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Gary L. Buchschacher, Jr. Lentiviral Vector Systems for Gene Transfer





MEDICAL INTELLIGENCE UNIT 31

Lentiviral Vector Systems for Gene Transfer

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LENTIVIRAL VECTOR SYSTEMS FOR GENE TRANSFER

Medical Intelligence Unit

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PREFACE

This volume is designed to summarize recent progress in the development of lentiviral vectors for in vitro gene transfer studies and for subsequent animal and human clinical studies. Although lentiviral vector systems may offer some advantage over previously studied viral vector systems for use in potential gene therapy applications, such vectors also present technological hurdles that must be overcome and raise unique concerns that must be addressed as the vector systems are developed and potentially applied to clinical gene transfer studies.

Applied gene transfer studies using viral vector systems are being undertaken by more and more people. For this reason, the first chapter presented here is designed to assist those interested primarily in the application of lentiviral vectors for gene transfer, but who may lack a background in basic retrovirology, by presenting some general information about retroviral replication and retroviral vector systems that will provide a basis for the topics that are discussed in detail in the remainder of the book.

The final section of this book, discussing ethical considerations relevant to lentiviral vector systems, is important for those studying either vector development or potential clinical gene transfer applications; it is recommended that this chapter be read first, so that topics discussed in other chapters can be thought of with these ethical considerations in mind.

Because the various lentiviruses share many features of replication and because human immunodeficiency virus type 1 (HIV-1) is the most thoroughly studied of the lentiviruses, one chapter is devoted to a discussion of HIV-1 replication. The following chapter discusses in detail the feature that makes lentiviral vectors attractive as gene transfer vehicles—the ability of lentiviruses to efficiently infect non-dividing cells. Subsequent chapters discuss progress in development of vector systems based on a variety of lentiviruses—HIV-1, HIV-2, SIV, FIV, EIAV and so on. Potential approaches to and issues regarding some clinical applications of the vector systems are then presented, along with discussions dealing with various safety issues that are raised when developing and considering these systems for clinical use.

It is hoped that the contents of the book will leave readers, either those involved in the study of basic virology or vector system development or those involved in gene transfer applications, with an understanding of both the current status of lentiviral vector technology and the issues which must be addressed as these systems are further developed and used. Clearly, regardless of how the various vector systems eventually might or might not be used for clinical applications, the value of study of the vector systems is evident by the amount of information on the basic science of viral replication, cell biology and gene transfer that continues to be obtained.

Gary L. Buchschacher, Jr., M.D., Ph.D.

CHAPTER 1

Introduction to Retroviruses and Retroviral Vectors

Gary L. Buchschacher, Jr.

Abstract

s various viral vector systems for gene transfer are developed, interest in using such systems in applied settings continues to grow. This Chapter is designed to provide background information for readers interested in learning about lentiviral vector systems for gene transfer applications but who lack a background in retrovirology. To assist those readers who are unfamiliar with retroviral vector systems, basic outlines of the retroviral replication cycle and of characteristics of retroviral vector systems are introduced here in order to present and define concepts and terms that are discussed in subsequent Chapters.

Introduction

The development of vector systems derived from the lentivirus genus of retroviruses has potential for possible clinical gene transfer applications.¹ Retroviral vector systems used previously in gene transfer applications have been derived from the oncogenic retrovirus genus. The oncoretroviruses, sometimes (though not necessarily correctly) referred to as "simpler" retroviruses in comparison to lentiviruses, had been studied for years and knowledge gained from those studies enabled development of vector systems based on the parent viruses. The concepts governing development of oncoretrovirus-derived vector systems also guide the development of lentiviral vector systems. Therefore, an understanding of the retroviral replication cycle and of the basic features of retroviral vector systems based on oncogenic retroviruses is necessary in order to fully understand the complexities and issues associated with the development of lentiviral vector systems discussed later in this book.

In this first Chapter, the retroviral replication cycle and concepts related to development of retroviral vector systems are outlined. Since a comprehensive review of the various retroviruses and vectors derived from them is obviously beyond the scope of this section, the information presented will describe generic features shared by retroviruses and vector systems in general, rather than focus on one or more particular viruses. Readers who are familiar with these concepts are advised to skip this introductory Chapter and to move on to Chapter 2.

General Background and Classification of Retroviruses

Retroviruses are single-stranded RNA viruses that replicate through a double-stranded DNA intermediate.² Various retroviruses have been found that infect a number of organisms, including humans and many other mammals. The earliest retroviruses studied were isolated from mice and birds. Examples of such retroviruses include murine leukemia virus (MLV) and

mouse mammary tumor virus (MMTV), and the avian pathogens Rous sarcoma virus (RSV), spleen necrosis virus (SNV), and avian leukosis virus (ALV). These viruses were discovered and were of interest because of their association with the development of tumors in their host organisms. Study of these viruses eventually led to the discovery and development of the oncogene theory of tumorigenesis: some of the viruses actually contained oncogenes within their genomes, while others interacted with oncogenes in either a direct or indirect way to contribute to tumor formation.^{3,4}

Over time, other retroviruses that had in common with the previously isolated viruses many features of their genome organization and overall replication strategy were discovered. Historically, because of their pattern of pathogenicity, these viruses were grouped into three subfamilies: 1) the acutely oncogenic retroviruses, or oncoretroviruses (such as those described above); 2) the lentiviruses (associated with "slow" diseases or those with long latent periods); 3) the spumaviruses ("foamy" viruses, named because of the pathogenic changes observed in infected cells). Viruses were also grouped (Types A-D) according to the electron microscopic appearance of their nucleocapsid structures.⁵ Further study of these viruses enabled detailed comparison of their genome structures and nucleic acid sequences, which resulted in a further refinement of the retrovirus classification system.⁶ This led to a revised classification of the Retroviridae family into seven genera: mammalian type B retroviruses, mammalian type C retroviruses, avian type C retroviruses, mammalian type D retroviruses, HTLV/BLV type retroviruses, lentiviruses, and spumaviruses. The remainder of this Chapter will focus on describing general features of the retrovirus replication cycle and retroviral vector systems, focusing on "simple" oncoretroviruses. Although vector systems derived from MLV are the most commonly used retroviral-based systems in gene transfer applications, this discussion will not focus on MLV per se, but will describe prototypical features characteristic of the retroviruses in general.

The Retrovirus Genome and Virion Structure

Genome Structure

The retrovirus genome contained within viral particles consists of two identical singlestranded RNA molecules (for this reason, retroviruses are referred to as being "pseudodiploid") of positive polarity that are replicated through a double-stranded DNA intermediate (reviewed in refs. 7, 8). The organization of the RNA and DNA forms of the genome are shown in Figure 1.

The 5' end of the genomic RNA begins with the "r" (for repeat) and "u5" (for unique 5' region) segments, followed by the viral genes *gag*, *pol*, and *env*. The 3' end of the genomic RNA terminates with the u3 (for unique 3' region) and r (identical to the 5' r region) regions and a polyA tail. Following reverse transcription of the RNA genome into a double-stranded DNA molecule, the DNA form of the viral genome is integrated into the host cell chromosomal DNA, where it is thereafter referred to as a "provirus." Because of the mechanism used for reading and utilizing the viral RNA template during reverse transcription,⁸⁻¹⁰ the u3 and u5 regions are duplicated such that the 5' and 3' ends of the proviral genome differ in structure from the ends of the RNA genome (Fig. 1).

Each end of the proviral genome is made up of regions called long terminal repeats (LTRs) which contain the proviral U3, R, and U5 regions. This rearrangement of both termini of the viral genome enables appropriate expression of the viral genes. The U3 region of the 5' LTR (copied from the u3 region at the 3' end of the RNA genome) contains the viral promoter and enhancers responsible for initiation of transcription of the viral genome at the 5' U3/R junction. The viral *gag* and *pol* genes are expressed from an unspliced transcript while the *env* gene is expressed from a spliced transcript (the splice donor is located between the 3' end of the *env* gene). The 3' end of the *gag* gene, with the splice acceptor located at the 5' end of the *env* gene). The 3' end of the genome contains the transcription termination signal, with the



Fig. 1. Genome structure of a prototypical retrovirus. The genomic viral RNA, represented by a single black line, is shown at the top of the figure, with the structure of the resulting provirus after reverse transcription below. The locations of the open reading frames *gag*, *pol*, and *env* are shown. Reverse transcription of the RNA results in rearrangement of the termini of the genome, resulting in the structures of the LTRs (long terminal repeats) as indicated. *Cis*-acting sequences of the viral genome are shown in more detail in Figure 3.

polyadenylation signal located in the 3' LTR. The *gag* gene encodes viral core structural proteins: matrix (MA), capsid (CA), and nucleocapsid (NC). Some retroviruses also encode various other small proteins or peptides within the *gag* open reading frame. The *pol* gene encodes the viral replication enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN). The *env* gene encodes the envelope glycoprotein (Env) which is processed into transmembrane (TM) and surface (SU) subunits.

Virion Structure

Retrovirus particles consist of the viral protein core and the surrounding viral envelope that is made up of a cellular membrane-derived lipid bilayer and viral-encoded envelope glycoproteins. The structural core of the virion contains the two RNA molecules (associated as a dimer) in association with the nucleocapsid protein that, in turn, is surrounded by a shell of capsid protein. The matrix protein is located outside of the viral capsid and appears to interface with the inner part of the viral envelope that surrounds the core. The viral-encoded replication enzymes, including reverse transcriptase and integrase, are located within the viral core.

The Retrovirus Replication Cycle

The retroviral replication cycle (Fig. 2) commences when a virion begins to infect a cell via the interaction of the envelope surface glycoprotein with a specific cellular receptor (or receptors). A subsequent conformational change in the viral envelope surface glycoprotein results in exposure of a fusion domain contained within the envelope transmembrane glycoprotein. Subsequent fusion of the viral envelope with the cellular membrane occurs, which results in release of the virus core into the cytoplasm of the cell.⁷ In most retroviruses, this membrane fusion occurs directly at the cell surface, but in some retroviruses the virion, after binding to the cellular receptor, is internalized via endocytosis; fusion of the viral envelope with the endosome then results in release of the viral core into the cytoplasm.

Exactly how the next series of steps occur in relation to each other is not completely clear. However, it is known that the viral genome is uncoated, reverse transcribed into double-stranded DNA, and integrated into the genome of the host cell where it resides permanently as the provirus. The provirus is then passed on to daughter cells following cellular division, just like a cellular gene would be. A brief description of the general steps of retroviral reverse transcription will be given here; the steps of reverse transcription of the HIV-1 genome are discussed in detail in Chapter 2 (refs. 7,8).

Viral reverse transcriptase, in order to initiate minus strand DNA synthesis using the RNA genome as a template, utilizes as a primer a cellular tRNA molecule that is bound to the primer binding site (pbs) located just downstream of the 5' LTR. The identical r regions of the



Fig. 2. The retrovirus replication cycle. Following recognition of a specific cellular receptor by the viral envelope glycoprotein and adsorption of the virion to the cell surface, fusion of viral and cellular membranes results in release of the viral core into the cell cytoplasm (usually at the cell surface, but for some retroviruses this occurs following endocytosis of virions). The viral RNA is uncoated and reverse transcribed into a double-stranded DNA copy. Following breakdown of the nuclear envelope, the DNA copy is integrated into a chromosome where it resides as the provirus. Transcription of the provirus results in production of copies of viral genomic RNA, which can either be translated to produce viral proteins or packaged by viral proteins to form new core particles. Cores form and mature while budding from the cell surface, during which time they obtain their envelopes consisting of cellular membrane and viral envelope glycoproteins (modified from ref. 1).

viral RNA genome facilitate a strand transfer of the reverse transcriptase and nascent minus strand DNA that is necessary for DNA synthesis to continue.⁹ Utilization of the polypurine tract (ppt) located near the 3' end of the genome to initiate synthesis of the DNA plus strand followed by a second strand-switching event results in the completion of reverse transcription and the existence of a full-length double-stranded DNA copy of the viral genome containing LTRs at each end. This genome is integrated, in an apparent random fashion, into a chromosome of the host cell where it resides as the provirus. The integration reaction^{11,12,13} is mediated by the viral integrase protein; the termini of the DNA genome are processed (two nucleotides on each end are removed), there is a break in and a short duplication made of the cellular DNA sequence, the viral DNA is inserted, and DNA repair enzymes complete the integration process. Because oncoretroviruses, unlike lentiviruses, lack a nuclear transport function to move the proviral preintegration complex into an intact nucleus, the integration process.¹⁴

Once integration of the viral genome is complete, the provirus is maintained in the cell just like any cellular gene. A cellular RNA polymerase uses the viral promoter/enhancer in the 5' LTR to initiate transcription of proviral DNA. RNA transcripts are polyadenylated and transported from the nucleus for translation as any cellular mRNA would be. The viral Gag and Pol proteins are translated from a full-length unspliced RNA that extends from the 5' LTR to the 3' LTR. Usually, during translation of the retroviral RNA, translation terminates after reading of the *gag* open reading frame by the ribosomal complexes, resulting in significant Gag production. However, about 5% of the time there is production of a Gag-Pol fusion polyprotein precursor [some studies have indicated that the stoichiometry of Gag and Pol production appears to be important for efficient and correct virion assembly^{15,16}]. The mechanism of production of Gag-Pol precursor varies among the different retroviruses: in some retroviruses, production of Gag-Pol during translation is a result of a ribosomal frameshift event that puts the *pol* gene into the same reading frame as the *gag* gene;¹⁷ in other retroviruses, Gag-Pol production results from a readthrough event mediated by a suppressor tRNA that prevents termination of translation at the end of the *gag* gene.¹⁸

The envelope protein is expressed from a spliced message. This protein precursor undergoes post-translational modification including extensive glycosylation and processing by a cellular protease to generate the two TM and SU glycoprotein subunits that make up the functional Env protein.

Full-length transcripts of the viral genome, in addition to being translated into viral proteins, also can be incorporated into newly forming viral particles.¹⁹ This "packaging" (or "encapsidation") of the viral genome into newly forming capsids is mediated by an RNA packaging (encapsidation) signal located on full-length viral transcripts. This predominate packaging signal, sometimes referred to as " ψ " or "E," is generally located near the 5' end of the genome between the splice donor and the *gag* start codon (Fig. 3). Because it is typically located within an intron, the packaging signal is removed during splicing, insuring that only fulllength viral transcripts and not spliced transcripts (containing only *env*) are incorporated into progeny virions.

Although the locations of retroviral packaging sequences are generally thought to be as described above, further study of the packaging signals of various retroviruses has revealed that the actual situation is, not surprisingly, more complex.^{20,21} In some cases, sequences upstream of the splice donor have been shown to be important in RNA packaging. In other cases, sequences extending past the *gag* start codon have been shown to increase efficiency of RNA encapsidation (these "extended" packaging signals are oftentimes referred to as " ψ +" or "E+"). Still, in other cases, sequences distant from the major packaging signal have been suggested to affect encapsidation of viral RNA. It is generally believed that the secondary structure of the RNA within the packaging signal, and not the primary RNA sequence itself, plays the more important role in RNA encapsidation.

The process and relative timing of many events of virus particle assembly are not understood completely. Newly synthesized Gag proteins recognize RNA that is to be packaged into virions and incorporate the RNA into capsids formed of multimers of Gag molecules. Recognition of the viral encapsidation sequence is thought to be mediated primarily by the NC portion of the Gag protein precursor. Viral core formation is thought to be driven by intermolecular Gag-Gag interactions, with the Pol protein being incorporated into the forming virion via Gag molecules interacting with the Gag portion of the Gag-Pol precursor. Viral core assembly and processing of the Gag and Gag-Pol protein precursors to form mature, infectious virions appears to occur during and just after budding of virus particles from the cell.²² The viralencoded protease (PR, encoded by *pol*) self-cleaves the Gag-Pol precursor and also further processes Gag into the MA, CA, and NC proteins and Pol into the RT and IN proteins. The



Fig. 3. Retroviral vector *cis*-acting elements. In this example of a "typical" retroviral vector, the viral genes have been removed and replaced with a foreign gene of interest. The viral sequences that remain as part of the vector construct are necessary in *cis* during various steps of the retroviral replication cycle. These sequences are necessary for vector production and for successful reverse transcription and integration of the vector genome, followed by expression of the foreign gene. Although in the example shown the foreign gene is expressed directly from the LTR, other strategies for expressing foreign genes exist and are illustrated in Figure 4. *att*, attachment site; pbs, primer binding site; ppt, polypurine tract. Modified from ref. 1.

virion envelope, made up of cellular membrane and viral envelope glycoproteins, is obtained during budding of the virus from the cell.

Elements of Retroviral Vector Systems

General Concepts

Retroviral vectors are derivatives of viruses that have been engineered to carry a foreign gene of interest into a target cell. Generally, for studies involving gene transfer or examination of the basic viral replication cycle, the vectors are engineered to be replication-defective, being able to complete only a single round of the retroviral replication cycle (though in some instances vectors capable of continued self-propagation might be used; ref. 23). Because of the way replication-defective retroviral vectors are designed, virus particles containing vector genomes can be produced and can be used to infect target cells. The vector genome then undergoes reverse transcription and integration into the cell's genome, where it can express the foreign gene(s) of interest, but is unable to be replicated an additional time and spread to other cells; the vectors can undergo only a single round of replication. For studies of basic viral biology, this property enables researchers to study in detail many aspects of the replication cycle; for gene transfer studies, it enables foreign genes to be permanently introduced into cells without exposing them to replicating virus.

Building a replication-defective vector from the parental retrovirus necessitates separating the *cis*- and *trans*-acting sequences of the viral genome. In a practical sense, this entails removal of the *trans*-acting *gag*, *pol*, and *env* genes from the virus (and replacing them with a foreign gene of interest), leaving on the genome only those *cis*-acting regions that are recognized by viral and cellular proteins during the various stages of the viral replication cycle—reverse transcription, integration, transcription, encapsidation—as reviewed above. These *cis*-acting regions are shown in Figure 3.

Obviously, necessary viral proteins that make up the physical structure of the virion and that perform enzymatic functions need to be provided in order to produce infectious vector virus. This requirement can be satisfied by expression of the *gag*, *pol*, and *env* genes, removed from the parental virus during vector construction, in cells that also express the vector construct. In this way, Gag, Pol, and Env can be provided in trans but the *gag*, *pol*, and *env* genes will not be carried along with the vector when it is harvested and used to infect target cells. Various strategies²⁴ for expression of vector constructs and viral genes and for production of vector virus are discussed below.

Vector virus that is produced by this *trans*-complementation can then be used to transduce (infect and express a foreign gene in) target cells. For experiments using vectors to study a single round of viral replication, the foreign gene is generally some type of marker gene that can be used to screen for transduction and to quantify the vector virus that is produced; typical markers used to itter vector produced include *lacz* or genes encoding GFP or a molecule conferring antibiotic resistance.

Design of Vectors

Once the basic vector backbone has been constructed (Fig. 3), there are several different ways in which to express a foreign gene (Fig. 4). In the simplest case, the foreign gene is expressed directly from the promoter located in the 5' LTR of the vector construct. Two or more genes can be expressed from the LTR if the second gene is expressed from a spliced message or if the second gene is translated using an internal ribosome entry site (IRES) (ref. 25). Although using the vector LTR to drive expression of the foreign gene(s) is useful in a number of settings, at times other strategies are used in order to attempt to control or to increase the level of gene expression. By designing vectors that contain internal, heterologous promoters, oftentimes foreign gene expression can be increased greatly compared to levels that could be obtained using the promoter in the vector LTR (this is attributable to low LTR promoter activity in the target cell, which typically is not the natural target cell of the parental virus). Frequently, the heterologous promoter is another viral promoter, such as cytomegalovirus (CMV), or it may be a tissue-specific promoter.

Of course, the years of experience with retroviral vector development have made it clear that the design of vectors to deliver and express foreign genes in cells is often not as simple as one might think from the description above. For example, sustained expression of the foreign genes has been a great problem that can have many possible etiologies. For instance, although use of a heterologous promoter can increase foreign gene expression, addition of an additional promoter on the vector sometimes can actually decrease gene expression. This phenomenon, termed "promoter interference," is incompletely understood and not always predictable: often it can be affected by the precise or relative location of the promoters or even by the foreign gene itself.^{26,27} Efforts to overcome promoter interference have included attempts to express the foreign gene from a heterologous promoter/foreign gene cassette placed on the vector in an anti-sense orientation relative to the LTRs. This strategy has had mixed success, probably since the same number of promoters are still present on the vector and possibly because the production of anti-sense transcripts may interfere with vector production or gene expression.

The development of self-inactivating (SIN) vectors may decrease the problem of promoter interference and also offers a potential safety advantage over traditional retroviral vectors. Sin vectors are named as such because they are engineered to generate, following reverse transcription of the vector RNA into the DNA form, a defective, and thus inactive, promoter in the 5' LTR. This is accomplished by engineering constructs that have a defective u3 region at the 3' end of the viral RNA form of the genome (this defective u3 is duplicated as a defective U3 of the 5' LTR during reverse transcription; see above and Chapter 2). In this scenario, there would be no active promoter in the proviral 5' LTR to interfere with internal, heterologous promoters. It also, in theory, offers additional safety advantages in that it would be more difficult to re-generate a wild-type parental retrovirus via recombination and also it may decrease



Fig. 4. Vector design strategies for expression of foreign genes. Examples of various strategies for expression of foreign genes delivered by retroviral vectors are illustrated. Other combinations of the strategies shown here also can be used. A. The foreign gene is expressed directly from the promoter located in the vector LTR. B. Expression of two or more foreign genes might be expressed using the promoter in the vector LTR by utilizing a spliced message to express the second gene. C. A foreign gene can be expressed from a heterologous promoter located in the middle of the vector; sometimes this promoter/foreign gene cassette is placed in the anti-sense orientation relative to the vector LTR. D. An internal ribosome entry site (IRES) can be utilized to express a second foreign gene. LTR, long terminal repeat; S.D., splice donor; S.A., splice acceptor; Pr, promoter. Arrows indicate the location and direction of transcription initiation.

the chances of insertional mutagenesis by a promoter insertion mechanism after the provirus is integrated into the cell's genome.

Design of Packaging Systems

There are three basic strategies for expressing viral proteins for *trans*-complementation of a replication-defective vector. These include co-infection of vector-producing cells with wild-type virus ("helper virus"), transient transfection of cells with plasmids expressing vector and protein-coding constructs ("packaging plasmids"), and the use of cells ("packaging cells" or "helper cells") that stably express viral proteins.

If cells containing a vector genome are infected with replication competent wild-type virus, the wild-type virus will complete the viral replication cycle as described above. The viral proteins produced as part of this process, however, also will recognize the vector RNA and will incorporate vector RNA genomes into virus particles. Both wild-type virus and vector virus will be released from cells. This vector virus can be harvested and used to infect target cells; however, because these preparations would be contaminated with wild-type virus, any target cells transduced with the vector would also be infected with wild-type virus. Therefore, continued replication and spread of the vector to other cells would occur. In certain experimental situations this could be a useful phenomenon, but in most cases a mixture of vector virus and wild-type virus would confound experimental results and make them difficult or impossible to interpret. Obviously, the presence of wild-type virus in vector preparations would make them unacceptable for clinical gene transfer studies.

In order to limit vector replication to a single round, separate viral protein-coding constructs can be used to *trans*-complement the vector. The *gag*, *pol*, and *env* genes are removed from the virus and expressed on separate plasmids using heterologous promoters. These packaging and vector plasmids can be co-transfected into cells. The viral proteins produced will package vector RNA, and vector virus that is released from these transfected cells can be harvested for transduction of target cells. Because the helper packaging plasmids do not contain the *cis*-acting sequences necessary for propagation (see above), they will not be packaged or transferred to the target cells; therefore, there will be only a single round of vector replication and the foreign gene can be introduced into target cells in a relatively predictable manner.

In order to decrease the chances of the re-generation of wild-type virus (replication-competent retrovirus, RCR) during vector production via recombination between vector and packaging constructs, use of a "split genome" approach to expressing viral proteins generally has been adopted. In such an approach, the vector is expressed on one construct, the *gag* and *pol* genes on another, and the *env* gene on yet another. Separate expression of the *env* gene also enables the use of Env from heterologus viruses in place of the native Env. The use of Env from heterologous viruses, termed pseudotyping, is extremely valuable because it allows the cellular tropism of the vector virus to be different from that of the parental virus from which the vector was derived. In addition, different envelope proteins have different physical properties that can be an advantage during vector preparation.^{28,29} The split genome approach decreases the frequency of RCR generation, given that multiple recombination events would need to take place. However, it does not reduce it to zero, especially when using transient transfection of cells, an inherently recombinagenic procedure, to produce vector virus. For this reason, packaging cell lines often are used to generate vector virus.

Packaging cells are cell lines that have been engineered to stably maintain viral genes and to express viral proteins.³⁰ The viral genes are expressed from heterologous promoters and there are no *cis*-acting sequences associated with the viral genes, just as when packaging plasmids are used in transient transfections as described above; usually the split genome approach is used to express *gag*, *pol*, and *env*. When a vector construct is introduced into the packaging cells, usually by transfection, vector virus is produced and can be harvested (Fig. 5). Generally, the use of packaging cells was thought to be preferential to the use of a transient transfection protocol for vector production: packaging cells can be well characterized and it was thought that the chances of RCR formation through recombination was less than during a transient co-transfection of vector and packaging plamsids. The use of packaging cells was generally a more direct way to produce vector virus and yielded higher vector titers than those obtained from transient transfections; however, improvements in protocols for vector production have made this factor less of a concern.

One final word on vector production and design of packaging systems: the biggest safety concern³¹ during vector production is that RCR might inadvertently be generated.³² Therefore, improvements designed to reduce the amount of sequence homology among constructs used in vector and packaging systems has remained a priority. Still, all vector preparations used need to be extensively tested for the presence of RCR.

Summary

Vector systems based on oncoretroviruses have been important tools for understanding basic retroviral replication and for applied studies involving gene transfer. Development of these vector systems enabled detailed study of the basic biology of the parent retrovirus from which they were derived and vice versa, enabling advances in vector systems and gene transfer technology to be made. Use of retroviral vectors for gene transfer has been limited for a number of years by several problems including low vector titers, inability to achieve sustained foreign gene expression in target cells, inability to target vector virus to transduce the desired cell type, the theoretical possibility of insertional mutagenesis by the vector upon integration, and the possibility of the re-generation wild-type virus during vector production. Over time, advances in the understanding of retroviral vector design and gene transfer have occurred and progress has been made in overcoming what had been identified as system limitations.



Fig. 5. Vector production using a packaging cell line. Packaging cells are cell lines that have been engineered to stably express viral proteins from heterologous promoters; these viral protein-coding sequences lack *cis*-acting sequences that are necessary for propagation and are therefore not transmitted to other cells. Following introduction of a vector into the packaging cell, usually by transfection, vector RNA is produced and packaged into vector virus particles that are then released from the packaging cell. These vector virus particles can be harvested and used to infect fresh target cells. Following reverse transcription and integration of the vector genome, the foreign gene is expressed in the target cells. Because viral proteins are not produced in the target cells, further vector production and propagation does not occur (1).

Recently, vector systems derived from several different lentiviruses, which could offer some advantages over oncoretroviral vector systems, have been under development. At least some of these systems are anticipated to have use in future clinical gene transfer applications. Certainly, the immense experience obtained with oncoretroviral vector systems and the basic tenets that guided their development has benefited and will continue to benefit the development of lentiviral vector systems which, because of their more complex genome and their potential to be human pathogens, raise technical, practical, and ethical issues that must be addressed.

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