

# Overview of the HIV-1 Lentiviral Vector System

Replication-defective oncoretroviral vectors have been the most widely used vehicles for gene-transfer studies because of their capacity to efficiently introduce and stably express transgenes in mammalian cells (Hawley, 1996, 2001; *UNITS 9.9-9.14*). A limitation of oncoretroviral vectors, however, is that cell division is required for proviral integration into the host genome (Miller et al., 1990). By comparison, lentiviruses such as human immunodeficiency virus type 1 (HIV-1) have evolved a nuclear-import machinery that allows them to infect nondividing as well as dividing cells (Lewis et al., 1992; Lewis and Emerman, 1994). This unique property has led to the development of lentiviral vectors for gene delivery to a variety of nondividing or slowly dividing cells including neurons and glial cells of the central nervous system, cells of differentiated epithelial tissues, hepatocytes, muscle cells, retinal cells, and hematopoietic stem cells.

Since HIV-1 is a known human pathogen, the development of HIV-1-based lentiviral vectors has been especially challenging. Before they could be considered safe for general (BSL-2) laboratory use, lentiviral vector systems required numerous modifications to preclude the generation of wild-type or replication-competent retroviruses (RCR). The complex genome of HIV-1 was fully characterized to determine the minimal amount of viral sequences that could constitute a functional transfer vector. Production of replication-defective vectors is accomplished by providing only essential virion structural and enzymatic proteins *in trans*. The latest versions of HIV-1-based lentiviral vectors carry as little as 10% of the viral genomic RNA.

Lentiviral vectors based on HIV-2 (Poeschela et al., 1998), simian immunodeficiency virus (Kim et al., 2001), equine infectious anemia virus (Mitrophanous et al., 1999), feline immunodeficiency virus (Curran et al., 2000), and visna virus (Berkowitz et al., 2001) have also been developed. However, since HIV-1-based lentiviral vectors are the most advanced, they are the primary focus of this chapter. This unit is intended to provide an overview of HIV-1 molecular biology and an introduction to successive generations of HIV-1-based lentiviral vectors. In *UNIT 16.22*, detailed protocols outlining the methodology and techniques in-

involved in the construction and application of HIV-1-based lentiviral vector systems are presented. That unit also describes procedures that can be used to concentrate and purify high-titer recombinant lentiviral vector preparations, as well as protocols for transduction of adherent and suspension cells.

## MOLECULAR BIOLOGY OF HIV-1

Like other members of the *Retroviridae* family of viruses, HIV-1 reverse transcribes its RNA genome into a double-stranded DNA form, hence the prefix “retro.” HIV-1 belongs to the subfamily *Lentivirinae*, which comprises primate and nonprimate retroviruses that cause slow, progressive diseases affecting the immune system. In humans, HIV-1 infection ultimately leads to the development of acquired immunodeficiency syndrome (AIDS). The molecular biology of HIV-1 has been reviewed extensively elsewhere (Frankel and Young, 1998). The salient features of the structure and life cycle of HIV-1 relevant to lentiviral vector-mediated gene transfer are summarized below.

## HIV-1 Genes

The HIV-1 genome (Fig. 16.21.1) is an ~9.3-kb RNA that encodes nine open reading frames (ORFs). Three of these ORFs, *gag*, *pol*, and *env*, which are common to all retroviruses, specify the Gag, Pol, and Env polyproteins, respectively (Table 16.21.1). These polyproteins are subsequently cleaved into the following protein subunits: Gag is cleaved to form the structural proteins matrix (MA), capsid (CA), nucleocapsid (NC), P6, P2, and P1; Pol is cleaved into the replication enzymes protease (PR), reverse transcriptase (RT), and integrase (IN); and Env (or gp160) is cleaved to form the transmembrane (TM or gp41) and surface (SU or gp120) glycoproteins required for viral binding and entry into the cells. Of the remaining six genes, *tat* and *rev* code for the regulatory proteins transactivator of transcription (Tat) and regulator of expression of virion proteins (Rev). The other four genes *vif*, *vpr*, *vpu*, and *nef*, which are referred to as accessory genes since they are not essential for viral replication, encode the virion infectivity factor (Vif), the viral protein R (Vpr), the viral protein U (Vpu), and the negative factor (Nef), respectively. The accessory genes are also called the virulence factors,

**Table 16.21.1** HIV-1 Genes, Gene Products, and Their Function

Gene	Size of mRNA	Encoded protein(s)	Function
<b>Regulatory genes</b>			
<i>tat</i>	1.7-2 kb	Tat (16 kDa)	<i>Trans</i> -activation of gene expression
<i>rev</i>	1.7-2 kb	Rev (19 kDa)	Nuclear export of late mRNAs; promotion of polysomal binding to RRE-containing RNAs
<b>Accessory genes</b>			
<i>vif</i>	5 kb	Vif (23 kDa)	Enhancement of virus transmission
<i>vpr</i>	4.5 kb	Vpr (10-15 kDa)	Nuclear transport of proviral DNA, induction of G <sub>2</sub> arrest in dividing cells
<i>vpu</i>	4 kb	Vpu (15-20 kDa)	CD4 degradation; virus maturation and release
<i>nef</i>	1.7-2 kb	Nef (25-27 kDa)	CD4 and MHC-I down-regulation; enhancement of virus replication
<b>Structural genes</b>			
<i>gag</i>	9.3 kb	Pr55 <sup>gag</sup> : MA (p17), CA (p24), NC (p9), p6	Formation of viral particles, packaging of viral genomic RNA
<i>pol</i>	9.3 kb	Pr160 <sup>gag-pol</sup> : PR (p10), RT (p61/p52), IN (p31)	Reverse transcription, integration, and virion maturation
<i>env</i>	4 kb	gp160:SU (gp120), TM (gp41)	Binding and entry into the host cell

as they are associated with HIV-1 virulence and pathogenesis in vivo (Cullen, 1991).

### HIV-1 Virion

The HIV-1 virion (Fig. 16.21.1B) has a diameter of ~110 nm and consists of a nucleocapsid core surrounded by a lipid membrane (Aloia et al., 1988). Inserted into the lipid membrane are the viral SU and TM glycoproteins which are noncovalently linked (Willey et al., 1988). Proteins within the inner shell of a mature virion are the cleavage products of the Gag (Pr55<sup>gag</sup>) and Gag-Pol (Pr160<sup>gag-pol</sup>) precursors (Mervis et al., 1988). The condensed inner core is formed by the capsid protein (CA), p24. In the space between the inner core and the lipid membrane is the N-terminal myristylated matrix protein (MA), p17, which remains associated with the lipid membrane. The virion core contains two copies of the single-stranded genomic RNA to which the NC protein is bound. Also packaged into the virions are host tRNA<sub>3</sub><sup>Lys</sup>, RT, PR, IN, Vif (Liu et al., 1995), and Vpr (Cohen et al., 1990).

### HIV-1 Life Cycle

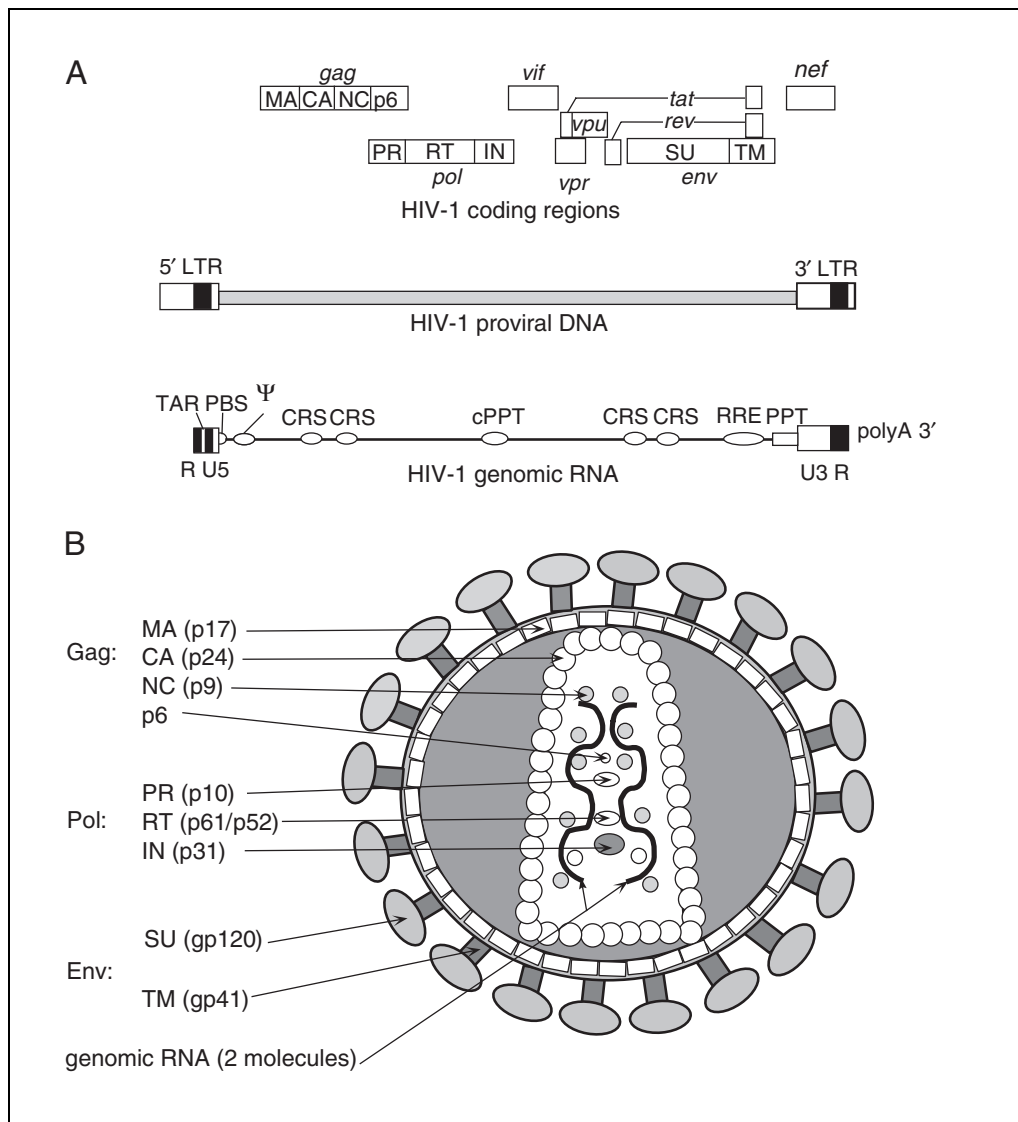
A schematic representation of the HIV-1 life cycle is shown in Figure 16.21.2. The HIV-1 replication cycle can be divided into the following steps: (i) virus entry; (ii) reverse transcription; (iii) nuclear localization and integration; (iv) viral RNA transcription; (v) RNA processing and viral protein synthesis; and (vi) viral particle assembly, release and maturation.

### Virus entry

HIV-1 infection is initiated by the binding of gp120 to the CD4 receptor which is found on the surface of T helper lymphocytes, macrophages, and glial cells (Dalglish et al., 1984; Maddon et al., 1986). Binding of gp120 to the CD4 receptor induces conformational changes that expose the N-terminal hydrophobic domain of gp41. This hydrophobic domain is then inserted into the cell membrane, initiating fusion in a pH-independent manner (Stein et al., 1987). Fusion of gp41 with the cell membrane has been shown to utilize a cellular coreceptor. The chemokine receptors CCR-5 (Alkhatib et al., 1996; Choe et al., 1996) and CXCR-4 (Feng et al., 1996) are the major coreceptors required for entry of macrophage-tropic and T cell-tropic strains of HIV-1, respectively.

### Reverse transcription

Following entry, the outer lipid membrane of the virion is removed and its core is delivered into the cytoplasm. Within the core, genomic RNA is reverse transcribed by RT into double-stranded DNA using tRNA<sub>3</sub><sup>Lys</sup> as a primer (Goff, 1990; Das et al., 1994). The tRNA<sub>3</sub><sup>Lys</sup> primer allows first strand DNA synthesis by binding to the PBS (primer binding site) located near the 5' end of the HIV-1 genomic RNA. As the minus-strand DNA synthesis proceeds to the 5' end, RNase H degrades the template from the RNA/DNA hybrid allowing the minus-strand to undergo an intermolecular strand switch ("jump") to complementary sequences



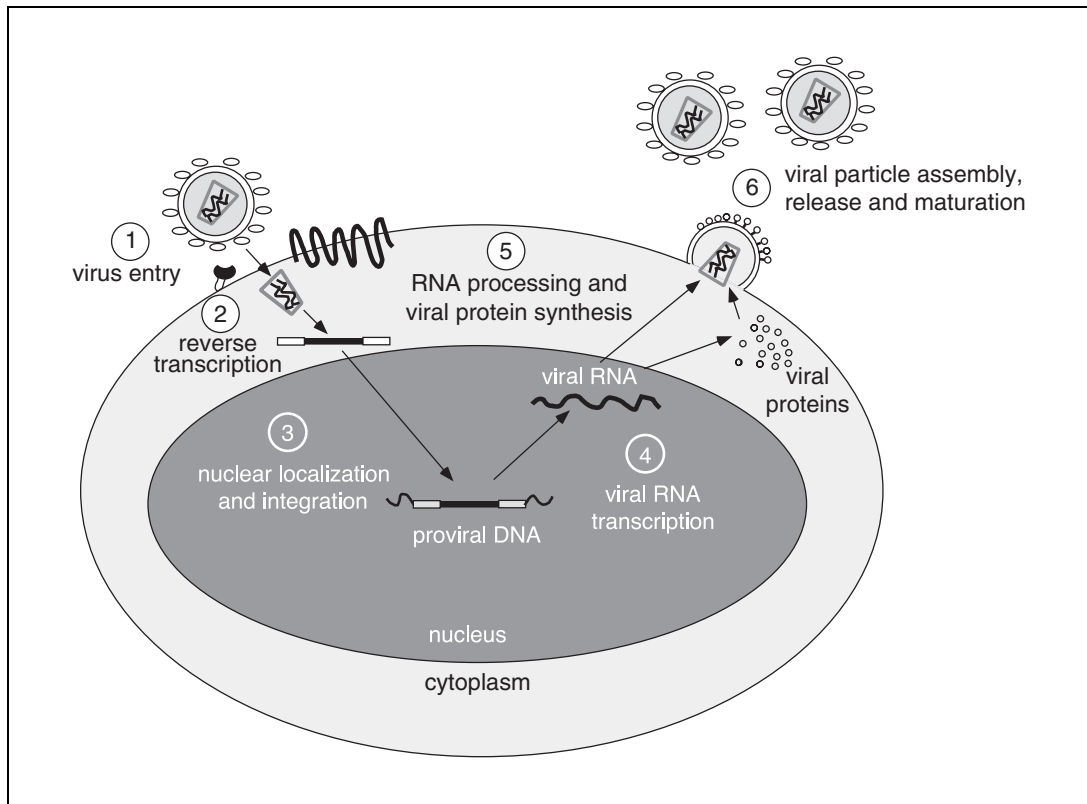
**Figure 16.21.1** (A) Structural features of HIV-1 proviral DNA and genomic RNA. The proviral DNA is flanked by LTRs which control the expression of the viral genes. The relative positions of genes that encode the structural and enzymatic proteins (Gag, Pol and Env), the regulatory proteins (Tat and Rev), and the accessory proteins (Vif, Vpu, Vpr and Nef) are indicated. The various *cis*-acting responsive sequences (CRS) within the *gag*, *pol*, and *env* coding regions that negatively regulate HIV-1 gene expression are shown on the HIV-1 genomic RNA. (B) Schematic diagram of the HIV-1 virion. The mature virion contains an inner core surrounded by a host-cell derived lipid membrane. Inserted within the lipid membrane are the viral Env glycoproteins. The condensed inner core is made of the capsid protein. The space between the inner core and the lipid membrane contains the MA protein, which remains attached to the lipid membrane. The inner core encompasses two copies of the genomic RNA which are bound by NC proteins. Also packaged within the viral particles are the RT, PR, IN, Vif and Vpr proteins, as well as the primer tRNA<sub>3</sub><sup>Lys</sup>, which is of the host origin.

at the 3' end of viral RNA (Panganiban and Fiore, 1988). RNase H activity also generates short template fragments at the 3' polypurine tract (PPT) located upstream of the unique 3' (U3) sequence and an internal PPT located at the 3' end of the *pol* gene (central PPT or cPPT) which serve as primers for plus-strand DNA synthesis (Charneau et al., 1992). A second jump (intramolecular) between the PBS se-

quences allows RT to extend to the ends of the templates, leading to complete synthesis of double-stranded HIV-1 DNA containing flanking long terminal repeats (LTRs).

#### Nuclear localization and integration

The newly synthesized linear double-stranded DNA is then translocated to the nucleus in the absence of mitosis while still part



**Figure 16.21.2** Schematic diagram of the HIV-1 life cycle. Following entry into the cell, HIV-1 genomic RNA is reverse transcribed; the DNA intermediate, as part of a nucleoprotein complex, is then transported to the nucleus where it is inserted into the cellular genome. The full-length 9.3-kb viral RNA is differentially spliced to give rise to various HIV-1 mRNAs. Virus assembly takes place at the plasma membrane, where two copies of HIV-1 genomic RNA are incorporated into the budding viral particles. The steps indicated by numbers 1 to 6 are described in the text.

of the nucleoprotein complex, which contains the viral proteins Vpr, RT, MA and IN (Farnet and Haseltine, 1991; Bukrinsky et al., 1993). Nuclear translocation is mediated via interaction with the cellular nuclear import pathway (Trono, 1995). Interaction involves Vpr, MA and IN, all of which contain nuclear localization signals (Heinzinger et al., 1994). As described below, studies of lentiviral vectors identified a “central DNA flap” formed during reverse transcription as a result of the cPPT that facilitates nuclear translocation of the HIV-1 preintegration complex in both dividing and nondividing cells (Follenzi et al., 2000; Zenou et al., 2000).

Within the nucleus, the linear double-stranded DNA is integrated into the host chromosome by the action of viral IN to form the provirus. IN recognizes short inverted termini at the ends of the viral DNA and cleaves a dinucleotide from each 3' end (LaFemina et al., 1991). The chromosomal DNA is also cleaved by IN, which subsequently ligates the 5' ends of chromosomal DNA to the 3' ends of the

resulting proviral structure (Bushman and Craigie, 1991).

### **Transcription**

Two identical direct 634-base pair (bp) repeats—the LTR sequences—flank the provirus at either end. The LTRs contain the enhancer and promoter elements for transcription of viral genes. Each LTR consists of the U3 region, the R (repeat) region and the unique 5' (U5) region. The U3 region contains binding sites for several cellular transcription factors including SP-1 and NF- $\kappa$ B (Cullen, 1991). The R region of the LTR contains the 5' untranslated leader sequence, which provides the binding site for a number of factors involved in transcription. In particular, the 5' untranslated leader sequence contains the *trans*-activation response (TAR) element, which is a 59-nt-long region forming a stem-loop structure at the 5' noncoding region of all HIV-1 RNAs (Selby et al., 1989). Transcription of the HIV-1 provirus initiates at the 5' end of the R region within the 5' LTR (Cullen, 1991), with the site of polyadenylation deline-

ating the 3' end of the R region within the 3' LTR (Valsamakis et al., 1991).

High-level transcription from the HIV-1 provirus requires the viral Tat protein (Feng and Holland, 1988). Activation of HIV-1 transcription by Tat relies on the TAR RNA element. In the absence of Tat, early transcriptional events generate mostly short transcripts. Interaction of Tat with TAR enhances formation of RNA polymerase II complexes. As the Tat protein accumulates, the efficiency of transcriptional elongation is enhanced as it suppresses random terminations and increases the processivity of RNA polymerase II (Keen et al., 1996). In addition to enhancing the transcription of viral RNAs, Tat is also known to block the expression of major histocompatibility complex (MHC) class II genes in HIV-1-infected cells (Kanazawa et al., 2000).

### ***RNA processing and viral protein synthesis***

Newly transcribed HIV-1 viral RNA is doubly spliced by the cellular splicing machinery, producing 1.7- to 2.0-kb mRNAs which code for the Tat, Rev, and Nef proteins. The Rev protein regulates transport of HIV-1 transcripts from the nucleus to the cytoplasm by binding to the Rev response element (RRE) located in the *env* coding region (Daly et al., 1989). By binding to RRE, Rev allows the RRE-containing viral RNAs to access a pre-existing cellular-export pathway. The nuclear-export signal of the Rev protein (Fischer et al., 1995; Wen et al., 1995) recognizes and binds to the nuclear-export factor exportin 1 (Ullman et al., 1997). As Rev accumulates in the nucleus, viral RNA transport from the nucleus to the cytoplasm is enhanced. This in turn increases the probability that the viral RNA will escape the splicing machinery, resulting in nuclear export of 4- to 5-kb singly spliced and 9.3-kb unspliced viral RNAs.

The Nef protein is encoded by an open reading frame that overlaps the 3' LTR. Nef is a myristylated plasma membrane-associated protein that is expressed early during virus infection from several multiply spliced mRNAs (Robert-Guroff et al., 1990). Nef reduces the cell surface levels of CD4 (Aiken et al., 1994). This may permit increased incorporation of Env into virions, enhancing viral replication and pathogenesis (Aiken and Trono, 1995). Nef has also been shown to down-regulate cell surface expression of MHC class I molecules (Swann et al., 2001).

Once in the cytoplasm, the singly spliced viral mRNAs are translated to produce the Vif, Vpr, Vpu and Env proteins (Malim et al., 1989). The Vif protein is localized at the inner face of the cytoplasmic membrane of infected cells (Goncalves et al., 1994). It is also incorporated within viral particles in association with the nucleoprotein complex (Liu et al., 1995; Khan et al., 2001). Vif plays a critical role in the production of infectious virions by affecting viral particle assembly or maturation (Gabuzda et al., 1992; Kishi et al., 1992).

Both Vpu and Env proteins are expressed from a singly spliced bicistronic mRNA due to leaky ribosome scanning at the upstream Vpu initiation codon (Schwartz et al., 1992). The viral Env protein is translated as a gp160 precursor polyprotein. Gp160 is directed to the endoplasmic reticulum by its hydrophobic N-terminal signal peptide where it is extensively glycosylated (Robey et al., 1985). Subsequently, gp160 is cleaved within the endoplasmic reticulum–Golgi apparatus by cellular proteases (Stein and Engleman, 1990) into the SU (gp120) and TM (gp41) subunits (Freed et al., 1989), which are then inserted within the plasma membrane. Vpu is a hydrophobic membrane-associated protein, which mediates degradation of CD4 complexes in infected cells (Chen et al., 1996), crucial for maturation and release of fully infectious viral particles (Klimkait et al., 1990).

The unspliced HIV-1 viral RNA is translated into the Pr55<sup>gag</sup> and Pr160<sup>gag-pol</sup> polyprotein precursors. The *gag* and *pol* ORFs overlap by 241 nucleotides, with the *pol* ORF in the –1 translational reading frame with respect to the *gag* ORF. The translation of the *pol* ORF thus occurs as a consequence of ribosomal frameshifting, resulting in the synthesis of a Gag-Pol fusion protein, Pr160<sup>gag-pol</sup> (Jacks et al., 1988). The N-termini of both Pr55<sup>gag</sup> and Pr160<sup>gag-pol</sup> polyprotein precursors are myristylated, which mediates their transport to the cellular membrane where they are packaged into the viral particles (Bryant and Ratner, 1990). Within the viral particles, the viral PR cleaves Pr55<sup>gag</sup> to generate the mature virion structural proteins, MA, CA, and p15 (Peng et al., 1989). Subsequently, p15 is cleaved to produce the NC (p9) and p6 proteins (Sheng and Erickson-Viitanen, 1994). The Pr160<sup>gag-pol</sup> polyprotein is also cleaved within the mature viral particles to produce PR, RT, and IN by the viral PR, which is itself derived from the Pr160<sup>gag-pol</sup> precursor (Debouck, 1991).

### ***Viral particle assembly, release, and maturation***

HIV-1 virus assembly initiates with interactions between the Pr55<sup>gag</sup> and Pr160<sup>gag-pol</sup> polyproteins, producing an immature virion core. In addition to serving as mRNA for Pr55<sup>gag</sup> and Pr160<sup>gag-pol</sup> synthesis, the unspliced 9.3-kb RNA also serves as HIV-1 genomic RNA. For efficient incorporation of HIV-1 genomic RNA into viral particles, a  $\psi$  packaging signal located within the 5' untranslated region and the *gag* ORF plus a 1.1-kb RNA sequence within the *env* ORF are required (Lever et al., 1989). Dimerization of HIV-1 genomic RNAs is mediated by the annealing of palindromic sequences within the dimer linkage structure located at the 5' end of the RNA (Skripkin et al., 1994). Packaging of two copies of HIV-1 genomic RNA into viral particles then ensues, a process that involves an interaction between the NC domains of the Pr55<sup>gag</sup> and Pr160<sup>gag-pol</sup> protein precursors and stem-loop structures within the  $\psi$  packaging signal (Clever et al., 1995; Rice et al., 1995). The RT and NC domains of the Pr160<sup>gag-pol</sup> polyprotein facilitate the incorporation of the cellular tRNA<sub>3</sub><sup>Lys</sup> primer into the virions (Mak et al., 1994). PR is activated during the final stages of budding within the newly released immature particles, leading to proteolytic processing of the Pr55<sup>gag</sup> and Pr160<sup>gag-pol</sup> polyproteins (Peng et al., 1989) and viral particle maturation.

### **REPLICATION-DEFECTIVE HIV-1-BASED VECTORS**

As with oncoretroviral vectors, generation of replication-defective lentiviral vectors requires segregation of *cis*-acting sequences necessary for the transfer of a functional viral genome to target cells and those sequences encoding essential viral structural and enzymatic proteins onto separate plasmids. The transfer vector consists of *cis*-acting sequences—the LTRs, the PBS, the packaging signal, the PPTs and the RRE—linked to a transgene of interest in the context of a transcriptional unit. Following cotransfection of the transfer vector together with packaging and envelope expression plasmids lacking most if not all of the *cis*-acting sequences into an appropriate “recipient” cell, viral proteins provided in *trans* assemble into virions encapsidating the replication-defective transfer vector RNA.

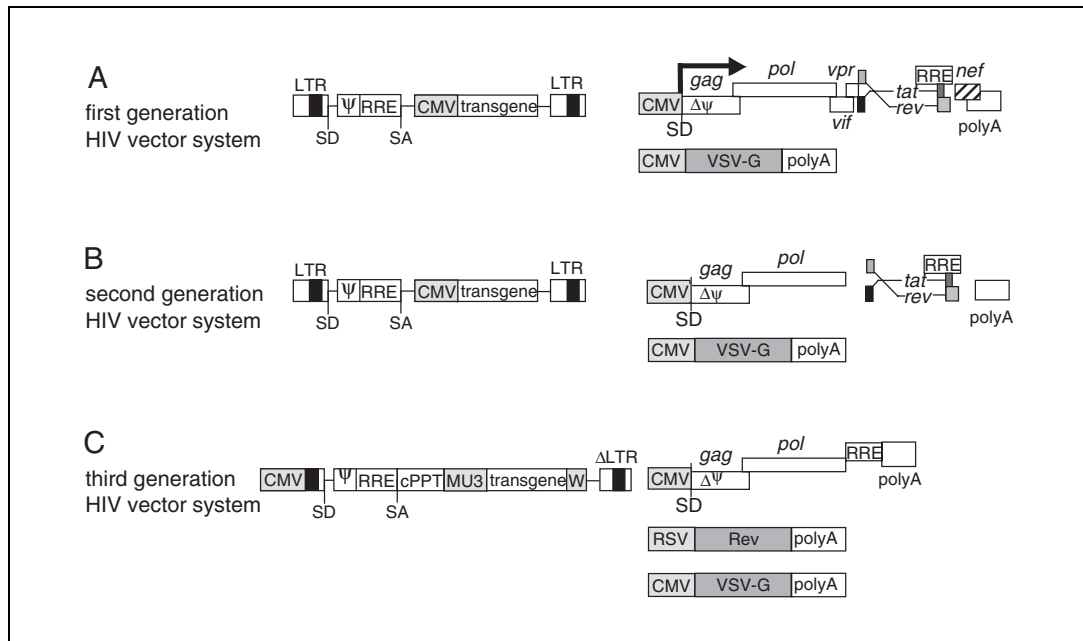
### **First-Generation HIV-1 Vector Systems**

Although earlier HIV-1-derived vector configurations have been reported in the literature

(Page et al., 1990; Landau et al., 1991; Poznan-sky et al., 1991), only the more recent safety-modified versions of HIV-1 vector systems will be described here. An example of a “first generation” HIV-1 vector system comprising three expression plasmids—a transfer vector, a packaging construct, and an envelope gene—is depicted in Figure 16.21.3A (Naldini et al., 1996a). The transfer vector contains intact HIV-1 LTRs and the *cis*-acting sequences described above—including 350-bp of *gag* encompassing the  $\psi$  packaging signal and *env* sequences encompassing the RRE—with the exception of the cPPT. The transgene is expressed from an internal human cytomegalovirus (CMV) immediate early region enhancer-promoter. The packaging plasmid encodes all of the HIV-1 proteins except Vpu and Env. It consists of the HIV-1 genome with the following modifications: the 5' LTR was replaced with the CMV promoter to drive expression of the viral proteins required in *trans*; the  $\psi$  packaging signal, the *env* gene, and the ORF encoding the Vpu protein were deleted; and the 3' LTR was replaced at the end of the *nef* ORF with a polyadenylation [poly(A)] site from the insulin gene. In the envelope-encoding plasmid, the G glycoprotein gene of vesicular stomatitis virus (VSV-G) is transcribed from the CMV promoter. Transient cotransfection of human embryonic kidney 293T cells (DuBridge et al., 1987) with the three-plasmid combination generates replication-defective VSV-G-pseudotyped vector particles with titers on the order of 10<sup>5</sup> transducing units (TU)/ml. The lentiviral particles efficiently transduced nondividing cells including human HeLa cells growth-arrested at G<sub>1</sub>-S or G<sub>2</sub> phases of the cell cycle, 4-day contact-inhibited rat 208F fibroblasts, and terminally differentiated adult rat neurons.

### **Second-Generation HIV-1 Vector Systems**

It was shown subsequently that none of the four HIV-1 accessory genes *vif*, *vpr*, *vpu*, or *nef* were required for HIV-1 replication in immortalized cell lines (Miller and Sarver, 1997) or for efficient generation of VSV-G-pseudotyped vector particles (Zufferey et al., 1997). This led to the development of a “second generation” of HIV-1 vector systems (Fig. 16.21.3B), which utilized a multiply attenuated packaging construct containing only the HIV-1 *gag*, *pol*, *rev*, and *tat* genes. Although elimination of all four accessory genes had no effect on the titers of VSV-G-pseudotyped vector particles produced in 293T cells, macrophage transduction effi-



**Figure 16.21.3** Schematic representation of various HIV-1 vector systems. **(A)** A first-generation HIV-1 vector system expressing all of the HIV-1 proteins except Vpu and Env. The vector particles are pseudotyped with the VSV-G envelope glycoprotein expressed from a separate plasmid. **(B)** A second-generation HIV-1 vector system having all of the accessory genes removed. **(C)** A third-generation Tat-independent HIV-1 vector system. The SIN transfer vector illustrated contains the cPPT for efficient nuclear import and utilizes an internal MSCV LTR promoter (MU3) and the WPRE (W) for high-level transgene expression. The two packaging constructs encode only the HIV-1 Gag, Pol and Rev proteins.

ciencies were reduced by ~50%, which was apparently due to the lack of the Vpr protein (Zufferey et al., 1997). Efficient transduction of resting hepatocytes, skin fibroblasts, and lymphocytes has also been reported to require HIV-1 accessory proteins (Kafri et al., 1997; Gasmi et al., 1999; Chinnasamy et al., 2000), suggesting that some of these accessory proteins might be necessary for efficient gene transfer of certain cell types.

### Third-Generation HIV-1 Vector Systems

The main concern regarding the use of any HIV-1 vector is the potential for generation of replication-competent retroviruses (RCRs). Numerous additional modifications have therefore been made to ensure the biosafety of current “third” generation HIV-1-based delivery systems (Dull et al., 1998; Kim et al., 1998; Miyoshi et al., 1998; Zufferey et al., 1998; also see Fig. 16.21.3C). Since the U3 region of the 3′ HIV-1 LTR serves as a template for the U3 regions of both LTRs in the resulting provirus, third-generation HIV-1 transfer vectors have been developed that contain a 400-bp deletion within this region. The consequence of this modification is that the 5′ LTR of the integrated

vector is almost completely inactivated (Miyoshi et al., 1998; Zufferey et al., 1998). Inability to efficiently transcribe full-length vector RNA by these so-called self-inactivating (SIN) HIV-1 vectors in transduced target cells considerably minimizes the possibility of RCR generation. The SIN configuration also reduces the possibility of oncogene activation by promoter insertional mutagenesis. By design, SIN vectors require an internal promoter for transgene expression. Fortunately, in contrast to what was observed previously for SIN oncoretroviral vectors, this modification does not result in a significant drop in vector titers.

To prevent reconstitution of U3 sequences within the deleted 3′ HIV-1 LTR by homologous recombination with the intact 5′ HIV-1 LTR during transient cotransfection of 293T cells, the U3 region of the 5′ LTR of third-generation transfer vectors has been replaced with the CMV or Rous sarcoma virus LTR promoters (Dull et al., 1998; Kim et al., 1998). Notably, titers as high as  $10^7$  TU/ml can be obtained from the chimeric LTRs irrespective of whether Tat is present, allowing deletion of the *tat* gene from the packaging construct. This finding permitted further refinement of the packaging system such that the *gag-pol* and *rev* genes can be

expressed from two separate nonoverlapping plasmids (Dull et al., 1998).

Efforts to eliminate the Rev dependence of HIV-1-based lentiviral vectors have generally involved replacing the RRE with autonomous RNA export signals termed constitutive RNA transport elements—typically from the simian type D retroviruses, Mason-Pfizer monkey virus, and the simian retroviruses type 1 and 2 (Bray et al., 1994; Ernst et al., 1997; Kim et al., 1998; Gasmi et al., 1999; Kotsopoulou et al., 2000; Mautino et al., 2000). Collectively, the studies have indicated that these elements can partially substitute for the Rev/RRE combination, although there is usually a reduction in vector titer when the Rev/RRE components are completely removed from the packaging system. For this reason, third-generation HIV-1 vectors generally retain the RRE sequence.

As noted above, after HIV-1 reverse transcription a 99 nucleotide-long overlap—the central DNA flap—is formed that is involved in normal import of the HIV-1 preintegration complex into the nucleus (Follenzi et al., 2000; Zennou et al., 2000). The sequences specifying the central DNA flap—a cPPT and a central termination sequence located within the *pol* ORF—were omitted from earlier generations of HIV-1 vectors. These sequences have been restored in more recent versions of third-generation HIV-1 vectors and shown to facilitate higher transduction efficiencies of several types of cells, both proliferating and growth-arrested.

For high-level transgene expression in a broad range of cell types *in vitro* and *in vivo*, a variety of viral and cellular promoters have been inserted into third generation HIV-1 vector backbones. In addition to the CMV promoter, other promoters that have been demonstrated to perform well include the murine stem cell virus (MSCV) vector LTR (Hawley et al., 1994), the gibbon ape leukemia virus LTR, the human elongation factor 1 $\alpha$  promoter, the CAG promoter (composed of the CMV immediate early enhancer linked to chicken  $\beta$ -actin promoter sequences) and the human X chromosome phosphoglycerate kinase 1 promoter (Ramezani et al., 2000).

Finally, other genetic elements have been identified that stimulate transgene expression post-transcriptionally. Chief among these is the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). This element has been postulated to exert a positive influence on transgene expression by augmenting 3'-end processing and polyadenylation (Loeb et al., 2000). Inclusion of the WPRE has been shown

to significantly enhance transgene expression levels from several types of promoters in both oncoretroviral and lentiviral vectors, and is thus routinely included in third-generation HIV-1-based gene delivery systems (Zufferey et al., 1999; Deglon et al., 2000; Ramezani et al., 2000).

### Packaging Limit of HIV-1 Vectors

Another advantage of HIV-1-based vectors over oncoretroviral vectors is their greater packaging limit. The packaging limit is the length of vector RNA that can be encapsidated into viral particles. Although an HIV-1 vector greater than 12 kb in size has been reported to give reasonable titers (Uchida et al., 1998), a systematic determination of the packaging limit of lentiviral vectors demonstrated that the viral titers decrease semilogarithmically with increasing vector length (Kumar et al., 2001). In that study, VSV-G-pseudotyped HIV-1 vectors with a proviral length of 10.5 to 11 kb yielded titers comparable to that of a control vector 5.5 kb in size. The third-generation vector illustrated in Figure 16.21.3C contains ~1.9 kb of *cis*-acting HIV-1 sequences, an MSCV-based internal promoter (~0.3 kb) and the WPRE (~0.6 kb). It can therefore readily accommodate transgenes of ~8 kb.

### HIV-1 Vector Packaging Cell Lines

HIV-1 lentiviral vectors with tropism limited to CD4<sup>+</sup> cells have been produced from stable packaging cell lines, albeit at titers of only ~10<sup>4</sup> TU/ml (Corbeau et al., 1996; Srinivasakumar et al., 1997). Because of the toxicity associated with the VSV-G envelope glycoprotein (Yang et al., 1995) as well as high-level constitutive expression of the HIV-1 PR (Kaplan and Swanstrom, 1991) and Vpr proteins (Planelles et al., 1995), the most common method of producing VSV-G-pseudotyped HIV-1 vectors has been by transient cotransfection. Titers in the range of 10<sup>6</sup> to 10<sup>7</sup> TU/ml can be obtained in this manner. Nonetheless, transient cotransfection of transfer, packaging, and VSV-G envelope plasmids can yield variable results and the process is labor-intensive when large amounts of vector stocks are required. Thus, although this method is satisfactory for most experimental applications, a stable producer cell line is highly desirable.

The requirement for a stable producer cell line has led to the development of inducible HIV-1-based packaging systems (Yu et al., 1996; Kaul et al., 1998; Kafri et al., 1999; Klages et al., 2000; Pacchia et al., 2001). Many

of these cell lines utilize a tetracycline-regulatable expression system to control protein synthesis from the packaging and VSV-G constructs (Fig. 16.21.4). Tetracycline-regulatable systems are based on the tetracycline resistance (*tet*) operon of *E. coli* (Gossen and Bujard, 1992). In one version of the system, commonly referred to as “tet-off” (Fig. 16.21.4A), the tetracycline repressor protein (TetR) is fused to the activation domain of the herpes simplex virus VP16 protein to generate a tetracycline-repressed transactivator (tTA). In the absence of tetracycline or tetracycline analogs such as doxycycline, tTA binds to and stimulates transcription from a minimal promoter sequence, usually derived from the human CMV immediate early region, linked to seven copies of the *tet* operon sequence (tetO). In the presence of low concentrations of tetracycline/doxycycline, tTA is prevented from binding to the promoter and transcription ceases. In the complementary “tet-on” system (Gossen et al., 1995), a “reverse” transactivator (rtTA) with the opposite properties of tTA binds to the minimal promoter and stimulates transcription only in the presence of doxycycline (Fig. 16.21.4B).

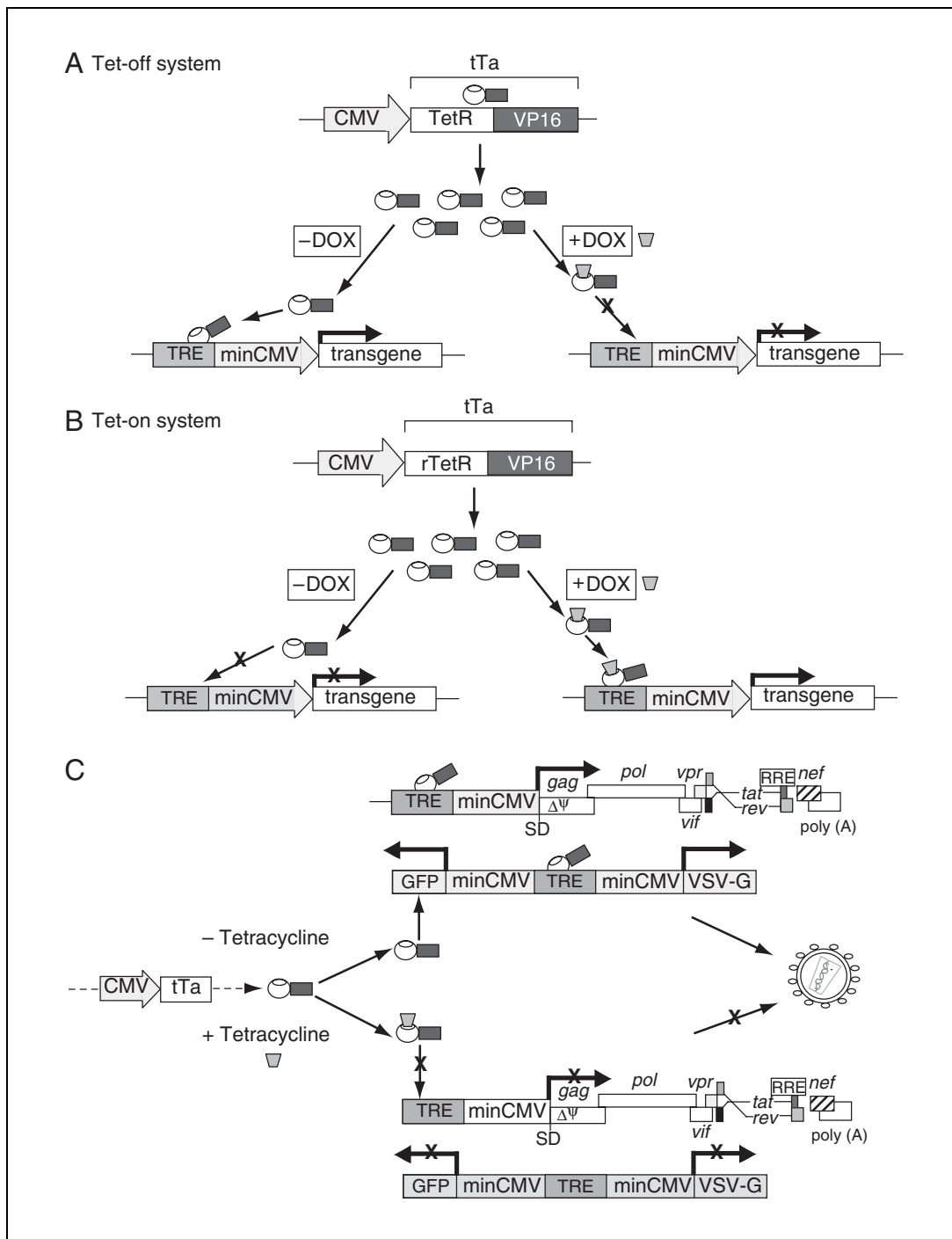
An example of a tTA-based HIV-1-packaging cell line that is capable of producing VSV-G pseudotyped vectors with titers on the order of  $10^6$  TU/ml for at least 3 to 4 days (Kafri et al., 1999) is illustrated in Figure 16.21.4C. The packaging cell line was generated by stable transfection of 293T cells with an expression plasmid that allows constitutive expression of tTA from the CMV promoter, a packaging construct containing all of the HIV-1 viral genes except the *env* gene under the control of a tetracycline-responsive promoter, and a VSV-G envelope plasmid also under tetracycline-responsive promoter control. More recently, a tTA-regulatable HIV-1 vector packaging cell line based on the third-generation split-genome, multiply attenuated packaging constructs has been developed (Klages et al., 2000). In this system, the HIV-1 proteins have been minimized to Gag, Pol and Rev. The Gag and Gag-Pol proteins are expressed from the CMV promoter. Advantage was taken of the fact that HIV-1 *gag/pol* transcripts are retained in the nucleus and rapidly degraded in the absence of Rev; consequently, Rev was placed under tetracycline-regulated control along with the VSV-G construct. Titers as high as  $5 \times 10^6$  TU/ml can be obtained after 7 to 10 days following removal of doxycycline, and the cells can be maintained in culture for about another week.

Another group has utilized the ecdysone-inducible expression system to regulate expression of the HIV-1 Gag, Pol, and Rev proteins from a packaging construct and the VSV-G protein from a second plasmid (Pacchia et al., 2001). The system is based on the ability of the ecdysone analog, ponasterone A, to induce transcription in mammalian cells through a modified heterodimeric insect ecdysone receptor (No et al., 1996). In the presence of ponasterone A, the insect ecdysone receptor, modified by fusion to the herpes simplex virus VP16 transactivation domain, forms a heterodimer with the retinoid X receptor. The heterodimeric complex activates gene expression from a hybrid ecdysone response element linked to a minimal promoter. Compared to the tetracycline-regulatable system, the ecdysone-inducible system appears to have a lower basal level of expression and faster induction kinetics (No et al., 1996). Accordingly, the peak production of HIV-1 vector particles in this system was achieved in 3 to 5 days, although the maximum titers obtained were only  $10^5$  TU/ml.

## TARGET CELLS OF HIV-1 VECTORS

An understanding of the molecular mechanisms mediating HIV-1 infection of nondividing cells has permitted HIV-1-derived lentiviral vectors to be designed to retain this property as well. As discussed previously, the nucleophilic properties mainly reside in the MA, IN, and Vpr proteins of the preintegration complex (Bukrinsky et al., 1993; Heinzinger et al., 1994; Gallay et al., 1997). Although the Vpr protein seems to be important for transduction of macrophages and hepatocytes (Naldini et al., 1996a; Kafri et al., 1997), it has been found to be dispensable for transduction of many other cell types (reviewed in Vigna and Naldini, 2000). Notably, however, while HIV-1 vectors do not require cell division, like HIV-1 they are unable to successfully transduce T lymphocytes that are in the  $G_0$  stage of cell cycle. Inefficient transduction of metabolically inactive T cells is due to blocks at the levels of reverse transcription (Korin and Zack, 1998) and nuclear import (Sun et al., 1997). By comparison, T cells in the  $G_1/S$  or  $G_2$  stages of cell cycle have permissive cytoplasmic conditions that allow HIV-1 and HIV-1-derived vectors to complete reverse transcription and nuclear localization.

As with oncoretroviral vector systems (Hawley, 1996, 2001), a major factor determining the host range and target cell type of HIV-1



**Figure 16.21.4** Schematic representation of a tetracycline-regulatable HIV-1 packaging system. **(A)** Principle of the “tet-off” gene expression system. The tetracycline-controlled transactivator (tTA) was generated by fusion of the tetracycline repressor protein (TetR) to the activation domain of the herpes simplex virus VP16 protein. In the absence of tetracycline or doxycycline, tTA binds to the tetracycline-responsive promoter element (TRE) and stimulates transcription of the transgene. **(B)** Principle of the “tet-on” gene expression system. A mutant TetR (rTetR) was created by four amino acid changes in TetR and fused to the activation domain of the herpes simplex virus VP16 protein to generate a reverse tetracycline-controlled transactivator (rtTA). rtTA binds to the TRE in the presence of doxycycline and stimulates transcription. **(C)** An example of a tetracycline-regulated HIV-1 packaging system. The packaging cells constitutively express tTA which allows expression of the HIV-1 packaging construct and the VSV-G glycoprotein envelope gene in the absence of tetracycline. In order to monitor the induction process, a green fluorescent protein (GFP) reporter gene is coexpressed with the VSV-G envelope glycoprotein gene from a bidirectional tetracycline-responsive promoter.

vectors is the envelope protein. While the Env protein of wild-type HIV-1 limits target cell tropism to CD4<sup>+</sup> lymphocytes, macrophages, and glial cells, VSV-G-pseudotyped HIV-1 vectors have a greatly expanded tropism, and, with the exceptions noted above, can transduce virtually all types of cells. The expanded tropism of VSV-G-pseudotyped HIV-1 vectors is due to the ability of the VSV-G protein to bind to a phosphatidyl serine component of the lipid bilayer present in the cell membrane of most eukaryotic cells (Schlegel et al., 1983). Thus VSV-G-pseudotyped HIV-1 vectors have been used to transduce and mediate transgene expression in differentiated cells and tissues as diverse as neurons, liver, muscle, and retina (Naldini et al., 1996a,b; Kafri et al., 1997; Miyoshi et al., 1997; Kordower et al., 1999) as well as undifferentiated stem cells and embryos (Hamaguchi et al., 2000; May et al., 2000; Hawley, 2001; Pawliuk et al., 2001; Wolfgang et al., 2001; Lois et al., 2002; Pfeifer et al., 2002).

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