and FKBP12-rapamycin-associated protein (FRAP). FKBP contains the DNA-binding domain while the activation domain of NF-κB p65 is fused to FRAP. The promoter containing the target DNA sequence is only induced when the two are dimerized by the action of rapamycin or (preferably) the use of alternative analogues in vivo (Rivera et al., 2005; Ye et al., 1999). Ye et al. (1999) utilized two AAV vectors, one expressing the dimerizing transcription factors and the other an erythropoietin transgene under transcriptional control of the target promoter. When delivered at an equal ratio by intramuscular injection in mice, plasma levels of erythropoietin were inducible by up to 200-fold upon administration of rapamycin in a dose-responsive fashion at 6 months after injection. Administration of vectors to nonhuman primates permitted 50-fold induction of erythropoietin that returned to baseline in 14 days. This maximal level of induction was comparable to the levels of erythropoietin expressed from a CMV promoter-driven vector.

3.4.2 Stimulus-Inducible Systems—Other types of regulatable promoters are those incorporating elements that are stimuli responsive, such as those inducible by heat, radiation, or metal-responsive elements. Of significant interest to several ischemic and cancer disease therapies are hypoxic response elements (HRE) that are activated in cells via hypoxia-inducible factor-1, which permit the selective induction of gene expression in a hypoxic environment. Many groups have utilized HREs to generate synthetic hypoxia-inducible promoter constructs. In studies by Ruan et al., a rAAV was generated in which the transgene can be regulated by hypoxia in human brain tumors (Ruan & Deen, 2001; Ruan et al., 2001). A DNA fragment containing 9xHRE and the *LacZ* reporter gene were incorporated into the AAV vector. Under anoxic conditions, this vector produced 79- to 110-fold increase in gene expression. This hypoxia-regulated rAAV vector will provide a useful delivery vehicle for CNS cancer-specific gene therapy.

4. CONCLUSIONS

The biology of different viruses offers unique solutions to the challenges of gene therapy, such as cell targeting, integration rate, transgene expression, and vector production. For example, the AAV platform offers substantial benefits including efficient gene transfer, long-term transgene expression, low-inflammatory responses, minimal integration rate, and scalable manufacture for clinical applications (reviewed in (Grieger & Samulski, 2005), and in this chapter). However, the limited packaging capacity of 4.7 kb is too small for some genes and greatly restricts the use of complex genomic regulatory elements. AAV is also not suitable for prolonged expression in dividing tissues. Lentiviral vectors, both integrating and nonintegrating, are becoming prominent CNS gene transfer tools. Their increased capacity of 8–9 kb of foreign DNA while still maintaining a relatively safe profile and reasonably efficient gene transfer to neural cells gives them a distinct advantage in certain applications. Similarly, adenoviral and HSV vectors have very large packaging capabilities (>25 kb) but adenoviral vectors are limited by their higher propensity to induce inflammation. In certain applications HSV vectors have a strong advantage due to their natural ability for efficient axonal transport, but, in general, they have not undergone the same level of fine-tuned engineering that has been devoted to other vectors to optimize them as safe and effective gene transfer reagents. Overall, it would be safe to predict that it is unlikely that one vector system will be exclusively appropriate for all types of research or therapeutic gene transfer applications into the CNS.

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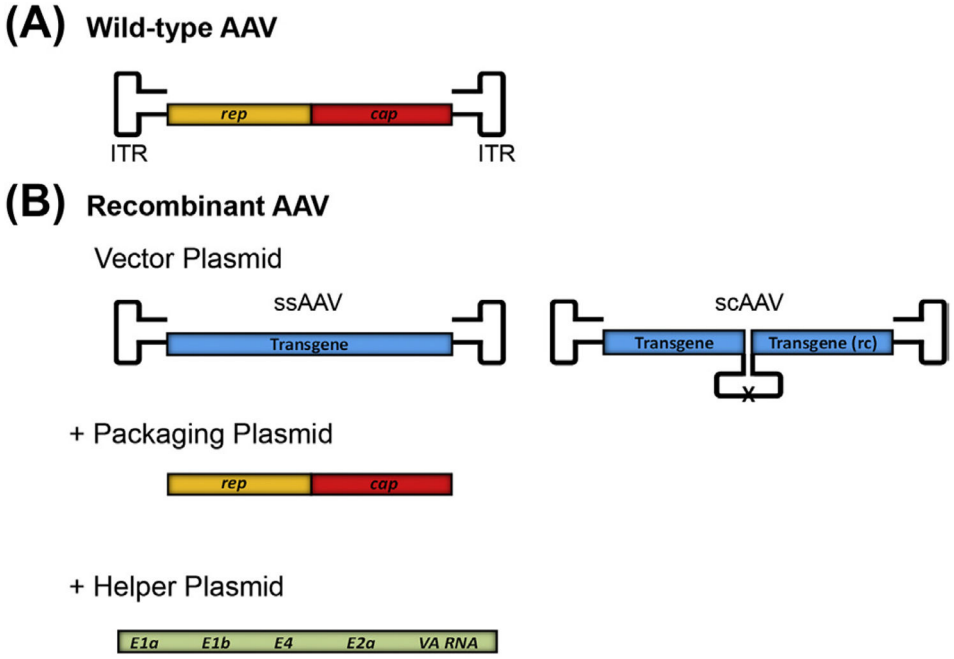

Figure 3.1.

Recombinant AAV vector genome design. (A) The wild-type AAV genome generally consists of the viral rep and cap genes between two inverted terminal repeats (ITRs). (B) Recombinant AAV vector is produced by cotransfection of a vector plasmid containing an ITR-flanked transgene cassette, a packaging plasmid that encodes the *rep* and *cap* genes of a specific AAV serotype and a helper plasmid that supplies the essential adenovirus helper genes (E1a, E1b, E2a, E4, and VA RNA). The vector plasmid may be either single-stranded DNA that encodes ~4.5 kb of novel transgene sequence or self-complementary (sc) DNA that can encodes ~2.2 kb of novel transgene sequence in duplex form. The sc AAV genome consists of the forward and reverse complement (rc) transgene sequences with wild-type ITRs at the extremities and a mutated ITR (X) at the axis of symmetry.

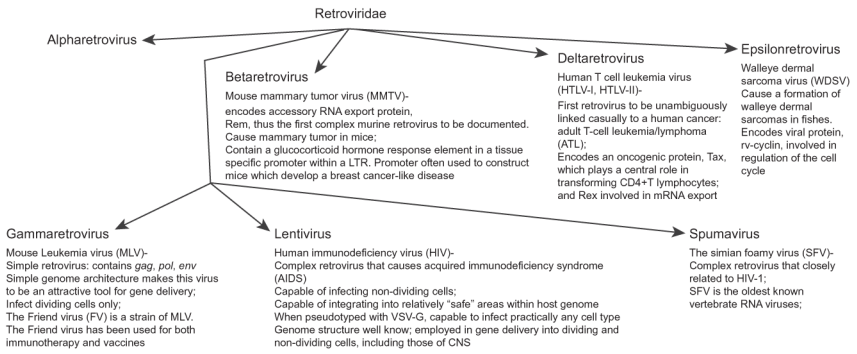

Figure 3.2.

Retroviridae family comprises seven major genera: alpha-retroviruses (prototype ALSV), beta-retrovirus (prototype MMTV), delta-retrovirus (prototype HTLV-I, HTLV-II), gamma-retrovirus (prototype MLV), epsilon-retrovirus (prototype WDSV), lentiretroviruses (prototype HIV-1), and spumaviruses (prototype SFV). Notable characteristics of the viruses are included.

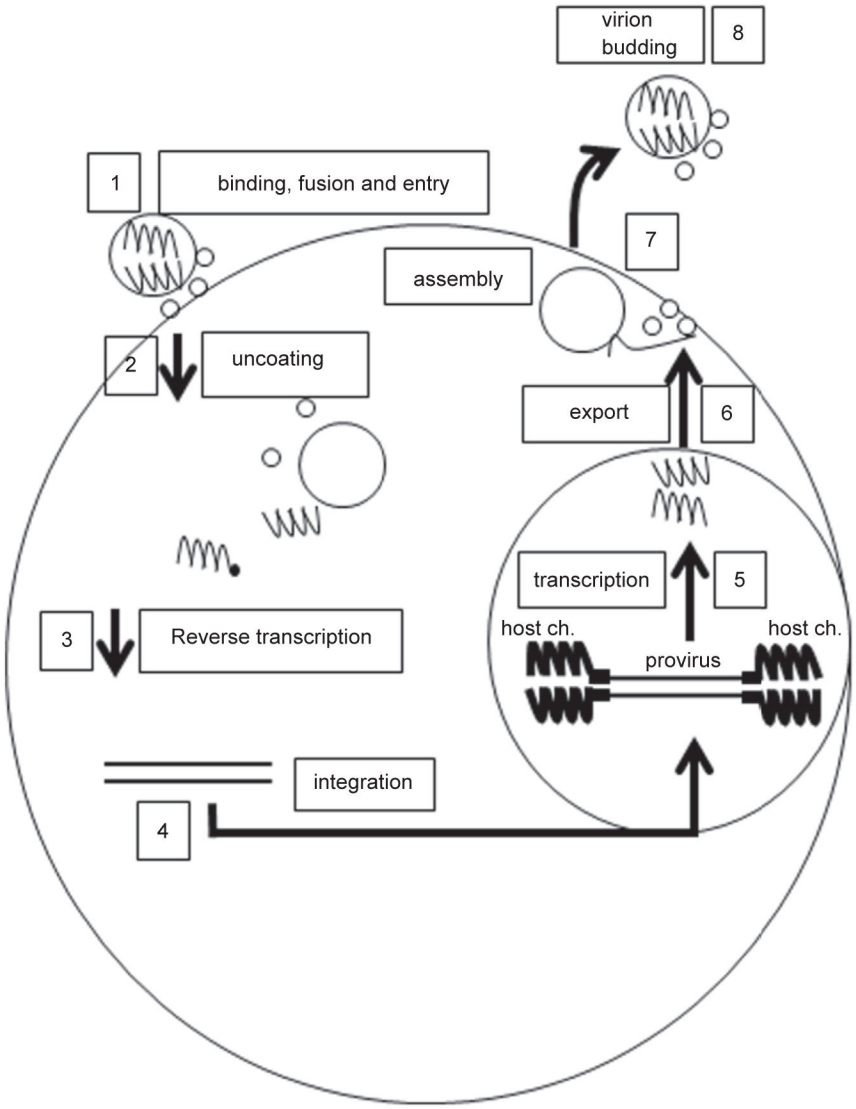

Figure 3.3.

Life cycle of the HIV-1 including binding-fusion, uncoating, reverse transcription, integration, transcription, nuclear export, assembly and virion budding are shown. The star represents reverse transcriptase. Steps of the life cycle labeled from one to eight.

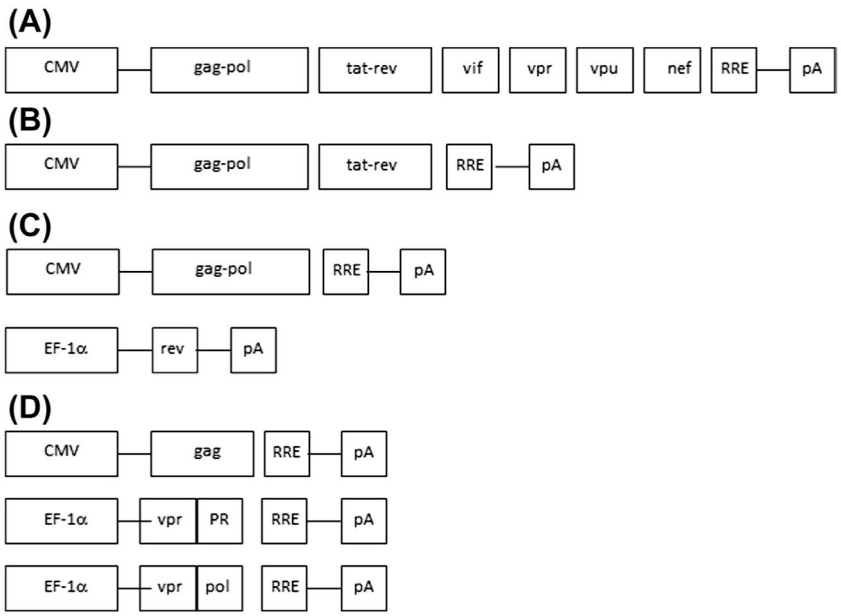

Figure 3.4.

Development of the packaging cassette. Four generations of the packaging cassette are shown. (A) First generation included all four accessory proteins, *Vpu*, *Vpr*, *Vif*, and *Nef* and the regulatory proteins, *tat* and *rev*. RRE stands for *rev* response element. Expression is driven from the CMV promoter; PolyA signal (pA) is shown. (B) Second generation excluded all four accessory proteins, *Vpu*, *Vpr*, *Vif*, and *Nef*, but included regulatory proteins, *tat* and *rev*. (C) Further split of the packaging cassette defined the third generation. (D) Four generation characterized by a further split of the cassette into three components: *gag* expressed from the CMV promoter; *Vpr*-protease expressed from the EF1-α promoter. *Vpr-pol* transcript is driven from the EF1-α promoter. All three packaging cassettes contain RRE and *pA* signals. CMV, cytomegalovirus; RRE, Rev response element.

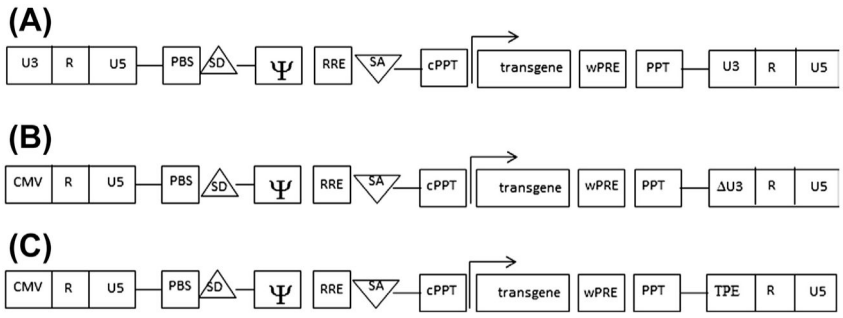

Figure 3.5.

Development of the expression (transgene) cassette. (A) First generation contains wild-typed 5′- and 3′-LTRs, primer-binding site (PBS), splice donor (SD), and splice acceptor (SA), central polypurine tract (cPPT) and polypurine tract (PPT), Rev response element (RRE), woodchuck hepatitis virus posttranscriptional regulatory element (wPRE), and the retroviral vector packaging element, psi (Y) signal. Expression of a transgene is driven from the promoter-of-choice shown by the arrows. (B) SIN vector is devoid of the parental enhancer/promoter sequences, located at the U3′ of the 3 ′-LTR (deletion is shown); thus, lacking the ability to transcribe full-length mRNA. CMV promoter incorporated in the 5′-LTR employed to generate full-length mRNA. (C) Inducible expression cassette drives from the tetracycline response element (TRE) incorporated in the U3 region of the 3′-LTR. This cassette named a conditional SIN cassette, expressing the genome in the presence of the *ttA*. SIN, self-inactivating; LTR, long terminal repeat; CMV, cytomegalovirus.

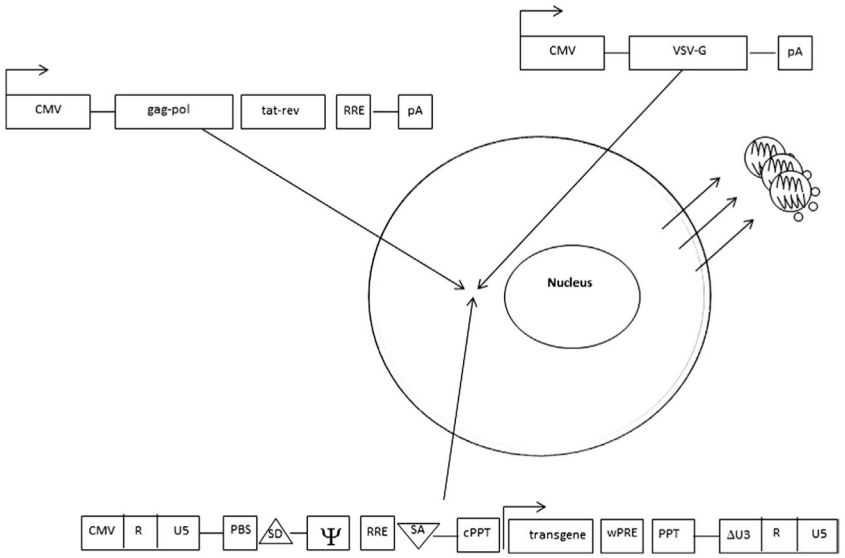

Figure 3.6.

Transient transfection protocol employed to generate lentiviral particles. 293T cells transfected with VSV-G, packaging, and transgene cassettes. Viral particles that bud out from the cell membrane contain full-length RNA of the vector (expressed from the transgene cassette). VSV-G, vesicular stomatitis virus G-protein.

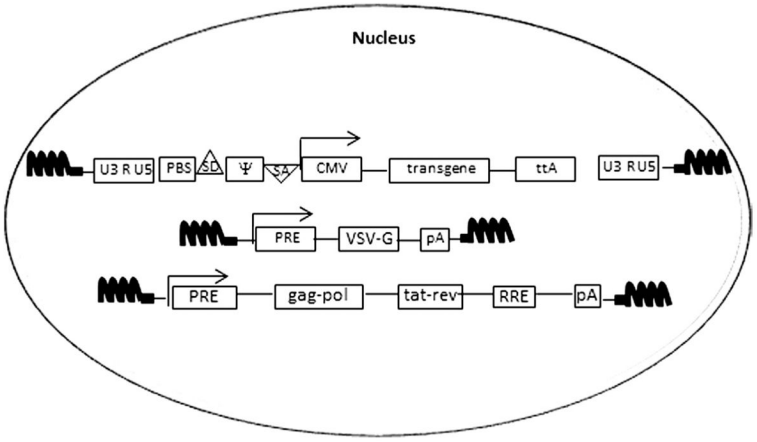

Figure 3.7.

Stable cell lines developed to generate lentiviral vectors. Transgene, envelope, and packaging cassettes introduced into vector's producer cells by stable transfection following by selection. *Tet*-inducible system is commonly employed to circumvent the toxicity associated with the viral proteins. Tetracycline *trans*activator, *ttA* can be expressed from the same cassette (transgene cassette) as does a transgene.

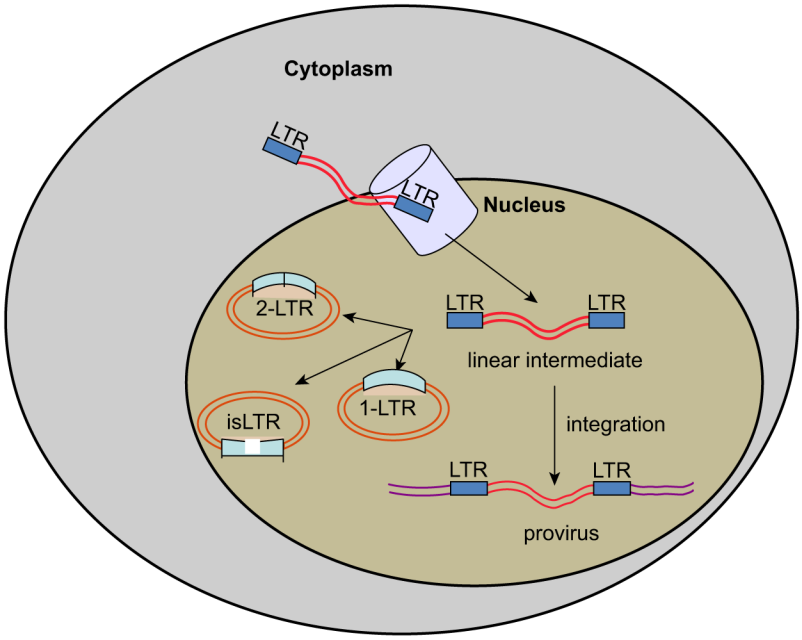

Figure 3.8.

Episomal forms generated during the HIV infection. Episomal genomes of the HIV-1 appear in four major forms: linear episomes: precursors for integration and for the rest of the episomes; 2-LTR (double-LTR) and 1-LTR (single-LTR), are aberrant circular forms, generated by the DNA repair machinery of the host, or through the aberrant reverse transcription reaction; the autointegrative forms are product of self-integration mediated by the host repair machinery. LTR, long terminal repeat.

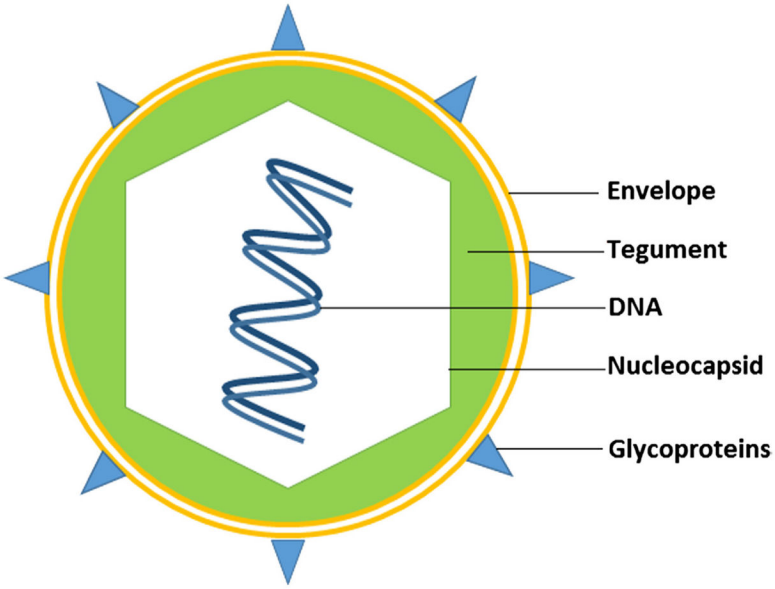

Figure 3.9.
HSV structure. HSV, Herpes simplex virus.

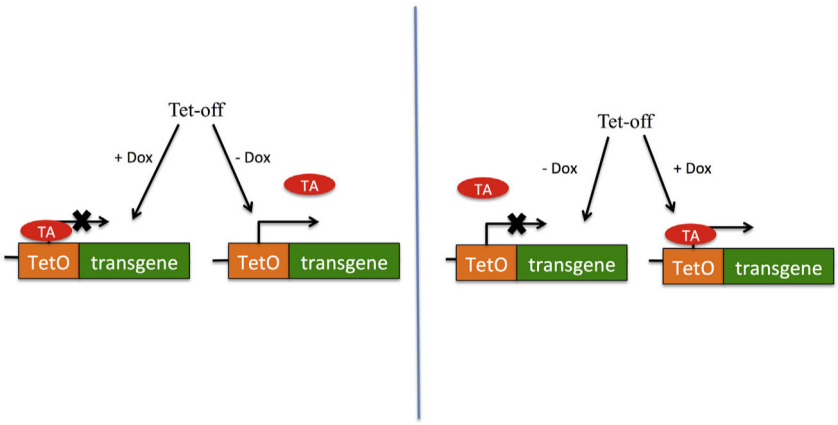

Figure 3.10.
Tetracycline-controlled transcriptional activation. Dox = doxycycline, TA = transactivator, TetO = tetracycline operator.

Table 3.1

Primary and Secondary Receptors for Common AAV Serotypes

Serotype	Primary Receptor	Secondary Receptor(s)
AAV1	N-linked α2,3-/α2,6-Sialic acid (Ng et al., 2010; Wu, Miller, Agbandje-McKenna, & Samulski, 2006)	Unknown
AAV2	Heparan sulfate (Summerford & Samulski, 1998)	Integrins αVβ5/α5β1, FGFR1 (fibroblast growth factor receptor 1) (Qing et al., 1999), HGFR (hepatocyte growth factor receptor) (Qing et al., 1999), and laminin receptor (Akache et al., 2006)
AAV3	Heparan sulfate (Rabinowitz et al., 2002)	FGFR1 (Blackburn, Steadman, & Johnson, 2006), HGFR (Ling et al., 2010), laminin receptor (Akache et al., 2006)
AAV4	O-linked α2,3-Sialic acid (Kaludov et al., 2001)	Unknown
AAV5	N-linked α2,3-Sialic acid (Kaludov et al., 2001; Walters et al., 2001)	PDGFR (platelet-derived growth factor receptor) (Di Pasquale et al., 2003)
AAV6	N-linked α2,3-/α2,6-Sialic acid (Wu, Miller, et al., 2006), heparan sulfate (Ng et al., 2010)	EGFR (epidermal growth factor receptor) (Weller et al., 2010)
AAV7	Unknown	Unknown
AAV8	Unknown	Laminin receptor (Akache et al., 2006)
AAV9	N-linked β1,4-Galactose (Shen, Bryant, Brown, Randell, & Asokan, 2011)	Laminin receptor (Akache et al., 2006)

Table 3.2

Heterologous Envelope Proteins Used for Psuedotyping Lentiviral Vector for CNS Applications

Envelope	Host Range	Cell Type	Stability upon Concentration
VSV-G	Mouse, rat, pig, dog, human	Ubiquitous	The most established protocol; stable, suitable for concentration
LCMV	Mouse, rat	Strong preference to astrocytes, glioma cells; neurons to a lesser degree	Stable, suitable for concentration
MV	Mouse, rat	Ubiquitous	Stable, suitable for concentration
Rabies-G	Mouse, rat	Preference to neurons; excellent axonal transport	Stable, suitable for concentration
MoMLV	Mouse, rat	Similar pattern to LCMV; capable of infecting neurons	Stable, suitable for concentration
RRV-G	Mouse, rat, human cells in vitro	Glial cells and neurons	Stable, suitable for concentration

Vesicular stomatitis virus G-protein (VSV-G); lymphocytic choriomeningitis virus protein (LCMV); Mokola virus G-protein (MV); Rabies virus G-protein (Rabies-G); murine leukemia virus envelope protein (MoMLV); Ross River virus G-protein (RRV-G).

Table 3.3

Typical Promoter Types Used to Drive Expression in Lentiviral Vectors in the CNS

Promoter	Strength	Cell Type
CMV	Strong but subject to transcriptional silencing over time	Ubiquitous
PGK	Strong	Ubiquitous
EF1-α	Strong	Ubiquitous
TRE	Strong, inducible by ttA or ttS+/−Dox, respectively	Ubiquitous
GFAP	Moderate-strong	Astrocytes
CaMKII	Strong	Postnatal neurons (strong after week 4–5 in mice and rats)
Synapsin I	Moderate-strong	Developing neurons (weaker after week 4–5 in mice and rats)
Thy-1,2	Moderate-strong	Prenatal and postnatal neurons

Cytomegalovirus (CMV); phosphoglycerate kinase 1 promoter, (PGK); Human elongation factor-1 alpha promoter, (EF1-α); The tetracycline response element-containing promoter, (TRE); Glial fibrillary acidic protein promoter, (GFAP); α-calcium/calmodulin-dependent protein kinase II, (CaMKII); Synapsin type I promoters, (Synapsin I); thymocyte differentiation antigen 1 and 2 promoter, (Thy-1,2).

Table 3.4

Representative Promoters for CNS Gene Transfer

Promoter	Specificity	Strength	Ref
Viral (CMV-IE, RSV-LTR, MoMLV-LTR, SV40)	Ubiquitous	+++	(Papadakis, Nicklin, Baker, & White, 2004)
Eukaryotic (EF1-α)	Ubiquitous	++++	(Xu et al., 2001)
Ubiquitin C	Ubiquitous	+	(Qin et al., 2010)
GUSB	Ubiquitous	+	(Husain, Passini, Parente, Fraser, & Wolfe, 2009)
PPE	All of CNS	+++	(Xu et al., 2001)
SYN1	Neurons	++	(Kugler et al., 2001)
NSE	Neurons	++++	(Smith-Arica et al., 2000)
GFAP	Astrocytes	+++	(Lee, Messing, Su, & Brenner, 2008; Smith- Arica et al., 2000)
MBP	Oligodendrocytes	++	(Gow, Friedrich, & Lazzarini, 1992)
F4/80	Microglia	++	(Cucchiarini, Ren, Perides, & Terwilliger, 2003)
MCH	MCH neurons	++	(van den Pol, Acuna- Goycolea, Clark, & Ghosh, 2004)
MeCP2 (MeP229)	Neurons	+	(Gray, Foti, et al., 2011)

CMV, cytomegalovirus; RSV-LTR, Rous sarcoma virus long terminal repeat; SV40, simian virus 40; EF1-α, Human elongation factor-1 alpha promoter; PPE, preproenkephalin; GFAP, Glial fibrillary acidic protein.

Table 3.5

Regulatory Elements to Modulate Expression

Element	Role/Effect	Reference
WPRE	Increases RNA stability, transgene expression	(Xu et al., 2003)
Intron	Increases RNA stability, transgene expression	(Wu et al., 2008)
PolyA	Affects mRNA stability, transgene expression	(Hager, Frame, Collins, Burns, & Maitland, 2008; Wu et al., 2008)
miRNA-binding site	Restricts expression from specific cells	(Xie et al., 2010)
Ligand-inducible system	Activate/deactivate transgene expression	(Blesch et al., 2000; Zoltick & Wilson, 2001)
Stimulus-inducible system	Regulates transgene expression based on physiological state	(Ruan & Deen, 2001; Ruan et al., 2001)
MAR	Increases long-term retention of the delivered DNA	(Girod et al., 2007)
Chromatin insulators	Blocks cross-communication between regulator elements; reduces variability of transgene expression	(Recillas-Targa,Valadez- Graham, & Farrell, 2004)

MAR, matrix attachment region.