Topic Introduction

Introducing Genes into Mammalian Cells: Viral Vectors

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Over the years, many different viral vector systems have been developed to take advantage of the specific biological properties and tropisms of a large number of mammalian viruses. As a result, researchers wanting to introduce and/or express genes in mammalian cells have many options, as discussed here.

BACKGROUND

Much of the early excitement about molecular cloning was fueled by a belief that cloned copies of mammalian coding sequences could be inserted into prokaryotic vectors, expressed to high levels in a bacterial host, and the expressed proteins purified in an active form and used not only in research projects but also therapeutically. Because of the wealth of knowledge of its physiology and genetics, its fast growth rate, and ease of handling, the host organism of first choice was *Escherichia coli.* The spectacular demonstration that *E. coli* could be used to clone and express small eukaryotic proteins, such as human growth hormone (Goeddel et al. 1979) and rat insulin (Ullrich et al. 1977), provided significant impetus for the rapid commercialization of DNA cloning technology in the late 1970s and early 1980s. Many of the early recombinant DNA companies were floated on the optimistic assertion that any difficulties encountered using *E. coli* to synthesize large quantities of biologically active mammalian proteins would be minor and that any problems could be solved reasonably quickly. However, this initial bubble of confidence dissolved when many eukaryotic proteins expressed in *E. coli* were found to be biologically inactive, denatured, and/or aggregated into insoluble inclusion bodies. Other eukaryotic proteins could be expressed only at very low levels, whereas a few either were not expressed at all or were toxic to their bacterial hosts. Purification of inclusion bodies and in vitro refolding of proteins offered a potential solution to this problem. But the conditions for refolding varied from protein to protein and the efficiency was generally low even after optimization.

These problems result from the inability of eukaryotic proteins expressed in *E. coli* to form the same folds, domains, and three-dimensional structures as are present in their natural hosts. As a rule, only small (<23 kDa) globular cytoplasmic proteins with a high content of charged amino acids and few contiguous hydrophobic residues can be expressed in soluble form in *E. coli.* In some cases, the solubility of the expressed protein can be improved by the addition of a hydrophilic tag (Dyson et al. 2004) or, more recently, by genetic selection (Lim et al. 2009). However, the majority of eukaryotic proteins are much larger than 23 kDa, have complex folds, and consist of several subunits. By the early 1980s, it had become clear that cloned cDNAs encoding mammalian proteins were best expressed in host cells that carry out the appropriate posttranslational modifications and are equipped with the specific chaperones, partner-binding proteins, and cellular trafficking systems required for accurate, rapid folding and assembly of nascent eukaryotic polypeptides.

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The past 30 years have seen the development of increasingly sophisticated systems to express genes (or more often, complementary DNAs [cDNAs]) in cultured mammalian cells. These systems are of two types: (1) those that involve transient or stable expression of transfected DNA (see Introduction: **Introducing Genes into Cultured Mammalian Cells** [Kumar et al. 2019]) and (2) those that involve the use of viral expression vectors, which take advantage of the multiplicity of mechanisms that viruses use to gain entry into mammalian cells and once there to harness the cellular machinery to express their cargo of genetic material.

Over the years, many different viral vector systems have been developed to take advantage of the specific biological properties and tropisms of a large number of mammalian viruses. Some systems have not yet progressed beyond an early exploratory stage; others have been designed to solve specific biological problems; and a few require detailed knowledge of, and sometimes affection for, the biology of the parental virus.

In their natural state, the virions of some vectors contain DNA as their genetic material, and others contain RNA. But whatever the natural state of the vector's genome, recombinants are constructed (1) using standard in vitro techniques to introduce an expression cassette into a cloned DNA copy of the vector's genome and (2) by recombination in cells transfected with the vector and an expression cassette. If the foreign DNA replaces viral sequences that are not essential for viral growth, the recombinant DNA can be transfected into an appropriate cell type and the recombinant virions can be harvested a few days later. If the segment of foreign DNA replaces one or more viral genes that are essential for growth, the missing functions must be provided in *trans*—either from a helper virus or, in some cases, by a cell that carries an integrated copy of the essential gene(s).

FACTORS TO CONSIDER WHEN CHOOSING A VIRAL VECTOR

Mammalian viruses display an extraordinary variety of genetic structures and biological behaviors. As a result, researchers wanting to introduce and/or express genes in mammalian cells have many options, and the choice among them is not always clear. Factors to consider when selecting a vector include the following.

The Purpose of the Experiment

The requirements made of a viral vector depend on the goals of the experiment. If the goal is to produce a biologically active protein, major factors to consider include the following:

1. The amounts of protein that will be required and the yields that can be expected from the various available vectors.
2. The size of the expression cassette, which must fit comfortably within the space available in the vector's genome.
3. The assays that will be used to monitor protein production and measure the biological activity of the protein (see Box 1).
4. Whether purification of the protein will be required. This becomes an important consideration if a major goal is to express and assay a large number of mutant forms of the target protein.
5. The scalability and estimated cost of the project (in both time and materials). In some cases (e.g., when assaying the activity of a series of mutant proteins), the cheapest and fastest option may be to use transfection systems rather than a viral vector. And, depending on the experience and skills available, the best option may be to buy a commercial viral expression kit, should a suitable one be available.

If the goal of the experiment is to stably express a transgene in cultured mammalian cells, the best option is to use a retrovirus or lentivirus vector, because they integrate into the genome without killing the host cells.

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BOX 1. ASSAYS DONE IN TRANSDUCED CELLS

1. Preparation of vector stocks.
2. Transduction of target cells at different MOIs or vector dose per target cell:

   Adenovirus vectors: 100–10,000 virus particles/target cell

   Adeno-associated virus vectors: 10,000–100,000 virus genomes/target cell

   Lentivirus vectors: MOI 5–500
3. Assess transgene expression at 3–5 d posttransduction.

   For *vector-encoded proteins*, a number of different approaches can be used to assess transgene expression in transduced cells and this depends on the type of transgene, subcellular localization, availability of suitable antibodies, or bioassays.

- If the vector encodes a fluorescent marker protein, assess transduction using a fluorescence microscope equipped with appropriate filters for visualization of the specific marker. Proceed to the next step.
- Extract total cellular proteins and analyze transgene expression by western blot or ELISA quantification.
- Analyze transgene expression using fluorescence activated cell sorting (FACS). This method will quantify both the percentage of positive cells and the intensity of transgene expression.
- Analyze expression and subcellular localization using immunofluorescence or immunocytochemistry.

i. To distinguish from endogenous protein, or for immunoprecipitation experiments in the absence of effective primary antibodies against the protein of interest, include a protein tag (Table B1) at the amino or carboxyl terminus of the vector-encoded protein. Alternatively, a small tetracysteine peptide can be used to perform live cell imaging of protein expression and localization in the presence of biarsenical compounds that become fluorescent after specific binding to proteins carrying this tag (Griffin et al. 1998).

ii. The potential effect of these tags on protein biochemistry and function should be carefully considered and analyzed. As an alternative, vectors can be used to transduce cells that do not express the particular protein, and thus, experiments are performed in a null background.

iii. Measure the biological effect of the vector-encoded protein in cultured cells or in vivo (e.g., use transgene-specific enzymatic assays, monitor changes in current regulation in cells transduced with ion channels such as the cystic fibrosis transmembrane conductance regulator).

For *vector-encoded shRNA/miRNA*, the following approaches can be used.

TABLE B1. Amino acid sequence of epitope tags

Tag name	Amino acid sequence
HA	YPYDVPDYA
c-MYC	EQKLISEEDL
His_6	HHHHHH
FLAG	DYKDDDDK
AU1	DTYRYI
EE (Glu–Glu)	EYMPME
IRS	RYIRS
Tetracysteine	CCPGCC[a]
GFP	—

Inclusion of a few Gly residues between the protein of interest and epitope tags may be important.

HA, hemagglutinin; IRS, insulin receptor substrate; GFP, green fluorescent protein.

[a]This tag allows for specific labeling of recombinant proteins in live cells incubated with biarsenical compounds that fluoresce on binding. Note that in vectors commercialized by Life Technologies insertion of this tag at the carboxyl terminus of a protein is done in the following context: GAGG**CCPGCC**GGG.

Continued

- Extract total cellular proteins and assess effect on target protein expression by western blot, ELISA, FACS, or immunostaining of transduced and control cells.
- In the absence of antibodies, extract total RNA and measure target gene mRNA level in naïve and vector-transduced cells by real-time PCR using a housekeeping gene (GAPDH, β-actin, or 18S rRNA) as reference.
- Biological effect of target down-regulation.

The Design of the Expression Cassette to Be Inserted into the Vector

Mammalian viruses have become a fruitful source of functional elements (promoters, splicing signals, transcriptional terminators, poly(A) addition sites, etc.) that can be used to construct synthetic cassettes containing the elements required for expression of proteins at different levels and in different types of host cells. The various control elements required for construction of expression cassettes are available as precloned modules from a number of the large commercial suppliers, many of whom also sell prefabricated, empty cassettes.

Most of the vectors designed for protein production use strong promoters cloned from viral genomes. These promoters are much more compact and far more powerful than promoters from eukaryotic genes, and many of them are indifferent to the species and tissue of origin of the host cell. Viral promoters commonly used in expression cassettes include the cytomegalovirus immediate-early promoter (CME-1E), the SV40 early promoter (SV40-E), and the long terminal repeats (LTRs) of murine and avian retroviruses. Other control elements commonly used in expression cassettes include a poly(A) addition site (usually from SV40) and an internal ribosome entry site (IRES) that allows two open reading frames to be translated from a single messenger RNA (mRNA) (for review, see de Felipe 2002).

For cell-type or tissue-specific expression, the best option is to use a promoter and associated *cis*-acting elements from a gene that is expressed strongly and specifically in the target tissue of cell type. For a useful catalog of tissue-specific promoters, see Papadakis et al. (2004). Boxes 2 and 3 show diagrams of commonly used expression cassettes.

Whether the Capacity of the Vector Is Sufficient to Accommodate the Segment of Foreign DNA

Many viruses used as vectors (e.g., adenoviruses and SV40) have strict genome size requirements for effective packaging into viral particles. Trying to circumvent packaging limits that have been established and optimized over long periods of evolutionary time is futile.

Whether the Appropriate Cell Lines Are Available

No single cell line exists that is permissive for all viral vectors. For example, many retroviruses show strong species specificity and show a marked preference for dividing cells. On the other hand, adenoviruses and adeno-associated viruses (AAVs) can infect a variety of mammalian cell types with high efficiency, in sharp contrast to SV40, which has a very strong preference for the cells of its natural simian hosts. (For more details, see descriptions of the individual vector systems below.)

To increase the amount of space available for insertion of an expression cassette, genes necessary for replication or other viral functions may be deleted from the vector's genome. Recombinants constructed in such vectors require the essential functions to be supplied in *trans*, either from a helper cell, a helper virus, or from cotransfection of plasmids encoding those functions. For further information about helper-dependent vectors and cells that supply functions in *trans*, see the sections below describing adenovirus vectors and retrovirus vectors.

Time Constraints and Skills

Generating a stock of recombinant virus is a lengthy process that involves choosing an appropriate vector, designing and constructing a modular expression cassette, inserting the cassette into the

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BOX 2. TRANSGENE EXPRESSION CASSETTES

The most commonly used promoters in viral vectors are ubiquitous promoters, cell-type-specific promoters, and polyadenylation signals.

Ubiquitous Promoters

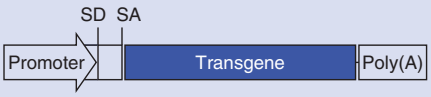
- human CMV immediate-early promoter
- murine stem cell virus (MSCV) LTR promoter
- CAG hybrid promoter carrying the CMV enhancer fused to the chicken β-actin promoter (also known as CGA, CBA, or CB; length varies among different versions)
- human EF-1α
- human phosphoglycerate kinase 1 (PGK1) promoter
- ubiquitin promoter

Cell-Type–Specific Promoters

- α1 antitrypsin promoter for liver-specific expression
- muscle creatine kinase (mCK) promoter for skeletal muscle-specific expression
- human synapsin-1 (SYN-1) or rat neuronal-specific enolase (NSE) promoter for neuronal-specific expression
- human Clara cell 10-kDa protein (CC10) promoter for lung-specific expression
- human interphotoreceptor retinoid-binding protein (IRBP) promoter for retinal-specific expression

Polyadenylation Signals Most Commonly Used in Viral Vectors

- bovine growth hormone (BGH)
- rabbit β-globin (RBG)
- SV40
- native signals present in LTR elements in retrovirus and lentivirus vectors.



Introns are commonly present in expression cassettes to enhance transgene expression. Artificial and native introns have been used for this purpose (e.g., RBG intron).

Insertion of posttranscriptional regulatory elements has been shown to increase transgene expression. The most commonly used element is derived from the WPRE (Donello et al. 1998).

Incorporation of miRNA target sequences in the 3′ UTR of viral vectors leads to transcriptional detargeting from cells where the particular miRNA is expressed at high levels. This approach has been successfully used

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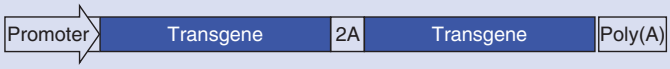
to prevent transgene expression in antigen-presenting cells in vivo and thus blunt transgene-specific adaptive immune response.

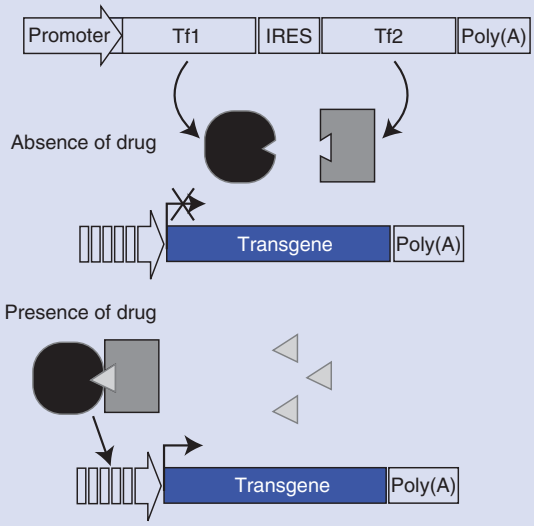
Insertion of a loxP–stop–loxP cassette allows for cell-type–specific transgene expression in transgenic mice expressing Cre recombinase in specific cell types.

The vector shown immediately above illustrates the expression of two transgenes from the same promoter. The efficiency of transgene expression is not necessarily identical on both sides of the promoter. This may not be the best approach to achieve identical expression of two genes (see other approaches) but is a useful design for studying miRNA regulation of gene expression (Brown and Naldini 2009). Inclusion of the miRNA target in the 3′ UTR of one transgene allows the study of miRNA regulation in the presence of basal transcription from the second transgene, which can be a marker gene (e.g., *GFP*).

In this vector, transgenes are expressed as separate proteins in their native state from a single transcript. Usually, expression of the transgene downstream from the internal ribosome entry site (IRES) is lower than that of the first transgene, but this aspect depends on the IRES element. The most commonly used IRES element is derived from the encephalomyocarditis virus. Newer IRES elements appear to be considerably more efficient (Chappell et al. 2000; Wang et al. 2005a). This cassette design is usually used to coexpress a transgene of interest and a marker gene that confers resistance to a toxic drug, such as puromycin, hygromycin, or neomycin, or a fluorescent protein that allows easy identification of transduced cells. Multimodal proteins that allow fluorescence and bioluminescence imaging have also been developed (Wurdinger et al. 2008; Tannous 2009).



In the example above, a single transgene encodes several proteins separated by 2A sequence/sec. The polyprotein self-processes into individual proteins during translation. Multiple proteins can be expressed from the same polyprotein (e.g., four transcription factors necessary to generate iPS cells [Sommer et al. 2009] and the simultaneous expression of antibody heavy and light chains [Fang et al. 2005]).



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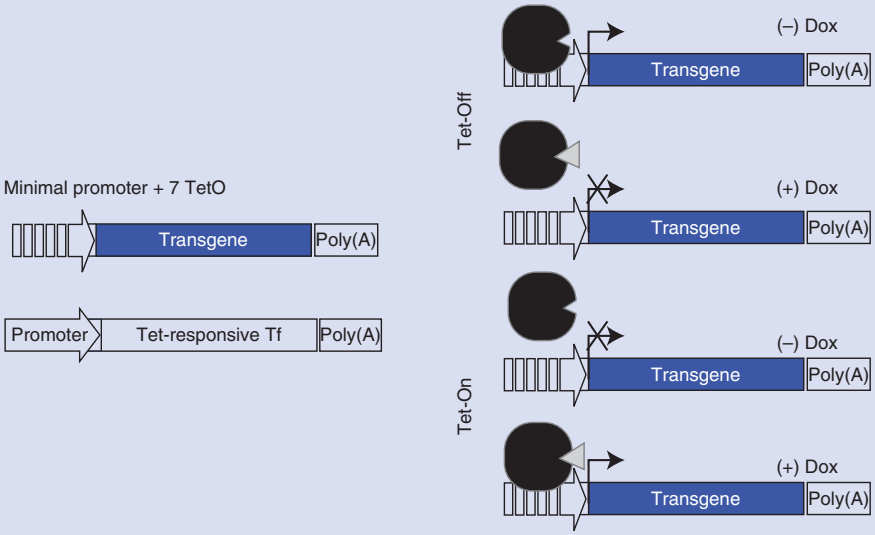
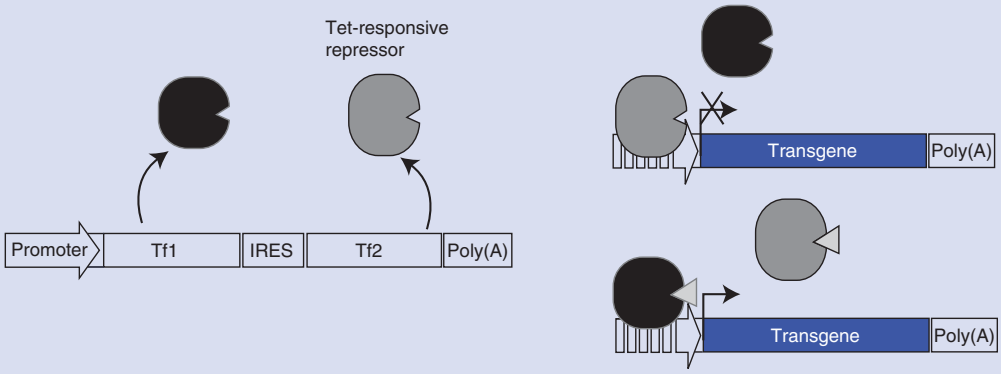
This system uses a bipartite transcription factor complex to regulate gene expression. One plasmid/vector carries a transgene under an inducible promoter composed of 12 ZFHD1-binding sites upstream of a minimal interleukin-2 promoter. Another plasmid/vector is bicistronic and encodes two fusion proteins that function as a transcription activator and a DNA-binding protein. The transcription complex forms in the presence of rapamycin via dimerization of human FKBP12 and FKBP-rapamycin-associated protein (FRAP) domains. The transcription activator (Tf1) consists of the FRB domain of human FRAP fused to an activation domain derived from the p65 subunit of human NF-κB. The DNA-binding fusion protein (Tf2) consists of the ZFHD1 DNA-binding domain fused to three copies of human FKBP12 in tandem (Ye et al. 1999; Rivera et al. 1999).



Tetracycline-regulated gene expression was one of the first systems to be developed for application in mammalian cells/organisms and continues to be the most widely used system for drug-regulated expression in biology. It uses a bacterially derived tetracycline-responsive transcription factor, composed of bacterially derived tetracycline binding domain (TetR) fused to the HSV-1 VP16 transcriptional activation domain. Two transcription factors have been developed, tTA and rtTA, that bind the cognate tet operator (tetO) in the absence (Tet-Off system) (Gossen and Bujard 1992) or presence (Tet-On system) (Gossen et al. 1995) of tetracycline, or derivative antibiotics such as doxycycline. Optimized versions of these Tet-responsive transcription factors—namely, rtTA2^{S}-M2—display lower residual binding to tetO and maximal induction at lower concentrations of doxycycline (Urlinger et al. 2000).



This Tet-regulated system combines a Tet-responsive transcriptional silencer (tTS^{Kid}) with an rtTA transcriptional activator leading to tight control of gene expression over several orders of magnitude (Freundlieb et al. 1999). In the off state, there is active repression of transcription by tTS^{Kid}. This approach is important in the context of viral vectors where other enhancer elements present in the vector may transactivate the Tet-

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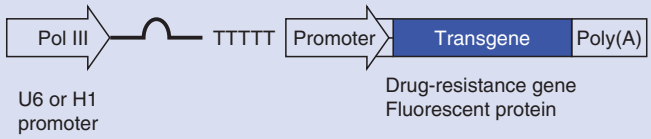
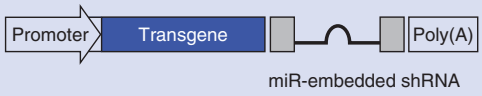
responsive promoter leading to high basal transgene expression. This approach has been successfully used to regulate gene expression in different viral vector systems (Pluta et al. 2005; Candolfi et al. 2007).



The most common approach for viral vector-based RNA interference (RNAi) is based on using RNA polymerase III (Pol III) promoters such as U6 and H1 (Sibley et al. 2010). The shRNA is followed by a stop sequence composed of a string of five Ts. The shRNA is processed by Dicer, which removes the 6-bp loop, leaving a 21-bp double-stranded molecule with 2-bp overhangs. Incorporation of tetO sequences into H1 or U6 allows for drug-regulated shRNA expression (Pluta et al. 2007).



Expression of shRNA in the context of a miRNA precursor molecule inserted in the 3′ UTR of a transgene expression cassette (Stegmeier et al. 2005) appears to have less toxicity in vivo (McBride et al. 2008; Boudreau et al. 2009). In addition, this design is compatible with RNA Pol II promoters used in the vast majority of transgene expression cassettes to accomplish numerous goals such as tissue-specific expression and developmentally or drug-regulated expression.

backbone of the vector, transfecting cells, harvesting the recombinant virus, generating and purifying a high-titer stock, and then confirming the genetic structure of the recombinant structure (best done by DNA sequencing). The entire process requires a wide range of skills and takes a minimum of a few weeks, even for people with experience in animal virology. First-time users of animal virus vectors might want to consider using one of the commercial kits that are available from several companies for the construction, purification, and titration of recombinants constructed in adenovirus vectors, lentivirus vectors, and adeno-associated viral vectors.

THE MAJOR TYPES OF VIRUSES CURRENTLY USED AS VECTORS

The power of mammalian virus vectors was amply showed in the early 1980s when SV40 was used to express high levels of the hemagglutinin gene of influenza virus (Gething and Sambrook 1981). The nascent subunits of the hemagglutinin were synthesized in large quantities, translocated into the endoplasmic reticulum, glycosylated, assembled into mature trimers, and transported efficiently to the cell surface. Since then, mammalian viruses of almost every type have been used as vehicles to express a wide variety of proteins. Some of them, including SV40, whose genome is too small to accommodate transgenes larger than a couple of kilobases, have limited applicability; others are still works in progress. But during the past decade, robust vectors have been developed from four types of viruses (adenoviruses, AAVs, lentiviruses, and retroviruses) that can satisfy most experimental needs. These four vectors differ dramatically in their biology and modus operandi. Adenoviruses and AAVs infect the host cells efficiently as episomal forms and express large quantities of the protein(s) encoded by their cargo of transgenes. Lentiviruses and retroviruses, in contrast, integrate the DNA form of their genomes into the host cell genome. These vectors, which do not kill the host cell, are used for stable transduction of mammalian cells. Although commercial kits have mitigated much of the grunt work involved in using these vectors, prior experience in culturing animal cells and in handling viral stocks remains a prerequisite.

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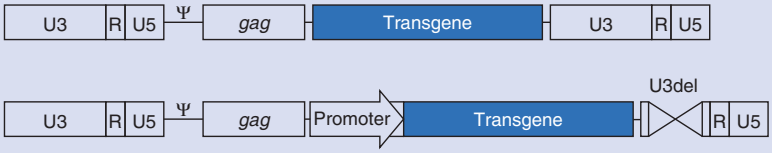
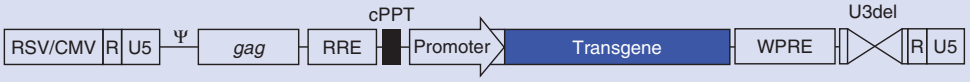
BOX 3. BASIC ELEMENTS IN VIRAL VECTORS

Retrovirus Vectors



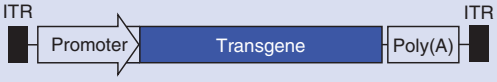
The most commonly used retrovirus vectors are based on the Mo-MLV. The first generation of vectors carried the transgene flanked by two intact LTRs composed of the enhancer (U3), R (defined as the start of transcription and present at both ends of the vector genome in the virion), and U5 region. Packaging of vector genome in virions is mediated by the packaging signal sequence (Ψ), but the adjacent *gag* sequence enhances its efficiency quite considerably. All retrovirus vectors carry an extended packaging signal that includes the Ψ sequence and part of the *gag* sequence, which has been modified to eliminate the start codon. In the first generation of these vectors, the LTR promoter was used to drive transgene expression (top vector in figure above). One of the issues with these vectors was promoter shutdown over time in vivo, and this spurred development of a new generation of SIN retrovirus vectors carrying a 3′-LTR with a nearly complete deletion of the U3 enhancer sequences and an internal mammalian promoter to drive transgene expression. During reverse transcription, the deleted U3 region at the 3′ end of the vector genome is duplicated to the 5′-LTR and thus generates an inactive LTR promoter.

Lentivirus Vectors



The vast majority of lentivirus vectors currently used in the laboratory and for gene therapy applications in humans are derived from HIV-1, and as such, their design features reflect safety considerations. These vectors carry an extended packaging signal (Ψ+*gag*) as in retrovirus vectors, are self-inactivating (U3-deleted 3′-LTR), and carry a chimeric 5′-LTR with enhancer sequences derived from either RSV or CMV immediate-early gene promoters. This type of design was also previously implemented in retrovirus vectors because it appeared to increase titers considerably. In addition, HIV-1 lentivirus vectors require the presence of the RRE, which is necessary for efficient transport of the vector genome to the cytoplasm and for efficient packaging. Similar to retrovirus vectors, lentivirus vectors also carry a polypurine tract (PPT) near the 3′-LTR, a site necessary to initiate plus-strand DNA synthesis during reverse transcription. One of the puzzling findings with the first generation of lentivirus vectors carrying a single PPT was the observation that transduction of postmitotic neurons in the adult brain was quite effective but not in adult nondividing hepatocytes (Park et al. 2000). Soon thereafter, it became apparent that a second PPT in a central location in the vector genome was key for efficient nuclear translocation of the preintegration complex in postmitotic cells (Follenzi et al. 2000; Zennou et al. 2000). Incorporation of the central PPT (cPPT), or central DNA flap, resulted in lentivirus vectors capable of transducing dividing and nondividing cells at high efficiency. Another element that is now part of most lentivirus vectors is the hepatitis virus woodchuck posttranscriptional regulatory element (WPRE), which has been shown to increase transgene expression fivefold to eightfold when placed downstream from the transgene in the sense orientation (Zufferey et al. 1999).

AAV Vectors



The majority of AAV vectors carry a transgene expression cassette flanked by AAV2 ITRs. No other genetic elements from the wild-type virus are present in the vectors. ITR elements from other AAV serotypes have been used as well (Desmaris et al. 2004; Hewitt et al. 2009). The major limitation to transduction by ssAAV vectors is their conversion to transcriptionally active dsDNA genomes in transduced cells. Self-complementary or dsAAV vectors carry an ITR with a deletion of the terminal resolution site that results in

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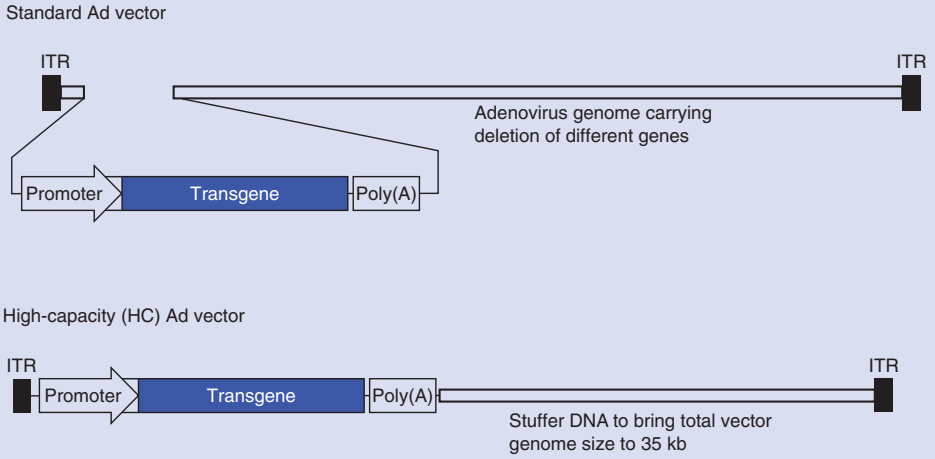
the packaging of double-stranded genomes that rapidly mediate transgene expression after transduction. The total packaging capacity of dsAAV vectors is ~2.4 kb, compared with 4.7–4.8 kb for traditional ssAAV vectors. The strength of these vectors remains their remarkable efficacy and stability of gene expression in vivo, which is not reproduced in cultured cells where other vectors are considerably more effective.

Adenovirus Vectors



Most adenovirus recombinants are based on Ad5 and carry a transgene expression cassette inserted in place of the E1 early transcriptional unit in the context of a mostly wild-type genome with one or more deletions of the other early transcriptional units. High-capacity adenovirus (HC-Ad) vectors carry only the adenovirus ITRs, a transgene expression cassette, and stuffer DNA to bring the total size to ~35 kb. These HC-Ad vectors do not carry any additional genetic elements from the wild-type adenovirus genome, and they do not have the same immunological complications associated with Ad-mediated in vivo gene delivery resulting from an adaptive response. Innate immunity to the capsid is likely to remain an issue.

Recombinant Adenovirus Vectors

The molecular biology of adenoviruses is known in great detail: The viruses are easy to handle and grow to high titer (10^9–10^{10} plaque-forming units [pfu]/mL of medium), they infect both dividing and nondividing cells, replication of the virus does not involve integration of the viral DNA into the cell's genome, and the viral particles can comfortably accommodate ~36 kb of DNA. With such attractive qualities, it is not surprising that adenoviruses have become vectors of choice for both florid expression of eukaryotic proteins and experimental forms of gene therapy (for review, see Hitt and Graham 2000; McConnell and Imperiale 2004; Palmer and Ng 2008; also see Box 4).

The more than 50 serologically different adenoviruses that infect humans can be classified into six species, A–F, based on antigenic relationship, oncogenicity, DNA homology, G/C content, and the patterns of the cleavage by restriction enzymes, and other properties. Virtually all adenovirus vectors are based on two closely related serotypes, adenovirus types 2 and 5, which belong to species C (Fig. 1). The molecular biology of these serotypes was worked out in the 1970s and 1980s after it became possible to dissect their genomes with restriction enzymes and hence to map biological functions and transcripts to specific sectors of the ~36-kb linear viral DNA. The DNA sequences of both of these viruses are available at GenBank.

First-Generation Recombinant Adenovirus Vectors

Particles of adenovirus 2 and 5 can, at most, accommodate ~37 kb of double-stranded DNA (dsDNA). The E3 region (78.5–84.3 map units) is not required for viral growth in cultured cells. E3-deleted mutants are viable and can accept DNA segments of ~4.4 kb in length, which is large

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BOX 4. ADENOVIRUS VECTORS

Adenoviruses belong to the Adenoviridae family of viruses, which consists of two genera: *Aviadenovirus* (bird adenoviruses) and *Mastadenovirus* (human, simian, bovine, equine, porcine, ovine, canine, and opossum adenoviruses). All of these viruses carry a linear dsDNA genome inside an icosahedral protein capsid, 70–100 mm in diameter. The ~36-kb genome is organized into four early transcription units (E1, E2, E3, and E4) to provide regulatory and replication functions, two delayed early units (IX and IVa2), and one major late transcription unit, which directs synthesis of capsid proteins, among others (Wold and Horwitz 2007). Clinical sequelae resulting from adenovirus infection in humans are primarily acute febrile respiratory syndromes (Wold and Horwitz 2007).

Adenovirus vectors based on human adenovirus serotypes 2 and 5 are the most widely used in the laboratory because of their broad cell-type and tissue tropism and excellent gene-transfer efficiency in culture and in vivo. In fact, these are the most efficient gene-transfer vehicles among dsDNA viral vectors (Amalfitano 2004). Most adenovirus vectors are rendered replication defective by deletion of the E1 region, to accommodate the transgene expression cassette. These vectors are routinely packaged in 293 cells, which carry a stably integrated E1 region of adenovirus serotype 5 that *trans*-complements the E1 functions necessary for replication and packaging of vector genomes. Adenovirus vector genomes reside in most target cells as nonreplicating episomes (Wold and Horwitz 2007).

The major drawback of using adenovirus vectors for somatic gene transfer in vivo is their ability to induce strong cellular immune responses directed against viral proteins and transgene products (Jooss and Chirmule 2003). Administration of an adenovirus vector elicits robust immune responses in the host on two levels: innate immunity directed to the viral capsid and adaptive T-cell immunity toward de novo expression of the remaining viral genes and transgene in the vector genome. Stimulation of innate immunity by viral capsid protein results in transient elevation of inflammatory cytokines such as IL-6 and IL-10, whereas adaptive T-cell immunity leads to clearance of transduced cells by cytotoxic T lymphocytes specific for viral and transgene proteins (Jooss and Chirmule 2003).

During the past decade, significant progress has been made in the development of new generations of adenovirus vectors with diminished T-cell immunogenicity by deletion of additional early genes in the vector genome. The most advanced generation of adenovirus vector is the gutless vector, in which all viral genes are void. The gutless adenovirus vector not only eliminates the cellular immune responses against de novo-synthesized viral proteins but also significantly expands its capacity for the transgene cassette. Because production of a gutless vector requires helper functions from coinfected E1-deleted adenovirus, it is critical to remove the helper virus efficiently from the gutless vector preparations (Altaras et al. 2005). It is worthwhile to note that there has been little progress in overcoming the innate immune response elicited by the viral capsid after adenovirus vector-mediated in vivo gene transfer.

enough to accommodate a small expression cassette but too small for most cloning projects. However, the cloning capacity of type C adenoviruses can be increased by deleting both the nonessential E3 region and the essential E1A region, which codes for a set of immediate-early proteins that activate transcription of the three other early regions (E2–E4; see Fig. 1). Mutants carrying deletions of E1 grow poorly or not at all in standard lines of cultured cells, rendering the virus replication defective. However, the E1 region becomes nonessential when the virus is propagated in the 293 or 911 helper lines of human cells that have been engineered to express the E1 region in *trans*. E1/E3 double-deleted recombinant adenoviruses can accommodate transgenes that are ~6.5 kb in length—enough for an expression cassette of average size. Although recombinants constructed in E1/E3 deletion mutants do not grow quite as well as wild-type adenoviruses, they can usually be propagated in permissive cell lines to a titer of 10^8–10^9 pfu/mL. The viral particles of E1/E3 deletion mutants are sufficiently robust to withstand the rigors of purification by standard methods (e.g., centrifugation through cesium chloride [CsCl] density gradients [Gerard 1995]). E1/E3 double mutants have become the adenovirus vectors of choice for production of many recombinant proteins because of their ease of handling and reasonable cloning capacity. Many first-generation vectors that differ from one another in detail have been described in the academic literature over the years; others are available commercially (for review, see Danthinne and Imperiale 2000).

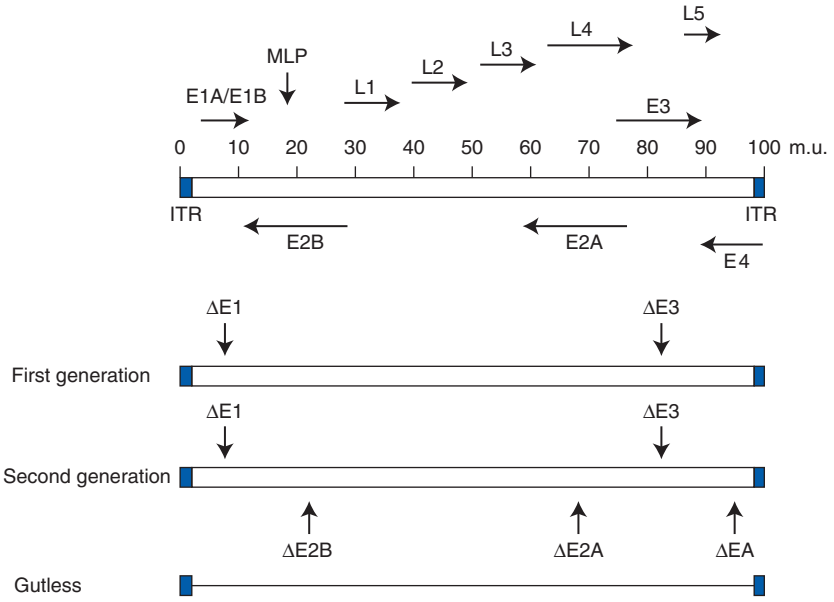

FIGURE 1. Transcription map of human adenovirus serotypes 2 and 5. (*Top*) The viral genome (~36 kb) is represented as a horizontal line with map units (m.u.). Transcription units are shown as arrows indicating direction of transcription. There are four early transcription units (E1–E4) and five families of late RNAs (L1–L5), which are the alternately spliced products of a common precursor expressed from a single major late promoter (MLP). The termini of the double-stranded genome consist of 103-bp inverted repeats (ITR) that are involved in viral DNA replication. Signals for encapsidation of the viral DNA during assembly of viral particles are located 190–380 nt from the left-hand end of the viral genome. (*Bottom*) Diagrams of the structures of three generations of adenovirus vectors (first, second, and gutless). (Downward arrows) Viral regions that are deleted in first- and second-generation vectors. All second-generation vectors lack early regions E1 and E3; some vectors also lack early regions E2A and E4. The only viral DNA sequences retained in gutless adenovirus vectors are the ITR and packaging signals. (Redrawn from Wu et al. 2001, with permission from Bentham Science Publishers Ltd.)

Second-Generation and Third-Generation (Gutless) Recombinant Adenovirus Vectors

Second-generation vectors lack three (E1, E2, and E3 or E1, E3, and E4) of the viral regions that are expressed early after infection (see, e.g., Gao et al. 1996; Amalfitano et al. 1998; Lusky et al. 1998; Moorhead et al. 1999). Although these mutants have a greater carrying capacity than first-generation vectors, they retain the ability to induce inflammatory and cytotoxic responses that lead to the elimination of the virally transduced cells from the body. To minimize this problem, recombinant adenovirus vectors that lack all viral genes have been generated. These vectors express transgenes in vivo to a high level for extended periods of time and have a greatly expanded cloning capacity (~36 kb) and a greatly reduced ability to stimulate the immune system (Schiedner et al. 1998; Kochanek et al. 2001). However, production of helper-virus-free gutless vectors is more challenging when compared with that of earlier generations of adenovirus vectors. For further information about the construction and use of these gutless adenovirus vectors, see Parks et al. 1996; Kochanek et al. 2001; Alba et al. 2005; Palmer and Ng 2008.

Expression cassettes can be cloned into the DNA of first-generation adenovirus vectors by ligating the expression cassette directly into plasmids (see, e.g., He et al. 1998; Mizuguchi et al. 2001) or cosmids (Giampaoli et al. 2002) carrying a full-length copy of an E1-deleted vector technique (see Protocol: **Construction of Recombinant Adenovirus Genomes by Direct Cloning** [Zhou et al. 2019]).

Expression cassettes can also be introduced into the backbone of an adenovirus vector by recombination in either mammalian cells or *E. coli*. In mammalian cells, recombinants are generated in cells that express an integrated copy of the adenoviral E1 region and are permissive to viral growth (see Table 1). These helper cells are cotransfected with (1) the genomic DNA of an adenovirus E1/E3 deletion and (2) a plasmid carrying an expression cassette flanked on both sides by tracts of DNA

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homologous to viral DNA sequences immediately upstream of and downstream from the E1 deletion. Homologous recombination between the plasmid and the viral DNA transfected into the helper cell line generates a recombinant in which the E1 region is replaced by the expression cassette (see Fig. 2).

Homologous recombination between the E1 sequences in the expression plasmid and the vector DNA results in formation of a recombinant with the expression cassette integrated into the E1 region of the vector. The recombinant virus particles are then plated on helper cells and the viral DNAs in individual plaques are screened by polymerase chain reaction (PCR) and/or cleavage with restriction enzymes. However, wild-type adenoviruses, generated by recombination between the vector and viral DNA sequences integrated into the genome of the helper cells, may also be present in the viral yield. Removing contaminating wild-type adenoviruses from the recombinant stock requires at least two rounds of plaque purification and screening. The frequency of wild-type contamination can also be reduced, but not entirely eliminated, by using cell lines such as 911 that carry a smaller segment of the adenoviral genome than 293 cells.

Constructing, purifying, and generating a high-titer stock of an adenovirus recombinant, by either direct in vitro insertion of an expression cassette into a second-generation vector or in vivo recombination, is a lengthy process that, even in experienced hands, may take several weeks to complete. If only one or two recombinants are required, it may be more effective, both for time required and for cost, to use a local core facility or a commercial kit. Commercial recombination systems are available from Clontech (AdenoX system) and Qbiogene (AdEasy system).

If your laboratory will have an ongoing need to produce adenovirus recombinants, the best option in the long term may be to establish an advanced in vitro recombination system or ligation-based direct cloning system for reduced wild-type contamination, using, for example, the *E. coli* or cre-loxP recombination systems (Chartier et al. 1996; He et al. 1998; Luo et al. 2007; Reddy et al. 2007) or standard molecular cloning techniques to directly insert transgene expression cassettes into the deleted E1 locus in a clone of infectious recombinant adenovirus genome (Mizuguchi and Kay 1998; Mizuguchi et al. 2001; Gao et al. 2003). The latter method is described in detail in Protocol: **Construction of Recombinant Adenovirus Genomes by Direct Cloning** (Zhou et al. 2019).

Adeno-Associated Viral Vectors

At first sight, AAVs seem to have few of the qualities required of a good vector. Their single-stranded DNA genomes are small (4.7 kb), and their replication depends on coinfection of cells with a helper virus (e.g., adenovirus, herpes simplex virus, papilloma virus). In the absence of helper functions—for example, those supplied by adenoviral proteins encoded by early regions E1, E2a, VA RNA, and E4—the wild-type AAV genome enters a state of latency by integrating at a single specific locus (19q13.3-qter) in the genome of cultured human cells. Recombinant AAV vectors express transgenes at high level in target tissues for extended periods of time, in some instances remaining unchanged for >10 yr. Other advantageous qualities include a lack of pathogenicity, an ability to infect a broad range of cell types (both proliferating and quiescent), and a capacity to be rescued from an integrated state by

TABLE 1. Examples of helper cell lines that support the growth of adenovirus E1 mutants

Name of cell line	Description	Reference(s)
293	Generated by transforming normal human embryonic kidney cells with sheared adenovirus 5 DNA. The transformed cells contain 4.5 kb from the left-hand end of the adenovirus 5 genome integrated into human chromosome 19 of the HEK cells.	Graham et al. 1977; Louis et al. 1997
911	Generated by transforming human embryonic retinoblasts with a plasmid containing sequences from the left-hand end of adenovirus 5 (bp 79–5789 of the Ad5 genome). Claimed to be better than 293 cells (faster plaque formation [4–5 d rather than 5–8 d] and higher virus yields).	Fallaux et al. 1996
N52.E6	Generated by transforming primary human amniocytes with adenovirus 5 E1 DNA.	Schiedner et al. 2000
PER.C6	Contains the E1A and E1B genes of adenovirus 5 under the control of the human phosphoglycerokinase promoter. Yields of superinfecting adenoviruses are equivalent to the yields from 911 cells.	Fallaux et al. 1998

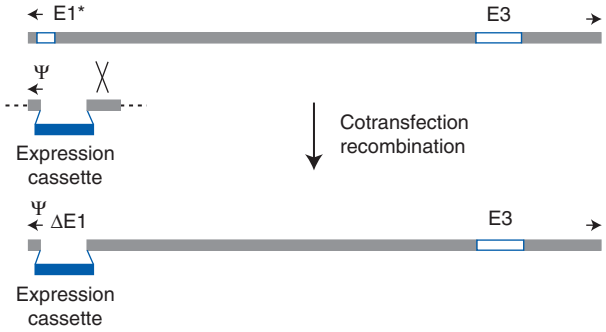

FIGURE 2. Construction of first-generation recombinant Ad vectors. E1-complementing cells (e.g., 293 cells) are cotransfected with a shuttle plasmid containing a transgene expression cassette flanked by sequences derived from the "left" end of the adenovirus genome together with genomic adenovirus DNA. The E1 region ("E1") of the genomic DNA, which can be either viral or plasmid derived, should be modified to reduce infectivity of the parental genome. Homologous recombination between the shuttle and genomic sequences results in a first-generation vector with the expression cassette replacing E1. (Gray bars) adenovirus sequences, (blue bars) heterologous DNA, (white bars) sequences of the adenovirus genome that are not required for the construction of first-generation vectors in E1-complementing cell lines. (Redrawn from Hitt and Graham 2000, with permission from Elsevier.)

superinfection with a helper virus or by transfection with plasmid vectors that supply helper functions in *trans* (for review, see Büning et al. 2008; Daya and Berns 2008; also see Box 5).

The majority of AAV recombinants are generated in AAV serotype 2, whose linear genome, which is 4675 nucleotides in length (Srivastava et al. 1983), consists of a long, single-stranded coding region bracketed between identical but inverted 145-bp terminal repeats. Each repeat can fold into a T-shaped, inward-facing, hairpin-like structure that contains information required to prime synthesis of a complementary-strand DNA. The product is a monomer-length duplex, one end of which is covalently closed. This cross-linked structure creates a replication origin that, after activation by the viral Rep protein, generates a rolling hairpin structure, which allows the replication fork to shuttle up and down the length of the viral genome. Progeny genomes are excised by the introduction of specific nicks at the closed circular terminus of each full-length duplex copy of the viral DNA. Both sense and antisense single-stranded viral DNAs are packaged efficiently into viral particles. For further information, see Cotmore and Tattersall (1996).

The coding region of the viral genome contains two open reading frames (*rep* and *cap*). *rep* encodes four proteins: (1) Rep 78, (2) its spliced variant Rep 40, (3) Rep 68, and (4) its spliced variant Rep 52 (see Fig. 3). *cap* codes for VP1, VP, and V3, the three viral capsid proteins, which differ in amino acid sequence only at their amino termini. Differences among the amino acid sequences of the capsid proteins account for the specific tissue tropisms displayed by different AAV serotypes and define the epitopes recognized by the host's immune system.

Construction of rAAV vectors is performed in standard plasmids by simple molecular cloning methods. rAAV vectors do not encode the Rep proteins necessary for integration and, as a result, long-term transgene expression depends on the persistence of extrachromosomal concatamers of vector genomes. For a discussion of in vivo applications of AAV vectors, see the In Vivo Expression section.

Retroviruses

Retroviruses have a long history as vectors for introducing and expressing ectopic genes in cultured mammalian cells and as exploratory vehicles for gene therapy. In retroviral particles, the viral genome is complexed with a nucleocapsid protein within a lipid envelope that is studded by viral glycoprotein protein. Interactions between the viral glycoprotein and receptors on the cell surface determine the tropism of the virus. The viral genome consists of two identical copies of single-stranded RNA (ssRNA) that are 8–10 kb in length. The viral RNA replicates through a DNA intermediate that becomes stably integrated into the genome of infected cells via a recombination reaction catalyzed by the virally coded integrase. Production of progeny viral particles requires transcription of the

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BOX 5. AAV VECTORS

Adeno-associated virus, the first and only ssDNA virus that has been engineered for the purpose of gene delivery, is a nonenveloped, icosahedral particle, 20–26 nm in diameter, encapsidating a linear ssDNA genome of 4.7 kb. Adeno-associated virus is one of the smallest mammalian viruses known, named after its initial discovery as a viral contaminant in adenovirus preparations. It is naturally replication defective in the absence of coinfection with helper viruses such as adenovirus and has not been associated with any disease in humans (Daya and Berns 2008).

Adeno-associated virus was first genetically engineered as a gene-delivery vector in the late 1980s. Since then, its vectors have become the most promising gene-delivery vehicle for effective and safe clinical application in gene therapy for chronic diseases (Daya and Berns 2008). Extensive studies have shown that AAV vectors transduce at exceptionally high efficiency dividing and nondividing/quiescent cells in a number of somatic tissues, including muscle, liver, heart, retina, and CNS. Importantly, adeno-associated virus-mediated gene delivery in vivo appears to be devoid of histopathological alterations or vector-related toxicity (Daya and Berns 2008).

Unlike adenoviral vectors, in vivo administration of AAV vectors in small and large animal models usually does not elicit host immune responses against transduced cells, which leads to long-term in vivo expression of the transgenes (therapeutic genes) (Jooss and Chirmule 2003). AAV vectors mediate stable gene expression by forming circular monomers and concatamers for episomal persistence in host cells (McCarty et al. 2004). A major drawback of adeno-associated virus is that it can accommodate vector genomes only up to ~5 kb (Daya and Berns 2008).

Recent investigations have revealed a diverse family of more than 120 novel primate adeno-associated viruses with unique tissue and cell-type tropisms and efficient gene-transfer capability (Gao et al. 2002, 2003, 2004, 2005). This critical advance in AAV technology has dramatically broadened the potential applications of AAV vectors. Another critical development in vector genome design is the incorporation of one wild-type ITR element and one modified ITR element to allow packaging of double-stranded or self-complementary genomes (McCarty 2008). These self-complementary AAV (scAAV) vectors are capable of initiating transgene expression immediately after capsid uncoating in the nucleus of targeted cells. This elegant vector genome design bypasses the rate-limiting step of AAV vector genome processing in the transduction process—the second-strand synthesis (SSS). This design enhances dramatically the transduction efficiency of AAV vectors both in culture and in vivo. However, the scAAV vector genome design reduces the transgene capacity to 2.5 kb, restricting its potential applications. Recently, Zhong and colleagues documented that mutations of critical surface-exposed tyrosine residues on AAV2 capsids circumvents the ubiquitination step, thereby avoiding proteasome-mediated degradation. This simple modification results in high-efficiency transduction by these vectors in human cells in vitro and murine hepatocytes in vivo (Zhong et al. 2008).

integrated viral DNA by host-encoded RNA polymerase II (Pol II). The resulting mRNA is processed, transported, and translated using machinery translation of the host cell. Progeny viral particles are assembled at, and bud through, the cell surface.

All retroviral genomes contain at least three genes (see Fig. 4). In murine retroviruses, these are the *gag* gene that encodes the Gag polyprotein that is cleaved into the viral matrix, capsid, and nucleocapsid proteins; the *pol* gene that encodes a protease, reverse transcriptase, and integrase; and the *env* gene that encodes a polyprotein that is cleaved by the viral protease into a surface glycoprotein (gp70) and a transmembrane protein (p15E).

The regions of the viral genome that regulate DNA synthesis and transcription are clustered in the two LTRs and consist of three functional regions, as shown in Table 2. The region downstream from the 5′-LTR contains a binding site for the transfer RNA that serves as a primer for initiation of DNA synthesis by reverse transcriptase. Finally, the sequences needed for packaging of the viral RNA are located between the primer-binding site and the *gag* open reading frame.

Most retroviral vectors in current use are derived from the Moloney strain of murine leukemia virus (Mo-MLV), which is amphotropic and has the ability to infect both murine and human cells. Recombinants are generated by replacing the viral *gag*, *pol*, and *env* genes in a cloned copy of the vector genome with an appropriately designed transgene. The maximum size of the transgene that can be

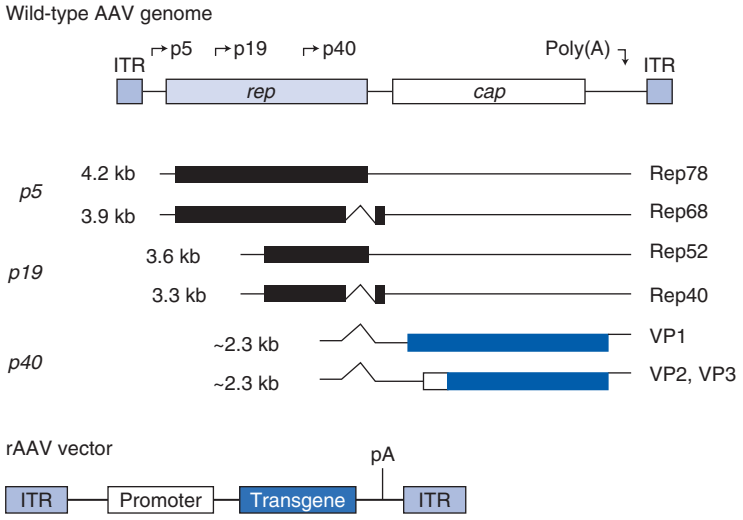

FIGURE 3. Structure of adeno-associated virus (AAV) vectors. The wild-type AAV consists of the viral genes *rep* and *cap* coding for the different rep (Rep78, Rep68, Rep52, Rep42) and cap (VP1, VP2, VP3) proteins, the AAV promoters (p5, p19, p40), the polyadenylation site (pA), and the inverted terminal repeats (ITR). In rAAV vectors, the viral *rep* and *cap* genes are replaced by a transgene cassette carrying the promoter, the transgene, and the pA site. (Redrawn from Walther and Stein 2000, with permission from Wolters Kluwer Pharma Solutions, Inc.)

efficiently cloned in a typical retroviral vector is ~6.5 kb. Expression of the transgene is driven either by the natural Mo-MLV control elements in the upstream LTR or by an interval promoter (e.g., the CMV promoter) cloned along with the transgene.

To generate a stock of recombinant virus, the DNA of the retroviral recombinant constructed in vitro is transfected into a packaging cell line that expresses the three viral genes necessary for particle formation and replication: *gag*, *pol*, and *env*. The backbone of the vector provides the signals (ψ) required in *cis* for packaging of the recombinant genomes into viral particles. The viral Env protein expressed by the packaging cell line determines the host range of the progeny viral particles. For example, the EcoPack 2-293 cell line sold by Clontech generates ecotropic recombinant viruses that can infect only rodent cells. In contrast, other cell lines, such as AmphoPack-293 cells and RetroPack PT67 cells (both Clontech), generate amphotropic or dual-tropic viruses, respectively, with the ability to infect a broad range of mammalian cell types. If necessary, the host range of the recombinant virus can be extended still further by producing the recombinant virus in a cell line (e.g., GP2-293 [Clontech]) that expresses the G glycoprotein of the vesicular stomatitis virus (VSV), which mediates viral entry through lipid binding and plasma membrane fusion rather than by attachment to a specific cell-surface receptor.

The virus stocks generated in helper cells are used to transduce actively dividing cultures of target cells that are of interest to the researcher. In contrast to lentiviruses (see below), recombinant murine retroviruses can transduce only actively dividing cells. This is because the murine viral preintegration complex cannot enter the nucleus of a nondividing cell. Once the recombinant provirus penetrates the nucleus, the viral genome integrates into the cellular genome, enabling long-term expression of the transgene. Each transduced cell carries the transgene at a different chromosomal location because integration is not site- or sequence-specific. The realization that integration of recombinant murine viral genomes at chromosomal sites close to cellular oncogenes (Montini et al. 2009) could lead to proliferation of clonal populations of T cells in patients treated by gene therapy for X-linked severe combined immunodeficiency (SCID-XI) (Hacein-Bey et al. 2001) is a considerable obstacle to the therapeutic use of recombinant murine retroviruses.

Lentiviruses, a genus of the Retroviridae family, are more complicated than murine retroviruses. In addition to the three canonical genes (*gag*, *pol*, and *env*), their genome encodes at least six additional proteins (tat, rev, vpr, vpu, nef, and vif) that perform a variety of regulatory functions involved in viral pathogenicity. However, lentiviral vectors derived from the human immunodeficiency virus type 1

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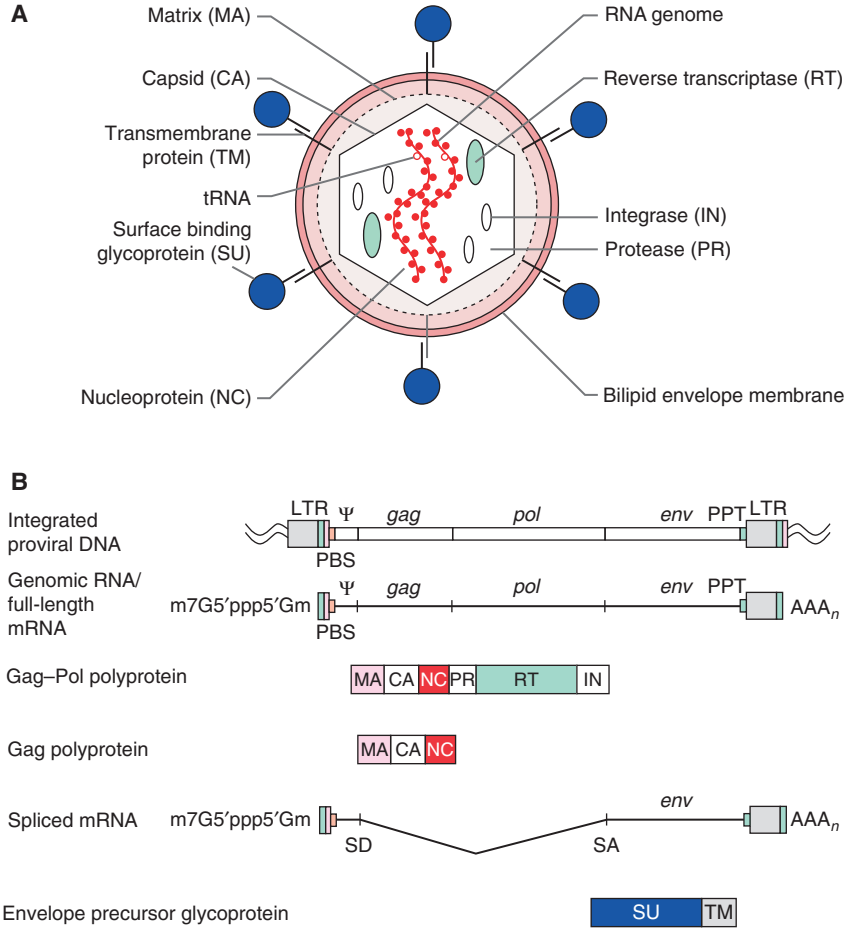

FIGURE 4. Retroviral particle and genome structure. (*A*) Retrovirus particle showing approximate location of its components using standardized two-letter nomenclature for retroviral proteins. (*B*) Genome organization and gene expression pattern of a simple retrovirus showing the structure of an integrated provirus linked to flanking host cellular DNA at the termini of its LTR sequences (U3-R-U5) and the full-length RNA that serves as genomic RNA and as mRNA for translation of the *gag* and *pol* open reading frames (ORFs) into polyproteins. *env* mRNA is generated by splicing and encodes an Env precursor glycoprotein. (LTR) (U3-R-U5) for proviral DNA, derived from R-U5 downstream from 5′ cap and U3-R upstream of 3′ poly(A) in genomic RNA; (PBS) primer binding site; (Ψ) packaging signal; (PPT) polypurine tract; (SD) splice donor site; (SA) splice acceptor site. (Redrawn from Pedersen and Duch 2003, with permission from Wiley.)

(HIV-1) have two advantages over murine retrovirus vectors: (1) Lentiviruses stably integrate the viral genome into the genomes of both dividing and nondividing cells (Naldini et al. 1996a,b) and (2) the genotoxic potential of lentiviruses appears to be considerably lower than that of murine retrovirus (Montini et al. 2009).

Recombinants constructed in lentivirus vectors can be used as simple expression plasmids for transient transfection of cells in culture or they can be packaged into high-titer viral stocks and used to

TABLE 2. Regions of the viral genome that regulate DNA synthesis and transcription are clustered in the two LTRs that consist of three functional regions

Region of LTR	Functions
U3 (unique-3′)	Transcriptional promoters and enhancers
R (repeat)	Reverse transcription and replication; polyadenylation
U5 (unique-5′)	Initiation of reverse transcription

LTR, long terminal repeat.

genetically modify cells in culture or in vivo. In addition, lentiviruses can be used to generate transgenic animals by transduction of murine embryonic stem (ES) cells in culture (Pfeifer et al. 2002), or by injection of vector stocks into the perivitelline space of single-cell mouse embryos (Lois et al. 2002).

Exceptionally useful libraries to probe gene function and regulation have been constructed in lentivirus vectors:

- the RNAi Consortium small hairpin RNA (shRNA) library, where shRNAs expressed from a U6 promoter target most mouse and human genes (commercialized by Sigma-Aldrich and Open Biosystems)
- a microRNA (miRNA)-adapted shRNA (shRNAmir) library, where shRNAs are expressed in the context of a miRNA precursor (shRNAmir) from a RNA Pol II promoter (Open Biosystems)
- a library of miRNA precursors (Open Biosystems) or cDNAs (Open Biosystems; GeneCopoeia)

The great advantage of lentivirus-based libraries is that they dramatically accelerate the ability to perform genome-wide, high-throughput screens of primary cultures of cells such as neurons that might not be susceptible to transfection by traditional methods. Importantly, lentivirus vector transduction does not appear to significantly change the gene expression pattern of target cells (Cassani et al. 2009).

Examples of lentiviral expression systems that have been designed for specific purposes include:

- bicistronic vectors, in which the transgene is coexpressed with drug-selection genes or fluorescent proteins (or both) to allow for selection and easy identification of transduced cells (for review, see de Felipe 2002)
- polycistronic vectors that encode the four transcription factors necessary to generate induced pluripotent stem (iPS) cells from somatic cells along with green fluorescent protein (GFP) (Carey et al. 2009) (Cell BioSystems)
- vectors that allow for drug-regulated gene expression of cDNAs (Pluta et al. 2005), shRNAs (Szulc et al. 2006; Wiznerowicz et al. 2006) (Open BioSystems; Addgene), and shRNAmirs (Stegmeier et al. 2005) (Open Bio-Systems)

Many of these lentivirus vectors are available through companies such as Life Technologies, Open Biosystems, Cell Biolabs, and GeneCopoeia, or at Addgene.org, where plasmids are deposited for distribution to other academic institutions. Figure 5 shows an example of a basic lentivirus backbone deposited at Addgene that includes all of the elements necessary to achieve highly efficient transgene expression in cultured cells or in vivo. The vector shown, pRRLSIN.cPPT.PGK-GFP.WPRE, was among the first to incorporate all of the design features that enhance transduction and safety of lentivirus vectors (Follenzi et al. 2000). Many other lentivirus vectors carrying a variety of different promoters are also available through Addgene.

Lentivirus vectors can be used to generate mammalian cell lines that produce high levels of recombinant mammalian proteins. Because lentivirus vectors stably transduce target cells, enhanced levels of recombinant protein can be achieved by sequential rounds of transduction and selection. Using a bicistronic vector in which GFP (or any other fluorescent marker protein) and the transgene of interest are placed under the control of the same promoter, the brightest cells can be selected by fluorescent cell sorting and reinfected until the required level of production of the target protein is reached.

For many laboratories, lentivirus vectors are the best choice as mammalian vectors. They can be manipulated using standard recombinant DNA techniques, they can be used as simple expression plasmids, and the production of vector stocks is straightforward and quick, requiring only a simple cotransfection of 293 cells with helper plasmids and harvesting of the recombinant virus in the supernatant a few days later. Thus, lentivirus vectors may be the best system to rapidly express and screen large numbers of mutant recombinant proteins.

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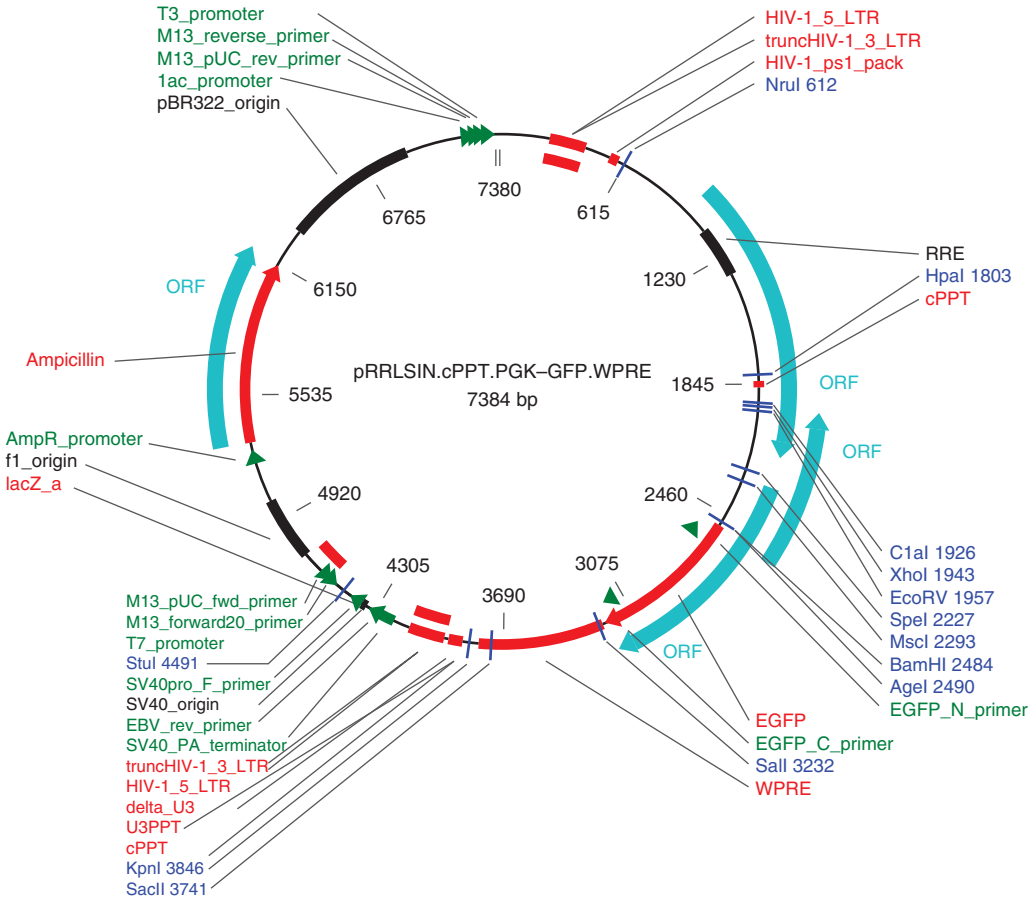

FIGURE 5. Vector pRRLSIN.cPPT.PGK-GFP.WPRE. Example of a lentiviral vector genome that includes all of the elements needed to achieve highly efficient transgene expression in cultured cells or in vivo. (Plasmid 12252 redrawn from Addgene, http://www.addgene.org/pgvec1?f=c&cmd=findpl&identifier=12252, and Follenzi et al. 2000.)

IN VIVO EXPRESSION

Despite the rapidly increasing use of lentivirus for genetic modification of cells in culture and in vivo, these vectors still have some limitations that include their total transgene capacity (~8 kb) and restricted transduction after systemic gene delivery in vivo. Vector systems with considerably larger transgene capacity (e.g., high-capacity adenovirus vectors [Schiedner et al. 1998] and herpes simplex 1 amplicons [up to ~150 kb] [Geller and Breakefield 1988]), have been used to deliver entire genomic loci to target cells to achieve regulated gene expression (Wade-Martins et al. 2001). However, these vectors are extremely difficult to produce at high titers and rarely achieve long-term expression of target genes.rAAV vectors share many of the attributes of lentivirus vectors; AAV plasmids can be easily manipulated by standard recombinant DNA techniques and they can be used as conventional expression plasmids. However, the most useful properties of AAV vectors are their exceptional efficiency and duration of transgene expression in vivo, which, in many cases, appears to last for the life span of the experimental animal. In rhesus macaques that received an intramuscular injection of an AAV vector encoding a primate transgene, expression remained unabated 11 yr later (G Gao, pers. comm.). Moreover, the in vivo gene-delivery efficiency of AAV vectors is such that a single intravenous infusion in adult animals is sufficient to achieve nearly complete transduction of liver, heart, and skeletal muscle as well as transduction in the central nervous system (CNS) (Gregorevic et al. 2004; Wang et al. 2005b; Inagaki et al. 2006; Duque et al. 2009; Foust et al. 2009; Hester et al. 2009). Despite these extraordinary

properties, AAV vectors are not the best choice for transduction of dividing cells in culture because they do not integrate into the target cell genome and are slowly lost over time.

ADENOVIRUS VECTORS

Recombinant adenoviruses are generated by ligating the gene of interest into a plasmid carrying a replication-defective adenovirus genome. This method was developed by Mizuguchi and Kay (1998) as a simple and efficient ligation-based platform, and it is commercially available under the trade name Adeno-X Expression System (Clontech). Gao et al. (2003) further modified this system with a convenient green–white selection feature that facilitates the isolation of transgene-positive recombinant adenovirus clones. The ligation-based direct cloning system is considerably faster and more efficient than traditional methods for generating recombinant adenovirus via homologous recombination. Direct ligation, although quicker and more efficient, is technically more challenging, requiring proficient skills to manipulate the large sizes of plasmids (>36 kb). Homologous recombination usually generates a mixture of recombinants, including replication-competent adenovirus (RCA), removal of which requires several time-consuming rounds of plaque purification and characterization.

Production of a recombinant adenovirus simply requires linearization of the infectious clone by digestion with the endonuclease PacI, followed by transfection into human embryonic kidney 293 cells or other packaging cell lines (see Table 1) with the necessary ability to complement viral genes deleted during construction of the vector backbone. Figure 6 depicts the entire process for generating a recombinant adenovirus vector using direct cloning with the green–white selection method. The process starts with cloning the gene of interest into the shuttle plasmid pShuttle-*pk-GFP* (pSh-*pkGFP*), followed by another cloning step to transfer the transgene expression cassette from the shuttle plasmid into the adenovirus clone pAd-*pkGFP*. Both pSh-*pkGFP* and pAd-*pkGFP* plasmids carry a prokaryotic green fluorescent protein (GFP) expression cassette for easy selection of transgene-positive/GFP-negative bacterial colonies. The next steps are to linearize the recombinant adenovirus plasmid by PacI digestion to expose the inverted terminal repeats (ITRs), followed by transfection into 293 cells for rescue and expansion. Finally, purified high-titer recombinant adenovirus stocks are prepared by cesium chloride (CsCl) gradient sedimentation, followed by detailed characterization of vector genome structure, vector particle titer, and infectious titer, as well as testing for the presence of RCA.

Protocol: **Construction of Recombinant Adenovirus Genomes by Direct Cloning** (Zhou et al. 2019) provides step-by-step instructions for creating recombinant adenovirus by this method. Protocol: **Release of the Cloned Recombinant Adenovirus Genome for Rescue and Expansion** (Su et al. 2019a) describes the process of virus rescue and expansion, and Protocol: **Purification of the Recombinant Adenovirus by Cesium Chloride Gradient Centrifugation** (Su et al. 2019b) describes the purification of adenovirus vectors by gradient sedimentation. Protocol: **Characterization of the Purified Recombinant Adenovirus for Viral Genome Structure by Restriction Enzyme Digestions** (Su et al. 2019c), Protocol: **Measuring the Infectious Titer of Recombinant Adenovirus Using TCID50 End-Point Dilution and qPCR** (Lock et al. 2019a), and Protocol: **Detection Assay for Replication-Competent Adenovirus by Concentration Passage and Real-Time qPCR** (Lock et al. 2019b) are supporting protocols for detailed characterization of recombinant adenovirus stocks.

Strategic Planning

Selection of Vector Backbone and Corresponding Packaging Cell Line

Several critical factors need to be considered in choosing the backbone, including the intended target (e.g., cells in culture or tissues/organs in intact animals), the impact of vector immunogenicity on the outcome of gene transfer, vector toxicity, transgene size, and desired level of transgene expression (Table 3). For simple gene-transfer experiments in cell culture where a high level of transient transgene expression is desired, a backbone with an E1 deletion only or with an E1/E3 double deletion is ideal (see Box 3). However, for systemic administration of an adenovirus recombinant, where liver

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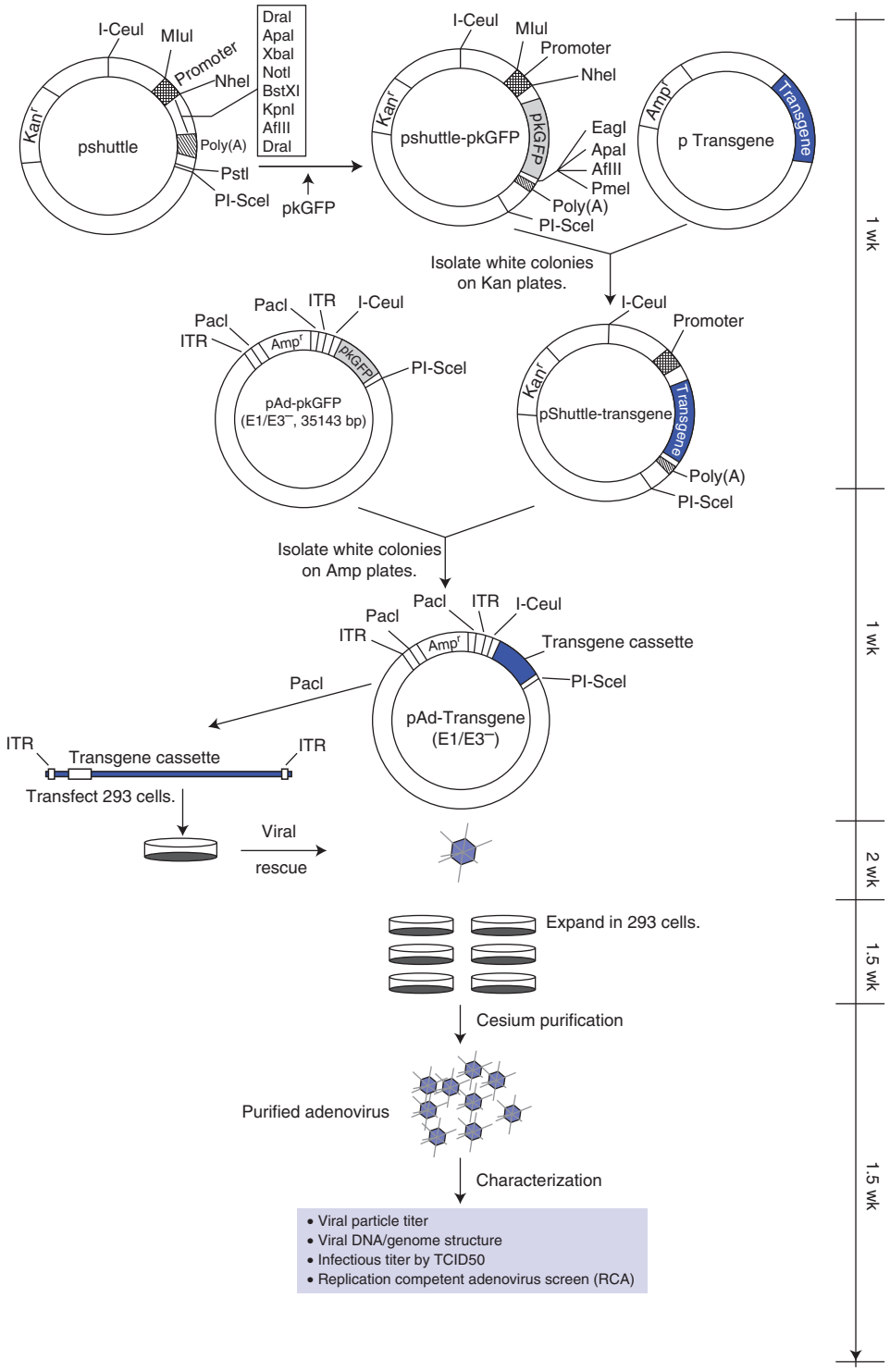

FIGURE 6. Schematic illustration of the process for adenovirus vector creation and production by direct cloning (Protocol: **Construction of Recombinant Adenovirus Genomes by Direct Cloning** [Zhou et al. 2019]).

toxicity and strong T-cell responses to viral and transgene products expressed in target cells may limit the interpretation of gene-transfer data, or when extended transgene expression is necessary, an E1/E4 double-deleted vector backbone may be a more appropriate choice.

TABLE 3. Comparison of major characteristics of four commonly used RNA and DNA viral vectors

Vector	Transgene capacity	Titer	Scale-up	Efficiency/ stability	Tropism and primary use	Host responsible	Genetic fate (genotoxicity)	Biosafety
Retro-	Up to 7 kb	10^6–10^8 IU/mL	Difficult	Low/stable	Dividing cells in vitro and ex vivo	Low immunogenicity and toxicity	Integrated[a]	BSL-II
Lenti-	Up to 7.5 kb	10^6–10^{10} IU/mL	Difficult	Moderate–high/ stable	Dividing/nondividing cells, envelope/ pseudotype-dependent tissue tropism in vitro and in vivo	Low immunogenicity and toxicity	Integrated[a]	BSL-II
Adeno-	Up to 35 kb	10^{12}–10^{13} virus particles/mL	Scalable	High/ transient	Broad in vitro and in vivo	Highly immunogenic and toxic	Episomal	BSL-II
AAV	Up to 4.5 kb	10^{12}–10^{13} GC/mL	Scalable	High/stable	Broad in vivo	Low immunogenicity and toxicity	Episomal	BSL-I

[a]See Box 6.

For applications where large transgene capacity is necessary, two types of backbones can be used. One is an E1/E3/E4 triple-deletion vector backbone with a transgene capacity of up to 8 kb; however, E1/E4 double-complementing cell lines, such as 10-3 cells, are required for recombinants. Because constitutive high-level expression of E4 in 293 cells is toxic, 10-3 cells carry an E4 orf6 expression cassette driven by the metallothionein promoter, which is inducible by heavy metals (Table 4). Alternatively, the transgene capacity of an E1/E3-deleted vector backbone can be increased by deleting most of E4, except for orf6. Because E4 orf6 is sufficient to provide the major functions of the E4 gene, an adenovirus backbone with E1/E3 full and E4 partial deletions can be rescued and grown in regular 293 cells and have a transgene capacity of up to 7 kb (Table 4).

Selection of Promoter for Transgene Expression

For most vector backbones listed in Table 4, strong viral promoters such as the cytomegalovirus (CMV) immediate-early promoter drive high-level transgene expression both in culture and in vivo. However, the CMV promoter appears to shut down over time after systemic infusion of E4-deleted adenovirus recombinants in animals (Armentano et al. 1997). In this case, strong constitutive cellular promoters should be considered in the vector design. Additionally, when adenovirus vectors carry genes that regulate the cell cycle, or are cytotoxic, introduction of a mechanism to regulate transgene expression during vector production should be considered. Otherwise, expression during production could lead to loss of transfected or infected cells, reducing vector yields significantly or preventing virus rescue and infection entirely (Bruder et al. 2000).

TABLE 4. Choices of backbones for clones of adenovirus vectors

					Immunogenicity			
						T cell		
Vector backbone	Viral gene expression	Transgene capacity/ expression	Packaging cell line	Vector yield	Innate (cytokine)	Viral	Transgene	Vector toxicity
ΔE1	Detectable	4 kb/strong	293 cells	High	IL6↑ and IL10↑	Strong	Strong	High
ΔE1 + E3	Same	6 kb/strong	293 cells	Same	Same	Same	Same	Same
ΔE1 + E3 +E4	Diminished	8 kb/reduced	10-3 cells	Reduced	Same	Diminished	Diminished	Diminished

A ↑ represents an increase in expression.

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BOX 6. LENTIVIRUS VECTORS

Lentiviruses belong to the Retroviridae family, and as all viruses in this family, the virions carry an ssRNA molecule that must undergo reverse transcription and integration into the host cell genome (provirus) for a productive infection. The distinguishing feature of lentiviruses in this family is the complexity of their genome and the cylindrical or conical shape of the nucleocapsid carrying the virus genome. The nucleocapsid is surrounded by a membrane bilayer carrying the envelope glycoprotein responsible for interaction with cell-surface receptors and entry into host cells. Retroviruses are major pathogens in almost all vertebrates. The human immunodeficiency virus type 1 (HIV-1) is responsible for acquired immunodeficiency disease syndrome (AIDS) in humans.

Lentivirus vectors based on HIV-1 were the first to be engineered as gene-delivery vehicles and shown to be highly effective for transduction of dividing and nondividing cells (Cockrell and Kafri 2007). For obvious reasons, HIV-1-derived lentivirus vector packaging systems (Fig. B1) have evolved over the years to minimize the number of HIV-1 genes present in the helper plasmids and thus decrease the probability of generating replication-competent lentiviruses (RCLs) during production. The first-generation packaging system was based on a single expression plasmid carrying an HIV-1 genome where the packaging signal (Ψ) and *env* gene were deleted, and the LTR elements were replaced by the CMV immediate-early promoter and a polyadenylation signal (Cockrell and Kafri 2007). Second-generation systems carry four HIV-1 genes, *gag* and *pol* (structural) and *tat* and *rev* (regulatory), in the complementing plasmid, whereas third-generation systems carry *gag*, *pol*, and *rev* split between two plasmids. Third-generation systems can only be used for production of lentivirus vectors carrying chimeric 5′-LTR, where the HIV-1 promoter is replaced by a CMV or RSV promoter, which renders production of vector genomic RNA necessary for packaging, independent of Tat (absent in third-generation systems). Most lentivirus vectors currently in use carry chimeric 5′-LTR elements (Cockrell and Kafri 2007).

The fact that retrovirus and lentivirus vectors integrate into the host cell genome is an advantage in achieving permanent genetic modification of target cells; however, it is also a source of safety concerns regarding their oncogenic potential through insertional mutagenesis. This issue became apparent in clinical

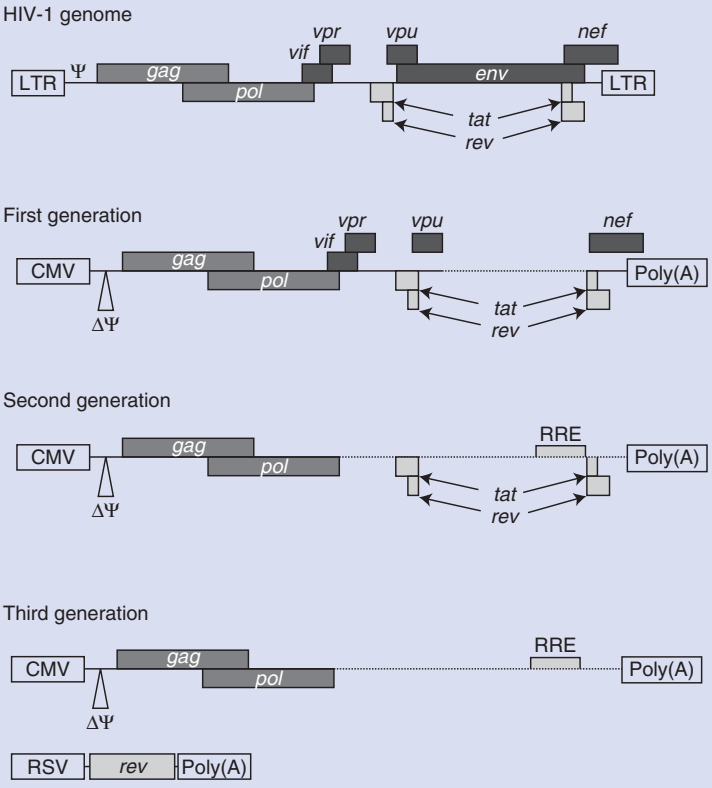

FIGURE B1. HIV-1–based lentivirus vector packaging systems. (*Top* two structures reproduced from Dull et al. 1998. *Bottom* two structures reproduced from Zufferey et al. 1997.)

Continued

trials for severe combined immunodeficiency (SCID), in which a number of patients receiving retrovirus vector-modified hematopoietic stem/progenitor cells developed leukemia. Apparently, MLV-based retrovirus vectors tend to integrate into promoter regions, leading to transactivation of oncogenes. Interestingly, the integration pattern of lentivirus vectors does not show this bias. Recent studies have shown that oncogenesis and abnormal transcription activity resulting from ex vivo modification of hematopoietic stem/progenitor cells with retrovirus or lentivirus vectors is related to the presence of transcriptionally active LTR elements in the vector provirus (i.e., vector integrated in the host genome). Lentivirus vectors carrying transcriptionally active LTR elements are nonetheless 10-fold less likely to induce tumors than comparable MLV-based retrovirus vectors. Self-inactivating retrovirus or lentivirus vectors, where the LTR elements are inactivated after integration into the host genome, have a markedly reduced/absent oncogenic potential (Montini et al. 2009).

ADENO-ASSOCIATED VIRUS VECTORS

To date, at least five different methods have been developed for the production of recombinant adeno-associated virus (rAAV) vectors (Zhang et al. 2009). These include (1) helper-free triple transfection of 293 cells, (2) infection of a stable rep/cap cell line with Ad-AAV hybrid, (3) infection of an rAAV producer cell line with wild-type adenovirus helper, (4) coinfection of 293 cells with two recombinant herpes simplex virus (rHSV) vectors, and (5) a recombinant baculovirus-based system. The method of helper-free triple transfection of 293 cells is the method most commonly used for production of rAAV vectors in the laboratory setting (Grieger et al. 2006). The process is simple and versatile and can be implemented in most laboratories. The simplicity and flexibility of this method facilitates the simultaneous production of rAAVs carrying different transgenes with the same or different capsids. Two drawbacks include difficulties in scaling up and the potential emergence of low levels of replication-competent AAV (rcAAV) particles in the vector preparations.

An overview of the rAAV production process using the helper-free triple-transfection method in 293 cells is summarized in Figure 7. In this production system, all genetic components that are necessary for the packaging of rAAV particles in 293 cells are provided by three plasmids: (1) the vector plasmid or pCis carries a transgene cassette of interest that is flanked by ITRs, the only viral elements from AAV (<4% of the AAV genome), (2) the packaging plasmid or pTrans expressing AAV Rep and Cap proteins for rescue and packaging of vector genomes, and (3) an adenovirus helper plasmid supplying all required helper functions from E2a, E4, and VA RNA genes except for the E1 gene, which is *trans*-complemented by E1 expression in 293 cells.

The production process of AAV recombinants starts with the generation of a pCis plasmid carrying an expression cassette for the transgene of interest. This is accomplished by subcloning the gene of interest, which could be a complementary DNA (cDNA), small hairpin RNA (shRNA), or artificial miRNA shuttle, into the pCis vector plasmid containing transcription elements of choice (promoter and polyadenylation signal) flanked by AAV ITRs (Fig. 7). Most AAV vectors carry ITR elements derived from AAV2. The second step is to select the packaging plasmid (pTrans) that expresses the AAV serotype capsid of interest. The third step is to perform triple transfection of the pCis, pTrans, and pAd helper plasmids into 293 cells for AAV vector genome rescue and packaging (Protocol: **Production of Recombinant Adeno-Associated Viruses (rAAVs) by Transient Transfection** [Su et al. 2020a]). This is followed by purification of rAAV particles from the crude lysate of transfected cells.

As shown in Figure 7, the methods used in the purification of rAAVs can be divided into three major categories: (1) gradient sedimentation, (2) column chromatography, and (3) combination of gradient centrifugation with column chromatography.

Gradient Sedimentation

For the first category of purification methods, either cesium chloride (CsCl) or iodixanol formed gradients can be used to purify rAAVs by ultracentrifugation (Protocol: **Purification of Recombinant Adeno-Associated Viruses (rAAVs) by Cesium Chloride Gradient Sedimentation** [Su et al. 2020b] or

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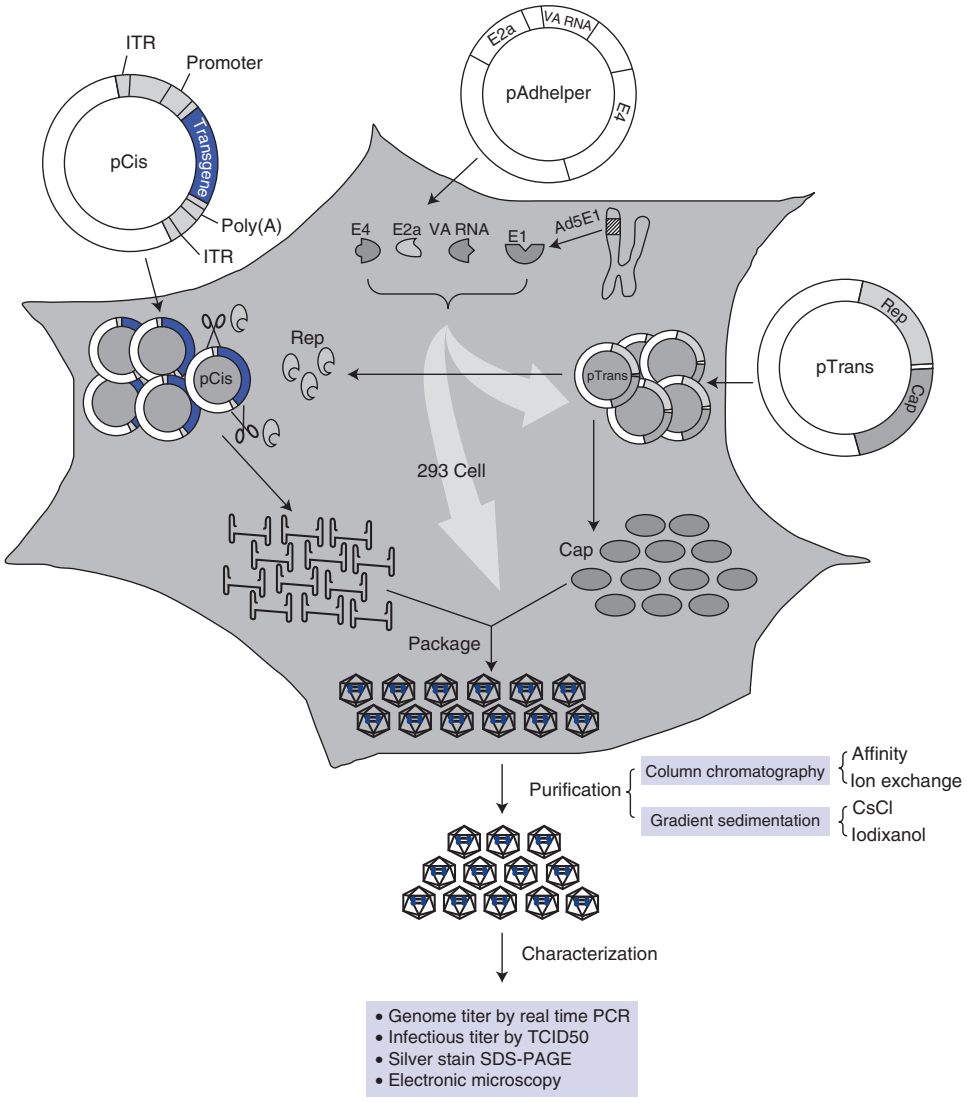

FIGURE 7. Schematic illustration of the process for production and purification of recombinant adeno-associated virus by 293 cell–triple-transfection method (Protocol: **Production of Recombinant Adeno-Associated Viruses (rAAVs) by Transient Transfection** [Su et al. 2020a]).

Protocol: **Purification of Recombinant Adeno-Associated Viruses (rAAVs) by Iodixanol Gradient Centrifugation** [Sena-Esteves and Gao 2020a]) (Grieger et al. 2006). The major advantages of gradient centrifugation-based methods are the ability to separate virions carrying vector genomes from empty viral particles and their applicability to all serotypes. CsCl gradient centrifugation is the most commonly used method for laboratory purification of AAV vectors; however, this method is time consuming and difficult to scale up. In addition, it requires an additional step to remove toxic chemicals used to generate the gradient and reformulate the vector stock in a physiological buffer.

Column Chromatography-Based Methods

The second category of purification methods is column chromatography-based and includes affinity chromatography (Protocol: **Purification of Recombinant Adeno-Associated Virus 2 (rAAV2) by Heparin Column Affinity Chromatography** [Auricchio et al. 2020]) and ion-exchange chromatography (Protocol: **Enrichment of Fully Packaged Virions in Column-Purified Recombinant Adeno-Associated Virus (rAAV) Preparations by Iodixanol Gradient Centrifugation Followed by Anion-**

Exchange Column Chromatography [Sena-Esteves and Gao 2020b]) (Grieger et al. 2006). Overall, the column chromatography-based methods are faster and more efficient in removing cellular and viral impurities. However, they also have shortfalls, including in developing a generic purification protocol for all serotypes and the inability to separate empty particles from vector preparations. An effective strategy to enrich the fully packaged virions in column-purified rAAV preparations is to combine gradient centrifugation with column chromatography, as described in Protocol: **Enrichment of Fully Packaged Virions in Column-Purified Recombinant Adeno-Associated Virus (rAAV) Preparations by Iodixanol Gradient Centrifugation Followed by Anion-Exchange Column Chromatography** (Sena-Esteves and Gao 2020b).

Finally, the genome of the purified rAAV should be quantitated by real-time PCR (Protocol: **Titration of Recombinant Adeno-Associated Virus (rAAV) Genome Copy Number Using Real-Time qPCR** [Su et al. 2020c]); the infectivity of the rAAV should be measured by infection of a rep-cap cell line and real-time qPCR (Protocol: **Sensitive Determination of Infectious Titer of Recombinant Adeno-Associated Viruses (rAAVs) Using TCID50 End-Point Dilution and qPCR** [Lock et al. 2020]). The morphology of the rAAV can be confirmed by electron microscopy (Protocol: **Analysis of Recombinant Adeno-Associated Virus (rAAV) Sample Morphology Using Negative Staining and High-Resolution Electron Microscopy** [Hendricks et al. 2020]), and its purity estimated by SDS-PAGE and silver staining (Protocol: **Analysis of Recombinant Adeno-Associated Virus (rAAV) Purity Using Silver-Stained SDS-PAGE** [Su et al. 2020d]).

Strategic Planning

Transgene Expression Cassette Design

The maximum size of the genome that can be efficiently packaged in most AAV capsids is 4.7–4.8 kb. This constraint on vector size dictates a minimalistic approach to the design of transgene expression cassettes in rAAVs, which should include only essential transcriptional accessories (e.g., promoter and polyadenylation signals of minimal size). The choice of promoter should be guided by, for example, whether ubiquitous or tissue-specific expression is desired (Le Bec and Douar 2006). In the former case, hybrid promoters such as CBA (also known as CAG or CB), which is composed of the cytomegalovirus (CMV) enhancer fused to the chicken β-actin promoter, mediates robust stable expression both in culture and in vivo in most cell types and tissues targeted by AAV vectors. Strong viral promoters such as the CMV immediate-early gene promoter works well for rAAV-mediated gene transduction in most target tissues except for liver, where CMV-directed transgene expression rapidly shuts off. Incorporation of tissue- or cell-type–specific promoters is reasonably effective in restricting expression to the desired target. Alternatively, tissue-specific expression can be achieved by incorporating into the vector mRNA target sequences that are recognized by miRNAs differentially expressed in target (absent) and nontarget (present) tissues. Finally, pharmacologically regulated rAAV-mediated transgene expression can be realized by incorporating the transcription regulator(s) and transgene cassettes separately into two vector genomes, packaging them individually, and coinjecting both vectors into the target tissue (Rivera et al. 1999; Ye et al. 1999).

Serotype Selection

Recombinant AAVs carrying the same transgenic genome (same AAV2 ITR-flanked expression cassette for a particular transgene), but with capsids derived from different AAV serotypes/strains, display dramatically different transduction properties in vivo (Gao et al. 2005). The AAV capsid determines the cell/tissue tropism of AAV vectors and other aspects of their biology. The choice of AAV capsid for a particular application should be guided by the ability to transduce the intended target tissue using a particular delivery route (Gao et al. 2005). Table 5 characterizes the major AAV serotype vectors with regard to their cellular receptor(s), optimal target tissues, and relative transduction efficiency in culture (293 cells) and in vivo. Production of AAV vectors with different capsids is accomplished by (1) *trans*-encapsidation of the same vector genome flanked by AAV2 ITRs, (2) capsid protein

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TABLE 5. Target tissue-specific AAV capsid selection

Capsid	Receptor	Optimal target tissues	Transduction (293 cells/in vivo)
AAV1	N-linked sialic acid	Skeletal muscle, CNS	Moderate/good
AAV2	HSPG αVβ5 integrin FGFR1 Laminin	Skeletal muscle, CNS	Good/poor
AAV4	O-linked sialic acid	CNS, eye/RPE	Poor/moderate
AAV5	N-linked sialic acid PDGFR	CNS, lung, eye/RPE/photoreceptor	Poor/moderate
AAV6	N-linked sialic acid	Skeletal muscle, cardium	Poor/good
AAV7	Unknown	Skeletal muscle, pancreas, liver	Poor/good
AAV8	Laminin	Liver, skeletal muscle, pancreas	Poor/good
AAV9	Laminin	Liver, lung, skeletal muscle, cardium, CNS (via both local and transvascular delivery)	Poor/good
rh.10	Unknown	Lung, CNS	Poor/good

AAV, adeno-associated virus; HSPG, heparan sulfate proteoglycan; FGFR, fibroblast growth factor receptor; RPE, retinal pigment epithelium; PDGFR, platelet-derived growth factor receptor; CNS, central nervous system.

expressed from a chimeric packaging plasmid carrying the *rep* gene from AAV2 (which is necessary to replicate AAV2 ITR-flanked genomes during packaging), and (3) the *cap* gene from an AAV serotype/strain of choice.

One paradoxical property of AAV recombinants is that they are generally inefficient for gene delivery to cells in culture but display remarkable transduction efficiency in vivo. The transduction efficiency of these vectors in culture, regardless of capsid type, is considerably (several orders of magnitude) lower than adenovirus or lentivirus vectors at comparable MOI values. Interestingly, AAV2 recombinants are generally the most effective for gene transfer in cell culture but are among the least efficient for many in vivo gene-delivery applications.

RETROVIRUS AND LENTIVIRUS VECTORS

Retrovirus vectors were among the first gene-transfer vehicles to gain wide acceptance in the scientific community as a tool for engineering cells in culture with genes of interest. Most retrovirus vectors currently in use are derived from Moloney murine leukemia virus (Mo-MLV), and numerous design improvements have been implemented over the years. The fastest and most efficient method to generate retrovirus vector stocks is by transient transfection of 293-based packaging cell lines such as Phoenix-ECO and Phoenix-AMPHO (Dr. Gary P. Nolan, Stanford University) or other commercially available cell lines such as Plat-A, Plat-E, and Plat-GP (Cell Biolabs); AmphoPak-293; EcoPak 2-293; and RetroPack PT67 (Clontech). For pseudotyping retrovirus vectors with other envelope proteins such as vesicular stomatitis virus glycoprotein (VSV-G), cell lines can be used that express Mo-MLV *gag–pol* genes only (Plat-GP cells, Cell Biolabs; GP2-293 cells, Clontech) cotransfected with the vector plasmid and an expression plasmid encoding the envelope of interest (e.g., see pVSV-G below). An alternative is triple transient transfection of 293 cells with the retrovirus vector plasmid and two expression plasmids separately encoding Mo-MLV *gag–pol* genes (e.g., pUMVC plasmid; Plasmid 8449 at Addgene.org) and an envelope protein (similar to the approach for production of lentivirus vectors, see below). The protocols for production and titration of retrovirus vector stocks by transient transfection of both 293-based packaging cell lines and 293 cells are the same as those used for lentivirus vectors.

The most efficient method for producing high-titer lentivirus vectors is by transient cotransfection of human embryonic kidney 293T cells with transfer vector plasmid, packaging plasmid(s), and an envelope expression plasmid. Lentiviruses are surprisingly tolerant of envelope proteins derived from other viruses, a process known as pseudotyping. In consequence, VSV-G is the most commonly used envelope for producing recombinant lentivirus vectors. This is mainly due to the broad tropism of VSV-G pseudotyped lentivirus vectors, which can transduce a wide range of cell types from many

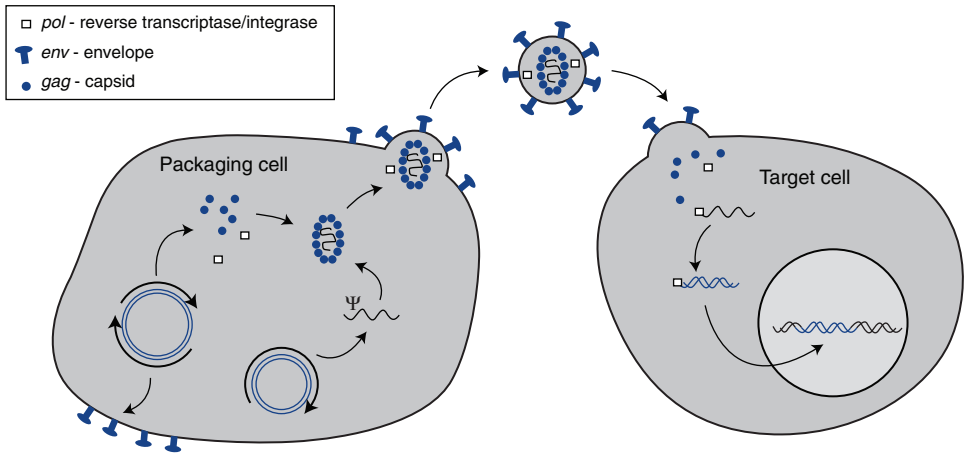

FIGURE 8. Packaging and infection by a lentiviral vector. During production, the lentivirus vector plasmid generates genomic RNA that is transported to the cytoplasm, and virion assembly takes place at the cellular membrane with structural proteins encoded by the helper plasmid (*left* panel). Upon entry into a target cell, the lentivirus genome is reverse-transcribed in a preintegration complex, which is transported into the nucleus of dividing and nondividing cells. Once in the cell nucleus, the viral integrase (part of the preintegration complex) mediates integration of the vector genome into the host cell genome. Integration into the host genome is not necessary for lentivirus vector-mediated transgene expression as integrase-deficient vectors are capable of mediating robust expression.

different organisms both in culture and in vivo. Moreover, these vectors can be concentrated by ultracentrifugation to titers in excess of 10^{10} TU/mL, which are useful for gene-transfer experiments in vivo (Fig. 8). Protocol: **Production of High-Titer Retrovirus and Lentivirus Vectors** (Sena-Esteves and Gao 2018a) describes the production of HIV-1–derived lentivirus vectors pseudotyped with a VSV-G envelope because these vectors have become the most widely used vectors of their class. Lentivirus vectors are highly effective for gene transfer both to dividing and to nondividing cells. Thus, they have largely replaced Mo-MLV-based retrovirus vectors, which require actively dividing cells as the tools of choice for gene function and regulation studies. Additional gene-transfer systems have been developed based on other lentiviruses such as equine infectious anemia virus (EIAV), simian immunodeficiency virus (SIV), and feline immunodeficiency virus (FIV). The method described here can be easily adapted for production of any retrovirus/lentivirus vectors and would differ only in the type of transfer vector and plasmid encoding helper functions.

After the production method (Protocol: **Production of High-Titer Retrovirus and Lentivirus Vectors** [Sena-Esteves and Gao 2018a]), we describe different approaches to determine the titer of a lentivirus vector (Protocol: **Titration of Lentivirus Vectors** [Sena-Esteves and Gao 2018b]) and, finally, a method to screen stocks for replication-competent lentiviruses (RCLs) (Protocol: **Monitoring Lentivirus Vector Stocks for Replication-Competent Viruses** [Sena-Esteves and Gao 2018c]). These protocols can also be adapted for other retrovirus/lentivirus vector systems.

Strategic Planning

Retrovirus Vector Design

Retrovirus vectors can accommodate up to 7–8 kb of foreign sequences. The Babe-Puro retrovirus vector (Morgenstern and Land 1990) is a prototypical classical design still in use. In this vector, the gene of interest is expressed from the Mo-MLV LTR promoter, and an internal immediate-early SV40 promoter drives expression of a drug resistance marker (puromycin, neomycin, or hygromycin) (Fig. 9). These vectors incorporate several modifications that reduce the risk of generating replication-competent retroviruses (RCRs) during packaging. Later versions of retrovirus vectors (Fig. 9B) carry internal mammalian or viral promoters in the context of a self-inactivating (SIN) design to prevent interference between the 5′-LTR and the internal promoter. During reverse transcription of

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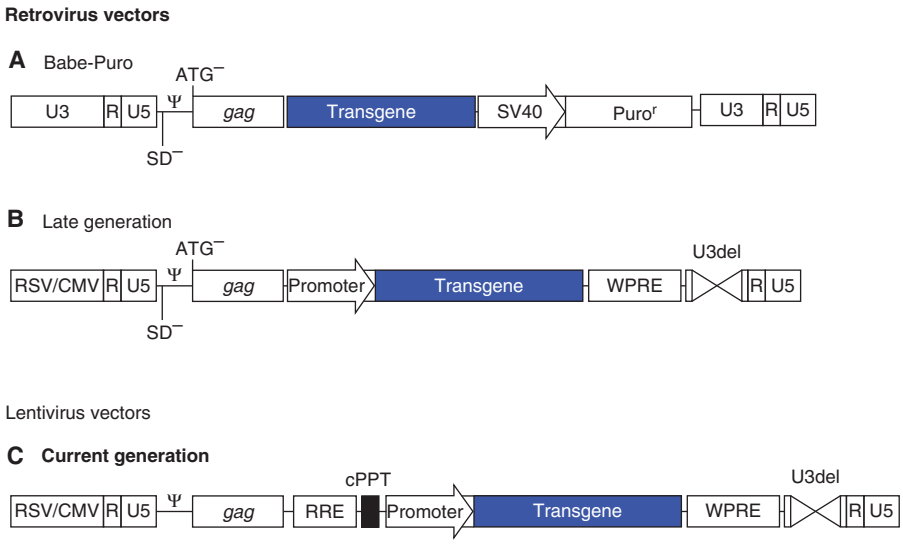

FIGURE 9. Basic structure of retrovirus and HIV-1 lentivirus vectors. (*A*) The Babe-Puro retrovirus vector is a classical design still in use today. The vector was optimized from previous generations to reduce the risk of generating replication-competent retroviruses (RCRs) during packaging. The gene of interest is expressed from the Moloney murine virus LTR promoter. The vector also carries a drug-resistance gene such as puromycin (other versions carry neomycin or hygromycin genes) under the immediate-early SV40 promoter to allow for selection of retrovirus vector-transduced cells in culture. (*B*) Later generations of retrovirus vectors carry internal mammalian or viral promoters in the form of a self-activating (SIN) design in which the promoter elements (U3 region) in the 3′ LTR are deleted, resulting in the inactivation of both LTR elements in the integrated provirus genome. Many of these vectors also carry the woodchuck hepatitis virus posttranslational regulatory element (WPRE) to increase transgene expression levels and may also carry a hybrid 5′ LTR composed of the CMV or RSV promoter in place of the native viral U3 promoter elements. (*C*) The design of lentivirus vectors is identical to late-generation retrovirus vectors. Most HIV-1-based lentivirus vectors are self-activating and carry a chimeric 5′ LTR in which the CMV and RSV promoters replace the HIV-1 native U3 promoter elements, making packaging of these vectors Tat independent and thus compatible with third-generation packaging systems. In addition to the packaging signal (Ψ) immediately following the 5′ LTR, all lentivirus vectors carry a portion of the *gag* gene. In addition, all vectors carry the Rev-responsive element (RRE) necessary for nuclear export of the vector RNA genome for packaging into virions. Inclusion of the central polypurine tract (cPPT) dramatically enhances transduction efficiency. WPRE is also commonly incorporated into lentivirus vectors. The choice of internal promoter to drive transgene expression is dictated by the specific experimental application.

retroviruses, the U3 region present only at the 3′ end of the genomic RNA is duplicated to the 5′-LTR in the provirus. As a consequence, deletion of the U3 enhancer elements in the 3′-LTR of a retrovirus (or lentivirus) vector plasmid leads to transcriptionally inactive 5′- and 3′-LTRs in the integrated provirus. It is interesting to note that the transcriptional status of the 3′-LTR in Mo-MLV retrovirus vectors is a key determinant of their genotoxicity (Montini et al. 2009). To reduce the risk of generating RCRs by recombination, the U3 promoter in the 5′ LTR has been replaced with another viral promoter such as the CMV immediate-early promoter or the Rous sarcoma virus (RSV) promoter. In addition, incorporation of posttranscriptional regulatory elements at the 3′ end of the expression cassette, such as the woodchuck posttranscriptional regulatory element (WPRE) or the constitutive transport element (CTE) from the Mason–Pfizer monkey virus, enhances transgene expression levels quite considerably. One of the drawbacks of Mo-MLV retrovirus vectors is their inability to transduce (infect) nondividing cells. This is because their preintegration complex can only gain access to the host cell genome after loss of nuclear membrane integrity during mitosis. Most of the design features first developed for retrovirus vectors have also been adopted for lentivirus vectors.

Lentivirus Vector Design

Lentivirus vectors can accommodate up to 7.5–8 kb of transgenic sequences and, as such, are compatible with an enormous variety of designs to accomplish different goals. There are, however, a number of basic components that have been shown to enhance safety, gene transfer, and expression

efficiency (Fig. 9): (1) The U3 promoter region in the 5′-LTR has been replaced with the RSV or CMV promoter, rendering packaging of these vectors independent of Tat expression. (2) Deletion of U3 promoter elements in the 3′-LTR renders the LTR element transcriptionally inactive after integration into the host cell genome. These vectors are known as SIN, and recent evidence indicates that this is a critical feature that significantly reduces genotoxicity (see Box 6). (3) Incorporation of a second polypurine tract in the vector genome, central polypurine tract, or central flap is critical for nuclear transport of the preintegration complex and dramatically increases transduction efficiency (Follenzi et al. 2000; Sirven et al. 2000). (4) Incorporation of a posttranscriptional regulatory element derived from the WPRE enhances transgene expression (Zufferey et al. 1999). Numerous lentivirus vector designs have been developed to achieve the following:

- tissue-specific expression by incorporation of tissue-specific promoters and/or miRNA targets to eliminate off-target effects (Frecha et al. 2008)
- expression of multiple proteins from a single vector using IRES, bidirectional promoters, or multiple open reading frames separated by self-cleaving 2A peptides (Carey et al. 2009)
- shRNA and miRNA expression
- generation of cDNA, shRNA (Moffat et al. 2006), and miRNA (Open Biosystems, Inc.) libraries
- drug-regulated gene expression

Envelope Selection

HIV-1–derived lentivirus vectors are permissive for incorporation of different envelope proteins in the virion. VSV-G pseudotyped lentivirus vectors appear to be highly effective for in vivo and ex vivo gene delivery. Lentivirus vectors pseudotyped with envelope proteins derived from other viruses or engineered to target specific cell-surface receptors have also been successfully used to increase transduction efficiency of specific cell types and to facilitate targeted transduction, respectively (Cockrell and Kafri 2007).

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Introducing Genes into Mammalian Cells: Viral Vectors

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